Soil Biology

Asunción Morte
Ajit Varma *Editors*

Root Engineering

Basic and Applied Concepts

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Soil Biology

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Foreword

Roots are highly complex systems. They are known to perform most diverse tasks targeted at root structure and function. In addition to the most obvious aspects such as structural support of the plant, uptake of water and nutrients, storage of food and nutrients, or vegetative reproduction, they are involved in phytohormone synthesis and regulation of plant growth. Furthermore, roots interact with fungi, to form mycorrhizas, and bacteria in root nodules to mention only the most intensively studied symbioses. Root–microbial interactions are often related to effective defense mechanisms directed against phytopathogenic agents (Chap. 17).

All these achievements were acquired during evolution within the last 400 million years, starting with the appearance of the first land plants. Considering this enormous period of time, it can be assumed that present-day roots are optimally adapted to their natural environments.

Nevertheless, there is growing interest in root engineering. In biology, root engineering is understood—in analogy to genetic engineering—as an alteration of root structure or function by means of genetic modification, but also breeding and manipulating root symbionts. In this way, it is hoped to adjust plants to man-made stress situations and rapidly changing environments or to adapt them to totally artificial conditions seen in root cultures to be used for pharmaceutical purposes and many other purposes, particularly in horticulture (Chap. 15). First attempts have been made with hairy root cultures transformed by *Agrobacterium rhizogenes*. Recent advances are reported in this book by the commercialization of hairy roots-based processes (Chap. 18). The identification of genetic markers for the selection of improved adventitious rooting performance will speed up this task. But an extension to different types of root culture is coming up only slowly.

The reason for slow progress in root engineering is lacking knowledge in crucial root functions, particularly at the molecular level. It is clear that without this understanding root engineering can be a futile undertaking. Therefore, an important goal of this book is to outline the latest achievements in structural aspects of root systems as well as physiological relations hoping that future efforts in root engineering will be based on a more solid ground. It is also recognized that pivotal steps

have to start at the level of interacting partners usually associated with roots, such as mycorrhizal fungi and/or bacteria.

Engineering of root system architecture is expected to support a second green revolution if crop performance under suboptimal water and nutrient supply is at stake (Chap. 3). Root growth models should help engineering of root architecture in order to fix trees in shallow soils, found in most urban landscapes (Chaps. 4 and 14).

It is hoped that this book is welcomed as an initiative for further efforts and investments in root engineering. Established scientists and academicians should enjoy the book for innovation in the future. Young scientists and students may find the book as a tool to undertake novel and new experiments to understand the functioning of diverse roots and for the welfare of mankind and at the same time sustain the environment.

München, Germany

Bertold Hock
Technische Universität München

Preface

Terrestrial root system is the most dynamic system in plants which regulates directly or indirectly the morphology, physiology, biochemistry, flowering, and the synthesis of secondary metabolites. It maintains a continuous conductivity link to stem and leaves. Any blockage in upward (xylem tissues) or downward (phloem) movement of water and solutes may damage the plants. Average scientists and plant biologists believe that it is an organ of anchorage into the soil and transportation of minerals and water. This is not true.

The prime aim and the objective of the book are to highlight the various essential roles of roots and their interaction with diverse microorganisms which are localized in the root system and/or in the vicinity, e.g., endophytes, rhizosphere (mycorrhizosphere), and non-rhizosphere. These are under the influence of root exudates (amino acids, sugars, and growth hormones). Microbial interaction has deep influence on plant growth, flowering, fruiting, production of secondary metabolites, and in combating biotic and abiotic stresses. In modern biology teachers and students are forgetting the role of root system which is subterranean, and the biomass is as enormous as that of aerial portion. They prevent soil erosion and play vital role in maintaining soil health.

The volume with 22 chapters, cleverly prepared by internationally recognized academicians, will serve and motivate the readers to value the root system and explore for better use of mankind and preservation of our ecosystem.

Murcia, Spain — Asunción Morte
Noida, India — Ajit Varma

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Chapter 1
Anatomy of Root from Eyes of a Microbiologist

Smriti Shrivastava, Ram Prasad, and Ajit Varma

1.1 Introduction

Microbial association with plants has various implications. Association with plant roots may be pathogenic, neutral, or beneficial. Bacteria and fungi of different strains show diverse method and site of attachment to the plant roots and rhizosphere. Soil microorganisms play an important role in soil fertility, e.g., the decomposition of organic matter entering the soil, increasing availability of minerals to plants, nitrogen fixation, production of phytostimulators, and many more. Few soil microbes are also pathogenic, causing damage to crops. In the present review, an intense study on specific attachment site and mode of various microbes studied has been reported. A major focus has been given on *Rhizobium* nodule formation and fungal mycorrhizae. Specific genes helping in microbial–plant root interaction have also been reported. Microbe–microbe communication and plant–plant microbe communication have been reviewed in details.

1.2 Soil Matrix and Rhizosphere

1.2.1 Soil Matrix

Soil is the surface layer of the earth that is exploited by roots. It is the natural medium for the growth of vegetation (including microorganisms) and has thickness that is determined by the depth of rooting of plants. Soil scientists generally agree that the soil comprises the first few feet of the earth's surface that influence and have been influenced by plant roots (exudates).

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The first scientific classification of soils was proposed in 1886 by the Russian scientist V.V. Dokuchaiev and was finalized in 1900. Dokuchaiev's work was translated from Russian into German in 1914 by Glinka and from German into English in 1927 by Marbut. It classified soils into normal (upland), transitional (meadow, calcareous, alkaline), and abnormal (organic, alluvial, aeolian). Soils have also been categorized into arid soils, dark-colored prairie soils, light-colored timbered soils, black swamp soils, and organic soils. At the same time that attempts were being made to sort soils into groups, the world of microorganisms was discovered (Hilgard 1911).

Soil matrix is the portion of a given soil that has the dominant color, or in other words the portion of that has more than 50 % similarity in color. Root-inhabiting bacterial communities and ribotype profiles are defined by soil type and host genotype, respectively (Bulgarelli et al. 2012).

Soil structure has lots of impact on soil fertility. Extensive uses of manure and fertilizers have deteriorated soil quality and created unfavorable conditions for beneficial soil microorganisms that may have important influence on the soil structure. Soil organic matter formed, decomposed, and transformed by microorganisms is of great importance to sustain soil fertility and soil structure (Hohl and Varma 2010).

1.2.2 Rhizosphere

The region of contact between root and soil is rhizosphere. This region is a cloud of microbes which literally surrounds plant roots and is vital for the plant's survival and growth. The term "rhizosphere" was coined by Lorenz Hiltner in 1904. Clark proposed the term "rhizoplane" for the external root surface and closely adhering particles of soil and debris. The influence of root exudates on the proliferation of soil microorganisms around and inside roots (Hartmann et al. 2008) and interactions between soil microorganisms, rhizosphere colonists, and plant hosts (Dennis et al. 2010; Friesen et al. 2011; Berendsen et al. 2012; Bacilio-Jimenez et al. 2003) has been widely studied. In rhizosphere, the microbial population differs both quantitatively and qualitatively from that in the soil. The term "mycorrhizosphere" was coined by Ajit Varma in 1995 (Varma and Hock 1995). As per the hypothesis, most of the plant roots are surrounded by mycorrhizae. Hence, it is appropriate to use the word mycorrhizosphere instead of rhizosphere.

Increasing demand of inorganic nutrients of plants is supported by wide variety of free-living and symbiotic nitrogen-fixing bacteria, as well as filamentous fungi. All these organisms are seen in abundance in mycorrhizosphere. Microbes are associated at root parts such as root hair, epidermis, cortex, vascular tissues, mycorrhizosphere, etc. As roots grow through soil, they release water-soluble compounds such as amino acids, sugars, and organic acids that supply food for

Fig. 1.1 Rhizospheric soil (The soil matrix is loaded with bacteria, actinomycetes and fungi, Normal garden soil, contains 10^{15} live organisms per g soil)

the microorganisms, and in return microorganisms provide nutrients to plants. These activities make mycorrhizosphere the most dynamic environment in soil. Mycorrhizospheres are differentiated from bulk soil due to the physical and chemical changes induced by roots moving through them (De Angelis et al. 2009) (Fig. 1.1). Interactions that are beneficial to agriculture include mycorrhizae, legume nodulation, and production of antimicrobial compounds that inhibit the growth of pathogens. Mycorrhizosphere microorganisms produce vitamins, antibiotics, plant hormones, and communication molecules that all encourage plant growth.

1.3 Root Anatomy

The root system is the entire belowground portion of the plant. Altitude of the root system is the longest single path through the root system. The architecture of root systems of a plant from dry habitat differs from those of wet habitat. Root of any plant is divided into three zones: meristematic zone, cell elongation zone, and cell maturation zone.

Plant roots live in firm teamwork with the surrounding microbes forming a unique self-regulating complex system. It defines the interface between a multicellular eukaryote and soil, one of the richest microbial ecosystems on earth (Tringe et al. 2005). Some mycorrhizosphere microorganisms leading to increase in crop yield are *Agrobacterium tumefaciens*, *Alcaligenes* spp., *Bacillus subtilis*, *Azospirillum brasilense*, *Pseudomonas* spp., *Tabaci* spp., *Trichoderma harzianum*, etc. (Sylvia et al. 2005). *Bacteroidetes* and *Actinobacteria* decreased in soil near the root tip compared to bulk soil but then increased in older root zones. Quantitative

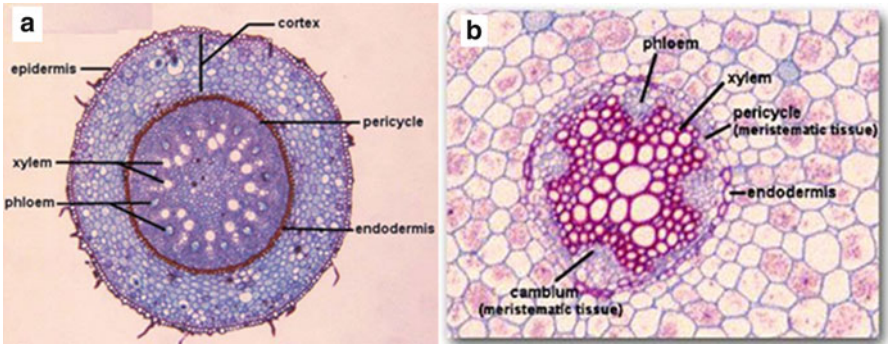

Fig. 1.2 Anatomy of typical (**a**) monocot and (**b**) dicot root

PCR have revealed mycorrhizosphere abundance of β-*Proteobacteria* and *Actinobacteria* (De Angelis et al. 2009).

1.3.1 Anatomy of Normal Root

Root is the part of a plant that attaches it to the ground or to a support, conveying water and nourishment to the rest of the plant. It typically lies below the surface of soil with aerial and aerating roots as exceptions. They may be broadly classified into dicot (primary tap roots) and monocot (fibrous root system) (Fig. 1.2).

1.3.2 Anatomy of Modified Roots

Some plants have fascinating root modifications with specific functions in addition to those of anchorage and absorption. Plant roots may also be present as specialized and modified roots (Fig. 1.3). Modified roots are of various types such as aerial roots, pneumatophores, contractile roots, parasitic roots, food storage roots, water storage roots, etc.

Minor modification in plant root could have important repercussions for soil microbial communities (Bressan et al. 2009).

1.4 Interaction of Bacteria with Plant Roots

Plant tissues interact with microbes with different degrees of dependence, and microbes range from plant beneficial (either nonsymbiotic or symbiotic) to pathogenic strains (Montesinos et al. 2002; Gewin 2010).

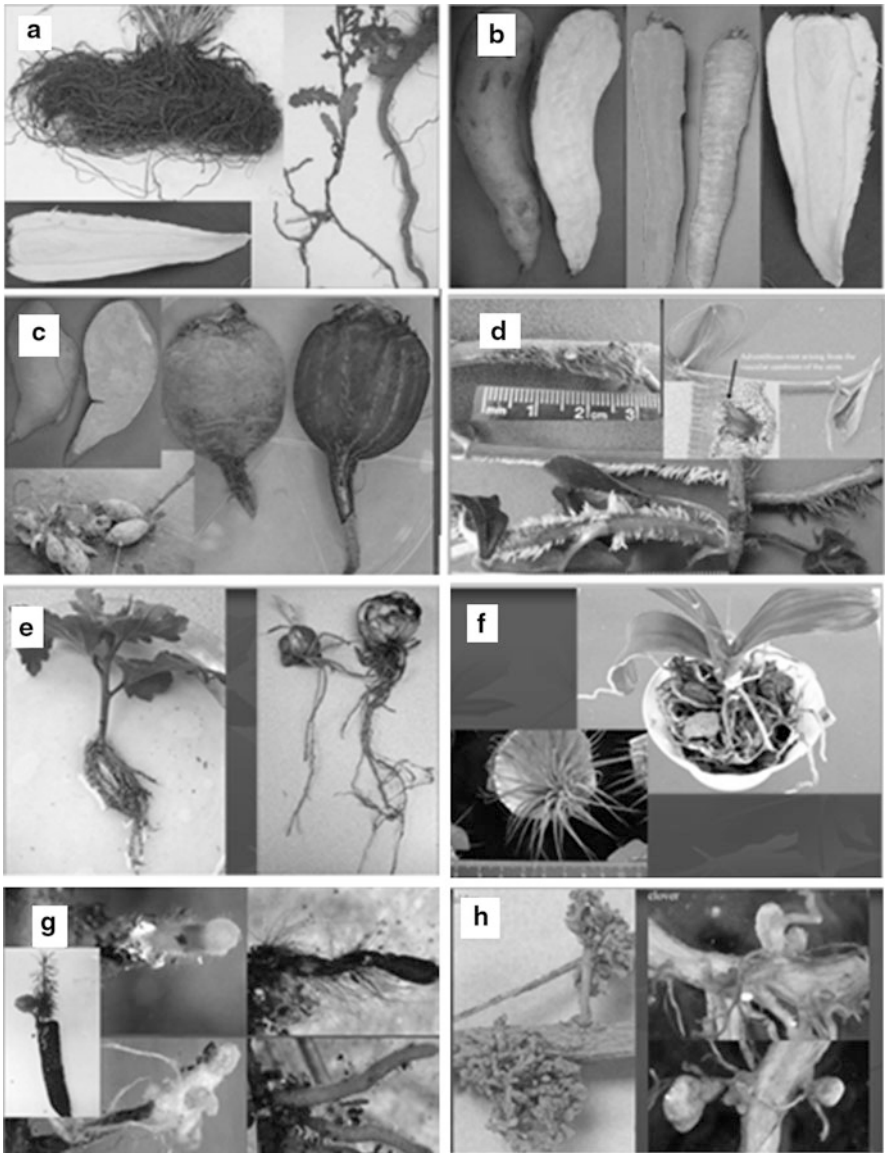

Fig. 1.3 Modified roots (**a**) Monocots fibrous roots and Dicot taproots (**b**) Storage root: Yam, carrot and radish (**c**) Tuberous root: *Dahlia*, beet root and sweet potato (**d**) Adventitious roots of *Hedera helix* (**e**, **f**) Aerial root: orchid and air plant (**g**) Ectomycorrhizae by fungi (**h**) Root nodules of Alder containing *Frankia* and Clover containing *Rhizobium*

The importance of soil bacteria in major plant nutrient cycles and relationship between plant roots and microbes was known in the nineteenth century. The importance of the whole plant microbiome (below- and aboveground, including

seeds) was demonstrated (Dennis et al. 2010; Friesen et al. 2011; Berendsen et al. 2012).

Pseudomonas, *Erwinia*, and *Xanthomonas* are general plant pathogenic bacteria that show hypersensitivity reaction (HR) and active pathogenicity (hrp) genes that control the capacity of bacteria to develop HR in nonhost plants (Montesinos et al. 2002). A complementary combination of avirulence gene (avr) in pathogen and resistant gene (R) in host plant triggers host defense mechanisms, and a noncomplementary combination of these genes results in infection (Baker et al. 1997; Flor 1955). The avirulence (avr) genes in bacteria code for most of the virulence-associated proteins (De Wit 1997). It has been found from few studies that plants can recognize pathogens (Chester 1933; De Wit 1997).

1.4.1 PGPR and BCA

Bacteria develop symbiotic, epiphytic, or endophytic association with plants. Nonsymbiotic bacteria are plant-growth-promoting rhizobacteria (PGPR) and biological control agents (BCA) (Montesinos et al. 2002). Soil bacteria multiply inside roots as benign endophytes and modulate plant growth and development (Hardoim et al. 2008), with implications ranging from enhanced crop productivity (Mei and Flinn 2010) to phytoremediation (Weyens et al. 2009). PGPR (*Pseudomonas*, *Serratia*, *Azospirillum*, *Bacillus*, etc.) inhabits mycorrhizosphere and enhances plant growth and limits growth of certain soilborne plant pathogens (Kloepper 1991). BCA are found either in the aerial plant part or in the root system and are colonized by an extremely abundant microbiota. Schulze-Lefert (2012) while studying *Arabidopsis* investigated root tissue, mycorrhizosphere, and unplanted soil in the surroundings of the test plants and reported that bacteria dominant in the root are *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Of these, *Actinobacteria* predominated in plant root (Schulze-Lefert 2012).

1.4.2 Endophytes

Endophytic colonization has been widely studied by microbe-associated molecular patterns (Rosenblueth and Martinez-Romero 2006), and structure of bacterial root endophyte has been determined by several other studies (Reinhold-Hurek and Hurek 2011). A comparative study of mycorrhizosphere and endophytic bacterial communities was done by Lundberg et al. (2012) and Bulgarelli et al. (2012) (Fig. 1.4). It was found that bacteria colonizing root surface enters the interior of the root and survives as endophytes. Roots of *Arabidopsis thaliana* are preferentially colonized by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Schulze-Lefert 2012). Among the root-inhabiting *Proteobacteria, Betaproteobacteria* are overrepresented in comparison to *Alphaproteobacteria* and *Gammaproteobacteria*

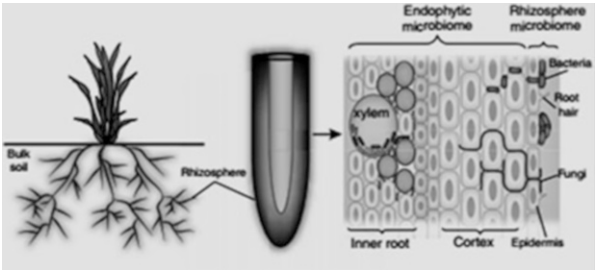

Fig. 1.4 Separating out root associated microbiomes for metagenomic analysis. The rhizosphere microbiome includes bacteria and fungi that are recruited from bulk soil and colonize the root surface. The endophytic microbiome includes species that infiltrate the rootcortex and live as endophytes until their release back into the soil upon root senescence [c.f. Bulgarelli et al. (2012)]

(Bulgaralli et al. 2012). Indigenous bacterial root endophytes show biocontrol activity against soilborne phytopathogens. *Pseudomonas fluorescens* PICF7 and *Pseudomonas putida* PICP2 are effective biocontrol agents (BCAs) against *Verticillium* wilt of olive (*Olea europaea* L.) caused by the fungus *Verticillium dahliae* Kleb. (Prieto et al. 2011). A study on difference in *Bacillus amyloliquefaciens* colonizing associated with *Zea mays*, *Arabidopsis thaliana*, and *Lemna minor* (Fig. 1.5) showed that *Bacillus* preferentially colonized root tips of *Arabidopsis* and fronds and roots of *Lemna* and was not seen in root tips of maize (Fan et al. 2011). *Corynebacterium flavescens* and *Bacillus pumilus* colonize region between epidermis and the mucilaginous layer of *Oryza sativa* (Fig. 1.6) (Bacilio-Jimeanez et al. 2001).

1.4.3 Bacteria and Root-Nodule Formation

Leguminous plants associate with microorganisms, ranging from largely nonspecific to very specific interactions. Plant-growth-promoting rhizobacteria (PGPR) helps in growth promotion and nutrient uptake and is an alternative source of N-fertilizer of nonleguminous crops (Mia et al. 2010) (Fig. 1.7). Studies have revealed that requirement of Nod factor for nitrogen fixation is facultative.

Flavonoids produced by the host plants induce rhizobial *nod* genes that lead to production of Nod factors. Infection thread passes the root cortex toward dividing cell that develops into root primordium for entry of microbes (Franche et al. 2009). Nodulation alters host developmental pathways (Desbrosses and Stougaard 2011). Example showing the mechanism of infection of *Glycine max* with *Rhizobium* has been illustrated in Fig. 1.8. Different stages of nodule formation of *Medicago truncatula* A17 and *Pisum sativum* cv. by *Sinorhizobium meliloti* have been illustrated in Fig. 1.9. During the process, the first root hair is deformed and curled followed by reactivating cell cycle in inner cortical cells. Bacteria then invade plant tissue through root hair infection (Haynes et al. 2004). In some cases, nodule

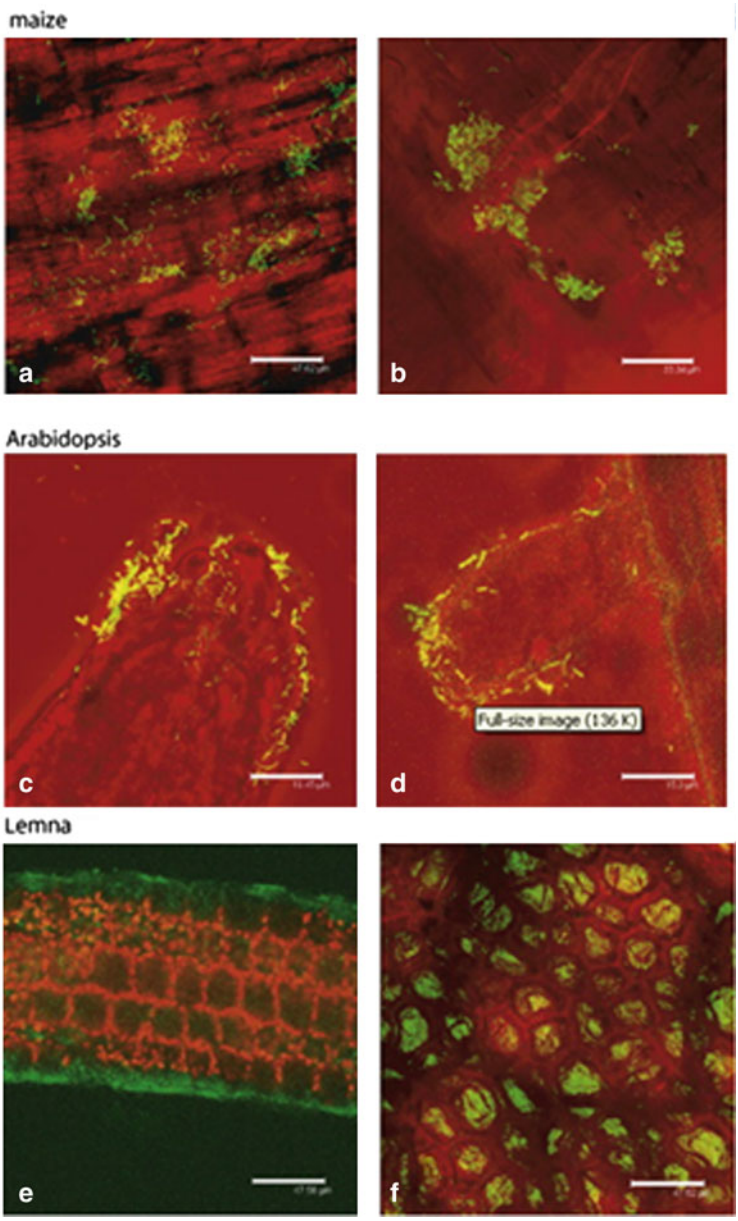

Fig. 1.5 Confocal Laser scanning Microscopy of BF01mut colonizing Plant Tissue (**a**) Surface of Maize roots, (**b**) Junction area, adjacent to emerging root hair of Maize root. The bacteria grew under root hair base, (**c**) *Arbidopsis* primary root tip, (**d**) *Arabidopsis* root hair, (**e**) *Lemna* root covered by FB01mut biofilm, (**f**) Lemna fronds root ventral side covered by FB01mut [c.f. Fan et al. (2011)]

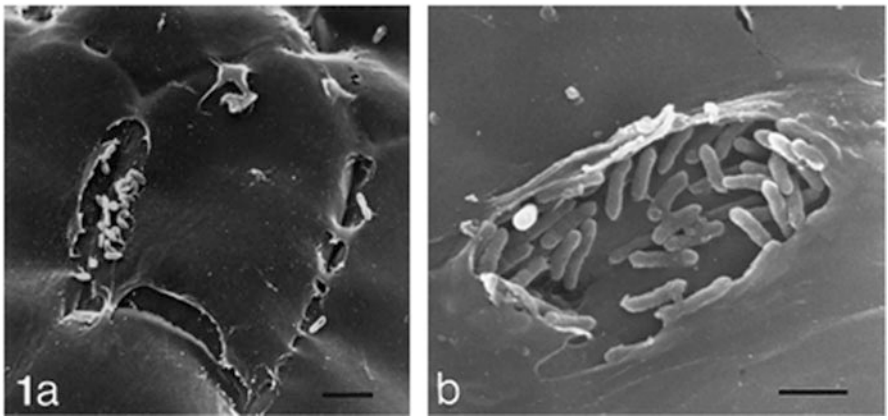

Fig. 1.6 Location of endophytic bacteria on the rice of non-inoculated plants. (**a**) Bacteria were observed in disrupted zones of the mucilage covering the grooves formed by the junctions among the epidermal cells. Bar = 5 μm, (**b**) high magnification of endophytic bacteria localized between the mucigel coat and the epidermis of secondary roots. Bar = 2 μm, SEM [c.f. Bacilio-Jimeanez et al. (2001)]

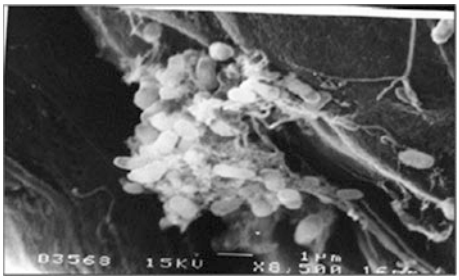

Fig. 1.7 Banana root surface colonization of plant growth promoting strain *Bacillus sphaericus* (strain UPMB10) isolated from oil palm roots [c.f. Mia et al. (2010)]

organogenesis is triggered by a calcium-dependent signaling pathway that leads to production of hormone cytokinin, which further leads to cortical cell division activating formation of nodule primordium (Fig. 1.10), (Oldroyd 2007).

Bradyrhizobium and *Casuarina glauca* could fix nitrogen without having Nod factor genes (Gewin 2010; Svistoonoff et al. 2003). This indicated the presence of other nitrogen fixation genes apart from the Nod factor.

1.5 Interaction of Fungi and Plant Roots

Similar to bacterial association with plants, fungi–plant association may be parasitic, neutral, or beneficial.

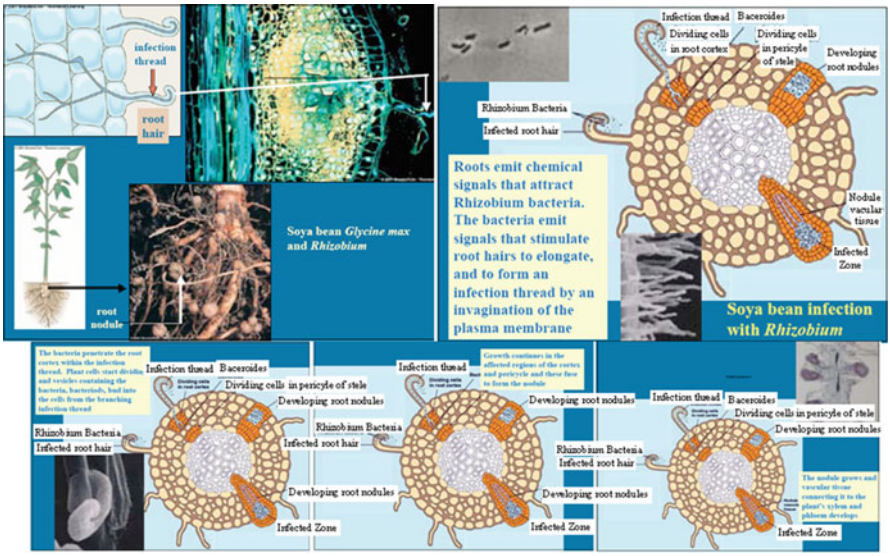

Fig. 1.8 Mechanism of infection of soyabean Glycine Max with *Rhizobium* [c.f. Desbrosses and Stougaard (2011)]

1.5.1 Mycorrhizae

Mycorrhizae (Greek words for fungus and root) are symbiotic relationship between a soil fungus and plant root. Mycorrhizal associations are omnipresent and nonselective and are seen in angiosperms and gymnosperms. They help in providing water and nutrients to plant, and the plant serves as carbon source. Mycorrhizal associations are broadly divided as ectomycorrhiza and endomycorrhiza. They can be differentiated depending upon their physical interface with plants (Fig. 1.11). Wherever mycorrhizae are formed, fungi are the dominant microbe in the region.

Ectomycorrhizae are generally seen in the roots of woody plants and form a dense hyphal covering over the root tip. From here, fungal hyphae grow into the intercellular spaces forming a net (Hartig's net) of hyphae around the root cortex cells but do not penetrate the cell walls. They are partially dependent on plants for growth. In endomycorrhizae, fungal hyphae grow into the root cortex and enter the cells forming fanlike, highly branched structure known as an arbuscule that remains separated from the cytoplasm by the plant plasma membrane (Harrison 2005). The endomycorrhiza can be further divided into the more widespread arbuscular mycorrhiza (AM) and the specialized orchid and ericoid mycorrhizas. The endomycorrhizae are wholly dependent on plants for their carbon (McNear 2013). Plant hormones (strigolactones) exuded by the plant roots stimulate fungal metabolism and branching and fungi release signaling molecules, "Myc factors," that trigger symbiotic root responses (Parniske 2008).

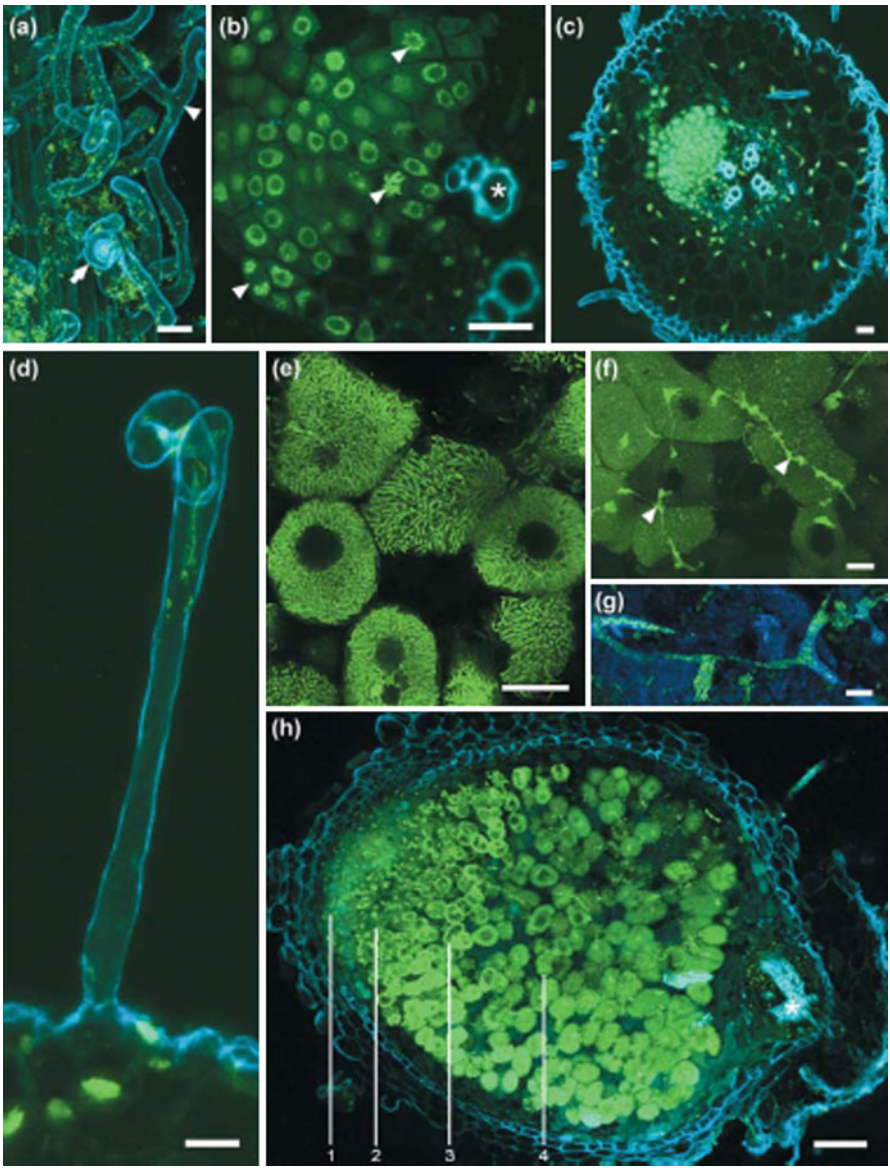

Fig. 1.9 Development of wild-type *Medicago truncatula* and *Pisum sativum* root nodules visualized using confocal microscopy. (**a**) *M. truncatula* A17 at 2 d post-inoculation (dpi) with *Sinorhizobium meliloti* 2011. Root hairs are branched (*arrowhead*) and curled (*arrow*). Bacterial cells are shown in *green*. Bar, 20 μm. (**b**) Nascent nodule meristem near the stele of a *Medicago* root at 2 dpi. *Asterisk*, vessel member; *arrowheads*, condensed chromatin in meristematic cells. Bar, 20 μm. (**c**) Cross-sectional view of *Medicago* root forming a nodule meristem (highlighted in *green*) at 2 dpi [c.f. Haynes et al. (2004)]. (**d**) High magnification image of curled root hair forming the classic "shepherd's crook" on *medicago* with an infection thread growing toward the root at 3 dpi. Bar, 20 μm. (**e**) High magnification image of *Medicago* nodule cells in the nitrogen-fixation zone at 9 dpi.

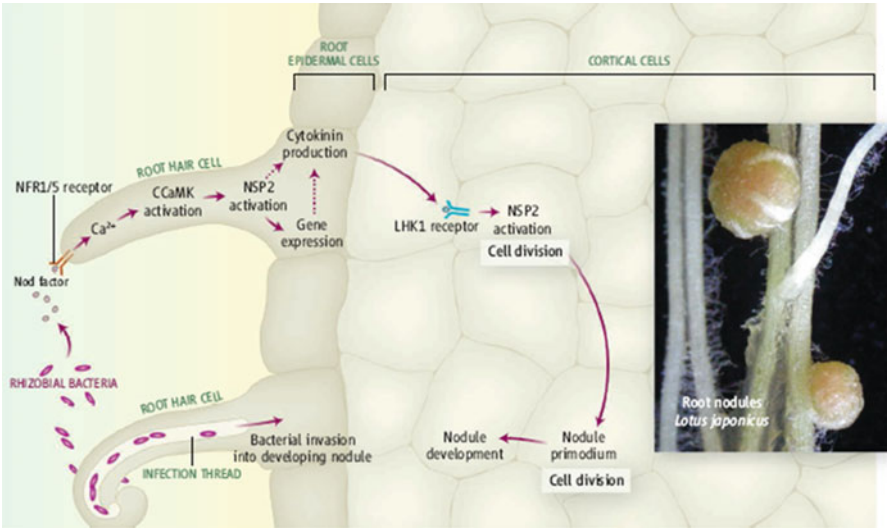

Fig. 1.10 Nodule organogenesis [c.f. Oldroyd (2007)]

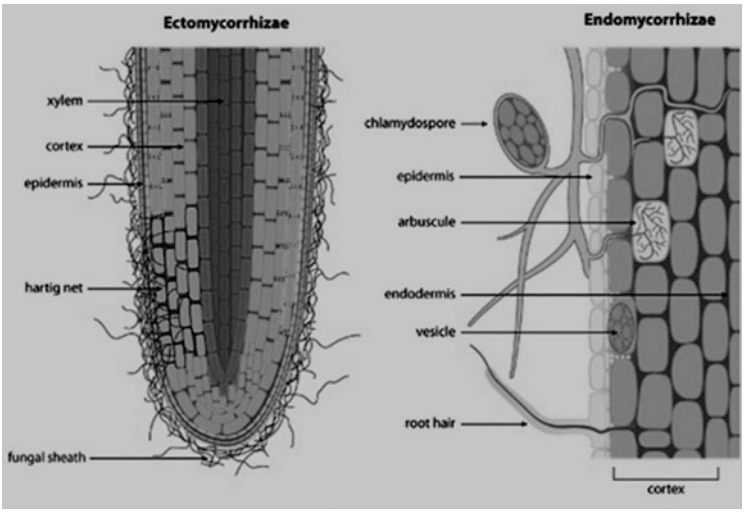

Fig. 1.11 Schematic showing the difference between ectomycorrhizae and endomycorrhizae colonization of plant roots. © 2013 Nature Education P. Bonfante and A. Genre. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nature communications* 1 doi:10.1038/ncomms1046

Fig. 1.9 (continued) Individual bacteroides are clearly visible. Bar, 20 µm. (**f**) Infected cells in the fixation zone of a mature *Medicago* nodule with remnant infection threads at 25 dpi. *Arrowheads*: Remnant infection threads. Bar, 20 µm. (**g**) High magnification image of a branching infection thread in a mature pea nodule at 31 dpi. (**h**) Longitudinal section of a mature *Medicago* root nodule at 20 dpi

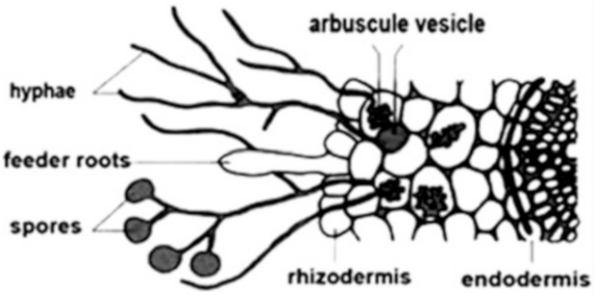

Fig. 1.12 Anatomy of root indicating the arbuscule, vesicle and spores. Note: the extra matrical hyphae in the soil and root cortex [c.f. Rootonic (2013)]

Colonization of *P. indica* (Fig. 1.12) in roots of different plants develops appressoria (Fig. 1.13a), and the roots are colonized intercellularly (Fig. 1.13b). The fungus also colonized cortex cells intracellularly, where it formed coils and branches (Fig. 1.13c) or round bodies (Fig. 1.13d), but a typical arbuscule such as that developed by the Glomales (Bonfante and Perotto 1995; Gianinazzi-pearson and Gianinazzi 1989) was not observed. The round bodies might function as storage organs (Walker 1995). *Piriformospora indica* does not invade the stelar tissue and does not traverse upward into the shoot.

Penetration of AM fungi through outermost root tissue of host plant requires early nodulin gene for formation of pre-penetration apparatus (Genre et al. 2005).

1.6 Algae in Plant Roots

Symbiotic association between cycads and filamentous cyanobacteria is established through specialized lateral roots "coralloid roots." Coralloid root is dichotomously branched, is greenish brown in color, and contains an algal (*Nostoc* and *Anabaena*) zone in the cortex. The cyanobacteria are present in a specific cortical layer inside the root, the so-called cyanobacterial zone. Cyanobacteria have heterotrophic mode of carbon nutrition due to their localization in dark coralloid roots.

Symbiosis between cycads and cyanobacteria results in mutualistic relation that is beneficial to biosphere (Fig. 1.14) (Vessey et al. 2004). Cycads have been widely studied by Lindblad (2009).

1.7 Communication Between Plant Roots and Microorganism

Root–root and root–microbe communications are continuous phenomenon in biologically active soil zone. Bais et al. (2004) reported that root exudates might initiate and manipulate biological and physical interactions between roots and soil organisms and thus play an active role in root–root and root–microbe communication. Root microbial interaction in mycorrhizosphere is influenced by edaphic factors and is more complex as compared to those interactions on soil surface (Mc Cully 1999) (Fig. 1.15).

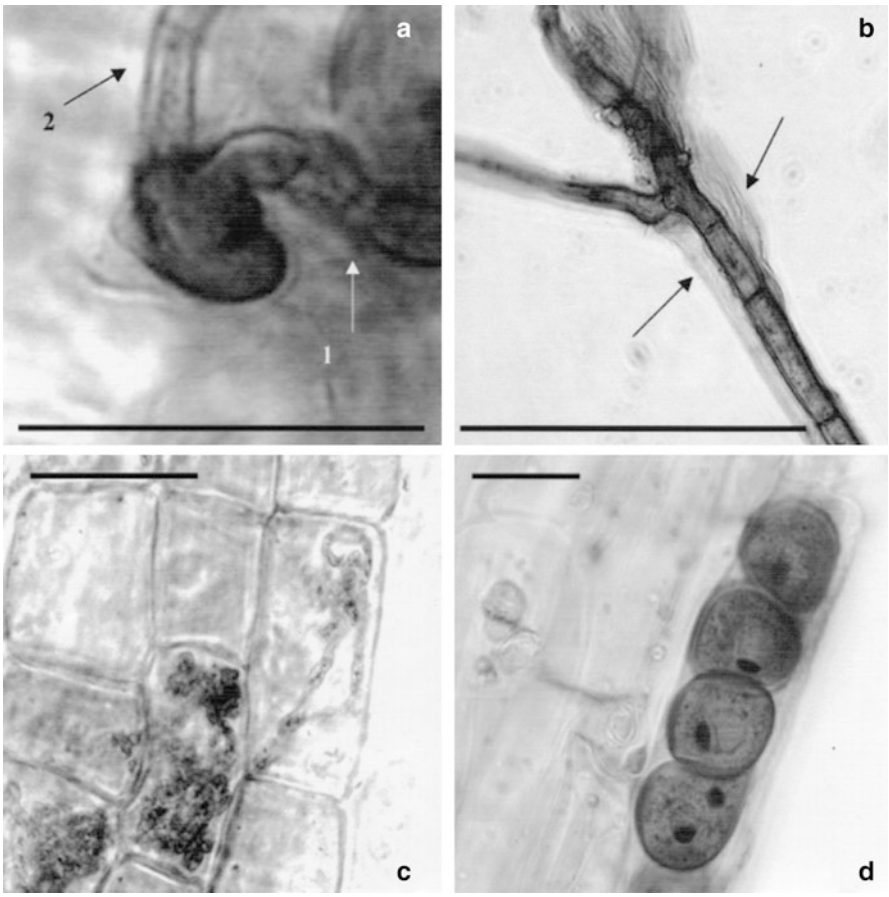

Fig. 1.13 Plant colonization by *P. indica*. Maize plants were inoculated with *P. indica*. Roots were harvested 4 (**a**), 7 (**b**), and 16 (**c, d**) days after inoculation, stained, and observed by light microscopy. Bars, 10 mm. (**a**) A fungal appressorium attached to the root surface. *Arrow 1* indicates a hypha leading to the appressorium; *arrow 2* indicates a hypha leading away from the appressorium into the plant. (**b**) A root segment showing intercellular hyphae. *Arrows* indicate plant cell walls. (**c**) Cortical cells with coiled and branched intracellular hyphae. (**d**) Cortical cells showing round bodies

1.7.1 Root Exudates

Root exudates include secretions (mucilages, etc., actively released from the root), diffuses (passively released due to osmotic differences between soil solution and cell), and lysates (released from autolysis of epidermal and cortical cells) (Bais et al. 2004). Root secretions mediate multipartite interactions in mycorrhizosphere that causes immediate alteration in environment (Badri and Vivanco 2009). They reported that root exudates serve as signals that initiate symbiosis with rhizobial and

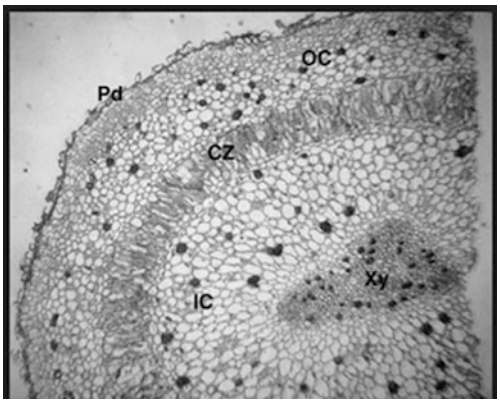

Fig. 1.14 Cross section of coralloid root showing cyanobacterial zone (CZ), triarch xylem (Xy), inner (IC) and outer cortex, and periderm (Pd). (c.f. Reference; courtesy: http://plantnet.rbgsyd.nsw.gov.au)

mycorrhizal fungi. A chemotactic response toward root secretions is the first step in root colonization (Zheng and Sinclair 1996). Plant root secretion is a passive process mediated through three separate pathways: diffusion, ion channels, and vesicle transport (Neumann and Romheld 2000; Bertin et al. 2003). Plant microbe relationships are influenced by soluble and non-soluble root exudates, border cells, and large polysaccharide layer surrounding roots (Hawes et al. 2000; Vermeer and Mc Cully 1982).

Various studies have focused on the effect of root exudates on plant microbial interactions. *Pseudomonas fluorescens* colonize tomato roots by chemotaxis driven by root exudates (De Weert et al. 2002). Increase in chemotaxis of endophytic bacteria has been observed in the presence of root exudates in rice (Bacilio-Jimenez et al. 2003). Isoflavonoids and flavonoids present in the root exudates of leguminous plants activate the *Rhizobium* genes responsible for the nodulation process, and they may also be responsible for arbuscular mycorrhiza colonization (Trieu et al. 1997; Peters et al. 1986; Becard et al. 1995; Phillips 2000; Mithofer 2002).

Apart from enhancing root–microbial interaction, root exudates also attribute to mechanical functions like maintenance of root–soil contact, lubrication of the root tip, protection of roots from desiccation, stabilization of soil microaggregates, and selective adsorption and storage of ions (Hawes et al. 2000; Griffin et al. 1976; Rougier 1981; Bengough and Mc Kenzie 1997). Root exudates help in adding phosphorus to phosphorus-deficient soil (Marschner 1995) with the help of acid phosphatases (ATases) (Raghothama 1999; Duff et al. 1994; Ascencio 1997) in form of soluble orthophosphate anions (Goldstein et al. 1987). Different carbon and nitrogen fluxes also operate in the mycorrhizosphere (Jone et al. 2009).

Electric signals can augment chemotaxis in mediating short-range tactic responses of oomycete zoospores at root surface (Van West et al. 2002).

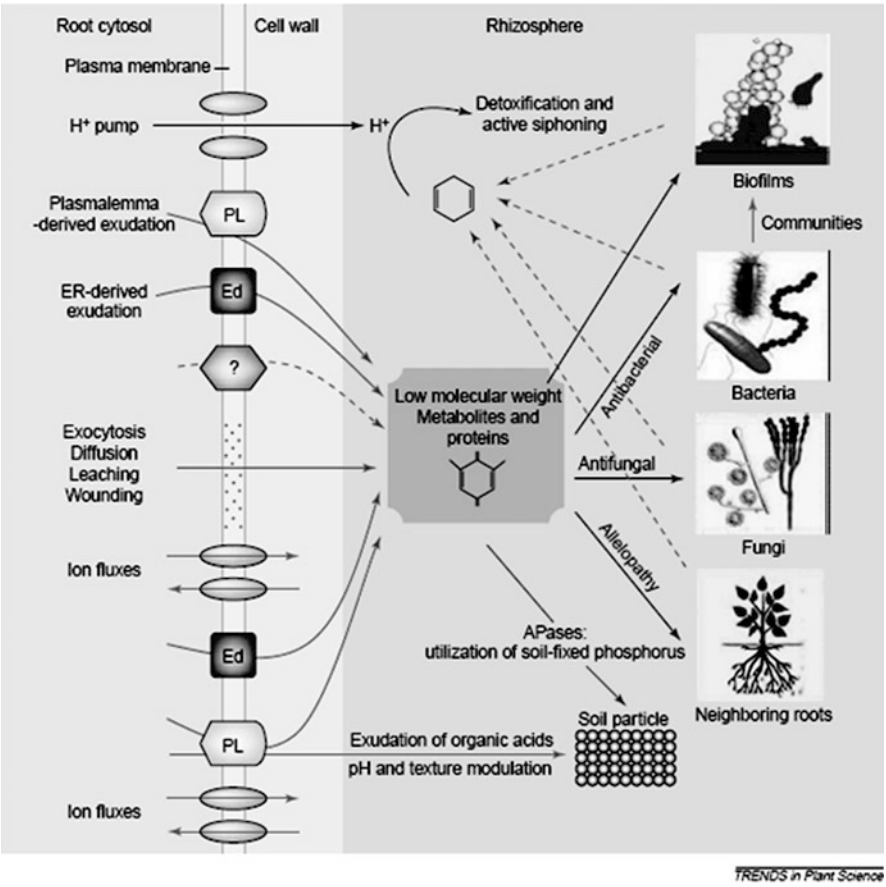

Fig. 1.15 Model showing plausible mechanisms of root exudation and active rhizospheric interactions. The hexagon component in the detoxification process depicts the low molecular weight toxins produced by bacteria and fungi during the pathogen attack. Plant roots adopt a proton (Hþ)-pumping mechanism to exclude the phytotoxins produced by bacteria and fungi. Broken arrow from biofilm, fungi, bacteria and neighboring roots depicts pathogen attack against the plant. All solid arrows shows response of host plant root to pathogen attack. Broken arrow from plasma membrane represents an unknown mode of root exudation and host plant response against pathogen attack. On the *right*, the biofilm panel depicts bacterial communities that are much more resistant to plant derived antimicrobials 3 than planktonic bacteria are. *PL* plasmalemma-derived exudation, *Ed* endoplasmicderived exudation [c.f. Bais et al. (2004)]

1.7.2 Root Exudates and Antimicrobial Activity

Root exudates also act as antimicrobials against mycorhizospheric microflora (Dixon 2001). The root-localized secondary metabolites are used for defense purposes (e.g., isoflavonoids in the *Leguminosae* and sesquiterpenes in the *Solanaceae*), by regular plant families (Flores et al. 1999; Dixon 2001). These

secondary metabolites have broad-spectrum activity (Van Etten et al. 1994; Bouarab et al. 2002; Flores et al. 1999; Dixon 2001). *Arabidopsis*, rice, corn, soybean, and the model legume *Medicago truncatula* are rich sources of the root-derived antimicrobials indole, terpenoid, benzoxazinone, flavonoid, and isoflavonoid (Dixon 2001).

1.8 Conclusions

Contribution of mycorrhizosphere bacteria to disease resistance and nutrient cycling in the plant system is well established. These studies can be applied for crop development and lead to approach for optimizing plant health, nutrition, and yield. Entophytes reside in various structural parts of plant root, and they play extremely important role in plant growth enhancement quantitatively as well as qualitatively. Though already studied, there is still vast scope for studying novel endophytes and exploring their utilization in agriculture and enhancing soil quality.

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Chapter 2
Role of *Phi* Cells Under Abiotic Stress in Plants

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2.1 Introduction

2.1.1 Phi Thickening Definition, Description and Discovery

The definition of *phi* thickening is not always present in the most consulted texts on plant anatomy. In many, the definition is not complete or is poorly defined. The last edition of the famous book on plant anatomy 'Esau's Plant Anatomy 3rd edition' defined *phi* thickening as '*Phi* thickenings are reticulate or band-like wall thickenings on cortical cells of certain gymnosperms (Ginkgoaceae, Araucariaceae, Taxaceae, and Cupressaceae (Gerrath et al. 2002)) and a few species of angiosperms such as *Ceratonia siliqua*, *Pyrus malus* (*Malus domestica*), and *Pelargonium hortorum* (Peterson et al. 1981)'. The reading of this definition suggests that *phi* thickening is an exception in the root anatomy. However, we have found in the literature 16 different families, covering more than 100 species, which present the *phi* thickening in the roots. All species described presenting *phi* thickenings are dicotyledons. However, there is one exception, the presence of *phi* thickenings in the rhizodermis of *Zea mays* (a monocotyledon) treated with a solid-waste slag. In Table 2.1, we show the different families and species that present *phi* thickenings in the roots. Some of these species are also very relevant for agriculture, e.g. *Prunus avium*, *Prunus dulcis*, *M. domestica*, *Pyrus communis*, *Brassica oleracea*, *Brassica napus*, *Raphnus sativus*, *Sinapis alba*, *Alnus glutinosa*, etc.

The *phi* thickening was first described by Van Tieghem in 1871 in roots of *Taxus baccata* (see Fig. 2.1). Van Tieghem called this cell wall thickening '*reseau sus-endodermique*'. However, the name '*phi* thickening' was first proposed by Russow in 1875, who commented that the transverse sections of the thick walls

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Table 2.1 List of families and species showing *phi* thickenings in the roots

Gymnosperms	Angiosperms	Angiosperms
Cycadaceae	***Rosaceae***	*Koniga* Van Tieghem (1888)
Cycas circinalis (Van Tieghem 1888)	*Prunus avium* L. (Soukup et al. 2004)	*Farsetia*
Fossil: Antarcticycas (Millay et al. 1987); *Radiculites type* (Strullu-Derrien et al. 2009)	*Prunus dulcis* × *P. persica* (Marin et al. 2009)	*Berteroa*
Ginkgoaceae	*Eriobotrya japonica Lindl.* (Nii et al. 2004; Pan et al. 2006)	*Vesicaria*
Ginkgo biloba (Gerrath et al. 2002)	*Malus domestica* (Mackenzie 1979; Weerdenburg and Peterson 1983)	*Hirschfeldia*
Taxaceae	*Pyrus communis* (Essau 1943)	*Thlaspi*
Taxus cuspidata (Gerrath et al. 2002), *T. baccata* (Van Tieghem 1871; Von Guttenberg 1961)	*Dryas integrifolia Vahl.* (Melville et al. 1987)	*Clypeola*
Torreya nucifera (Van Tieghem 1888)	***Fabaceae***	*Isatis*
Cupressaceae	*Ceratonia siliqua* L. (Pratikakis et al. 1998; Rhizopoulou 2004)	*Crambe*
Callitris quadrivalvis (Noelle 1910)	*Arachis hypogaea* (Tajima et al. 2008)	*Enarthrocarpus*
Chamaecyparis lawsoniana, C. obtuse, C. pisifera, C. sphaeroidea (Noelle 1910)	*Caesalpinia peltophoroides* (Henrique et al. 2010)	*Erucaria*
Cryptomeria japonica (Gerrath et al. 2002; Noelle 1910)	*Hedysarum pedicellare* (Van Tieghem 1888)	***Adoxaceae***
Cunninghamia lanceolata (Gerrath et al. 2002), *C. sinensis* (Noelle 1910)	***Geraniaceae***	*Viburnum* sp. (Schwendener 1874)
Cupressus macrocarpa, C. sempervirens (Noelle 1910)	*Pelargonium hortorum Bailey* (Haas et al. 1976; Peterson et al. 1981)	***Sapindaceae***
Juniperus virginiana (Gerrath et al. 2002), *J. communis, J. chinensis, J. excelsa, J. nana, J. sabina* (Noelle 1910)	*Geranium* sp. (Van Tieghem 1888)	*Sapindus saponaria* (Van Tieghem 1888)
Libocedrus decurrens (Noelle 1910; Wilcox 1962)	***Brassicaceae***	*Nephelium longana* (Van Tieghem 1888)
Sciadopitys verticillata (Noelle 1910)	*Thlaspi caerulescens* (Broadley et al. 2007; Zelko et al. 2008)	*Talisia* sp. (Van Tieghem 1888)
Sequoiadendron gigantea (Gerrath et al. 2002), *S. sempervirens* (Noelle 1910; Von Guttenberg 1961)	*Brassica oleracea* (Fernandez-Garcia et al. 2009)	***Berberidaceae***
Taxodium distichum (Noelle 1910)	*B. napus* (Enstone et al. 2003), *Brassica carinata, B. nigra* (Van Tieghem 1888)	*Mahonia aquifolium* (Van Tieghem 1888)
Thuja occidentalis (Gerrath et al. 2002; Van Tieghem 1888; Brundett et al. 1990), *T. orientalis, T. plicata, T. standishii* (Noelle 1910)	*Sinapis alba, S. allionii, S. hispida, S. dissecta, S. pubescens, S. abyssinica, S. laevigata, S. geniculata* (Van Tieghem 1888)	***Betulaceae***
	Cheiranthus cheiri (Van Tieghem 1888)	*Alnus glutinosa* (Massicotte et al. 1999)
	Lepidium sativum (Van	*Betula alleghaniensis* (Massicotte et al. 1988)
		Fagaceae
		Quercus coccifera L. (Christodoulakis and Psaras 1988)
		Orchidaceae
		Prosthechea alagoensis, P. bulbosa, P. caetensis, P. faresiana, P. fragrans, P. vespa (Pires et al. 2003)
		Encyclia alboxanthina, E. amicta, E. dichroma, E. linearifolioides, E. longifolia, E. megalantha, E. randii (Pires et al. 2003)
		Epidendrum crassifolium (Pires et al. 2003)
		Oncidium varicosum (Pires et al. 2003)
		Scrophulariaceae
		Bacopa salzmannii,

(continued)

Table 2.1 (continued)

Gymnosperms	Angiosperms	Angiosperms
Thujopsis dolabrata (Noelle 1910)	Tieghem 1888)	*B. monnierioides* (Bona and Morretes 2003)
Araucariaceae	*Alliaria officinalis* (Van Tieghem 1888)	
Araucaria heterophylla (Gerrath et al. 2002; Van Tieghem 1888), *A. brasiliana, A. excelsa, A. imbricada, A. cunninghamii* (Noelle 1910)	*Raphanus sativum, R landra* (Van Tieghem 1888)	
	Malcolmia intermedia, M. Africana, M. chia (Van Tieghem 1888)	
	Sisymbrium hirsutum, S. bursifolium, S. binerve, S. sophia (Van Tieghem 1888)	
	Cochlearia armoracia (Van Tieghem 1888)	
	Alyssum saxatile (Van Tieghem 1888)	

resembled the Greek letter *phi*. Van Tieghem (1888) classified them into three types based on their root cell location: type I, the most frequently found, *phi* cell layer is located in contact with the endodermis. *Phi* thickening could be observed in one or two cell layers, depending on the species and/or location in the root. Type II *phi* cell layer is located in contact with the epidermis and is frequently formed by only one cell layer. Type III *phi* cell layers are located in the inner cortical cells, but not in contact with either the epidermis or the endodermis; they can be composed of one or more *phi* cell layers. In general, we consider that this classification is up to date and can be used to describe the different locations of *phi* thickening in the root.

What is the origin of the cell wall thickenings? The occurrence of cell wall thickening in the radial cell walls of the cortical cells has been observed in fossil plants from the upper Triassic, Paleozoic and upper carboniferous periods (Millay et al. 1987; Strullu-Derrien et al. 2009; Cesari et al. 2012). Strullu-Derrien et al. (2009) observed in rootlets of the Radiculites type in Radiculites (upper carboniferous flora; samples were obtained from Grand Croix, France) the undoubted presence of *phi* thickenings. Similarly, Millay et al. (1987) have described the presence of *phi* thickenings in roots of the fossil family Antarcticycas. These fossils show that in young roots a single cell layer with *phi* thickenings is present in both the tangential and the radial walls and in direct contact with the endodermis. Mature primary roots show well-developed *phi* thickenings that may occupy about 25 % of the walls. These interesting findings in fossil plants demonstrate that *phi* thickening was present in the ancestors of the present flora.

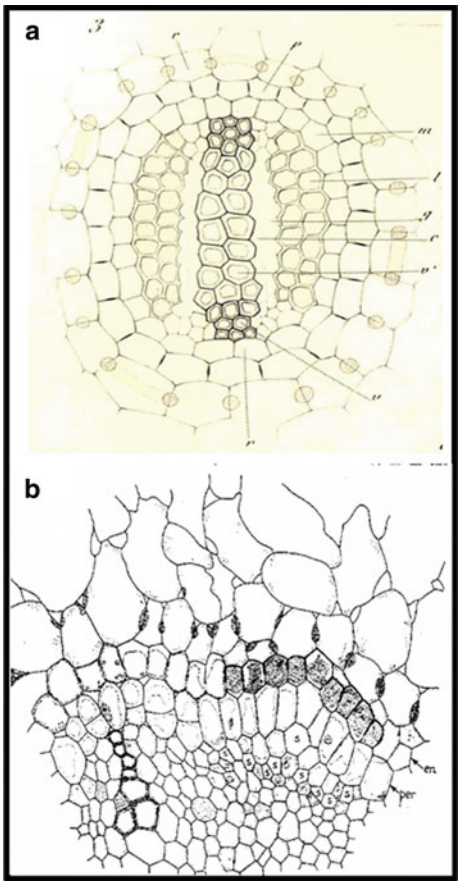

Fig. 2.1 (**a**) Original picture of root section of *Taxus baccata* showing the first description of *phi* thickening [Memoire sur la racine, Van Tieghem (1871)]. (**b**) Picture of transverse section of *Pyrus communis* root showing *phi* thickening [Vascular differentiation in the pear root, Essau (1943)]

2.2 *Phi* Cell Ultrastructure and Chemical Composition of *Phi* Thickening

The ultrastructure of the *phi* cells has been poorly described in the literature. To our knowledge, the first ultrastructural description of *phi* thickening was published by Haas et al. (1976) in *Pelargonium hortorum*. The *phi* cells presented abundant microtubules parallel to the long axis of the *phi* thickenings. In some cases, the authors observed that *phi* thickenings of adjacent cells are occasionally misaligned to some extent. In this work, the authors observed that plasmodesmata were absent in the thicker region of the *phi* thickenings but were observed in their periphery. The histochemical analysis demonstrated that the *phi* thickenings were intensively lignified in matured cells.

Recently, Fernandez-Garcia et al. (2009) have described the ultrastructure of *phi* cells in *Brassica oleracea*. In *Brassica oleracea*, the *phi* thickening is type I and can be composed of one or, less frequently, two cell layers (see Fig. 2.2). The *phi*

thickenings are present in the radial cell walls, and between two adjacent cells, they are frequently aligned, but in some cases, similarly to *Pelargonium*, they can also be misaligned. The ultrastructural study of the radial walls of the immature *phi* cells showed that these cell walls were rich in plasmodesmata connecting two neighbouring immature *phi* cells. Mature cells presented well-developed *phi* thickenings, and in many cases they were traversed by plasmodesmata. In some cases, the middle portion of the thickening seemed to be blocked, although it is highly probable that the plasmodesmata passed out of the plane of sectioning, as has been described in the literature. The *phi* cells of *Brassica oleracea* showed several cell wall ingrowths present only in the inner tangential cell walls (see Fig. 2.2). These cell wall ingrowths were never observed in the outer tangential cell walls of *phi* cells. In general, the cell wall ingrowths in the inner tangential cell walls were smaller in size compared with the *phi* thickenings (about 0.5 μm at the base and 0.5–1.0 μm long). The physiological role of these cell wall ingrowths is unknown. Fernandez-Garcia et al. (2009) have proposed on the basis of the differential distribution of the cell wall ingrowths, observed only in the inner tangential cell walls, that *phi* cells might present a possible polarity. Moreover, the increased number of the cell wall ingrowths supposes a significant increment of the plasmalemma in the inner cell wall of the *phi* cells. This may indicate different roles for the two cell walls. The authors suggest that, across the plasmalemma of the outer tangential cell walls, the *phi* cells can probably take up cations and anions actively for accumulation in the vacuoles, whilst the inner tangential wall can regulate the excretion of cations and anions to the apoplast between the *phi* cells and the endodermis.

Phi thickenings are structures of the secondary cell walls and could be lignified. Therefore, cell walls of *phi* cells may contain lignin and/or suberin. The chemical composition of *phi* thickening has been analysed using specific dyes for cell wall components, such as phloroglucinol for lignin or Sudan III for suberins. Some authors have observed that *phi* thickenings have abundant content of lignins (Haas et al. 1976; Soukup et al. 2004) and can be significantly lignified after environmental stresses (Fernandez-Garcia et al. 2009; Henrique et al. 2010). Suberin is typically found in the endodermal cells, but has not been found in the *phi* thickenings.

2.3 The *Phi* Thickening Is Altered by the Environment

Since its discovery, the function of *phi* thickening has been the subject of much speculation and experimentation, but it remains unclear. Van Tieghem (1888) proposed that the *phi* thickening can provide a mechanical support for the root. Although Weerdenburg and Peterson (1983) assumed that *phi* thickenings function primarily as supportive structures in the root cortex, they did not specify what force this mechanical support might oppose. Mackenzie (1979) suggested that they may act in a manner similar to a Casparian band.

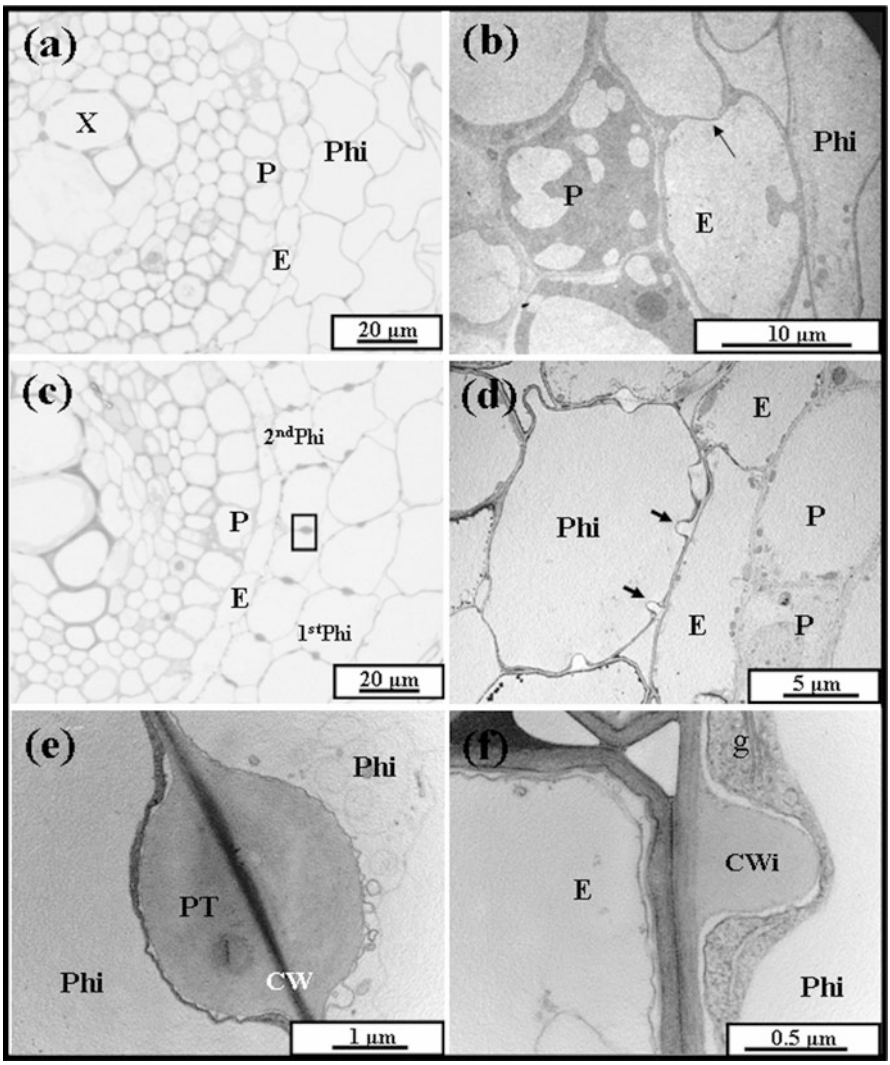

Fig. 2.2 (**a**) Semi-thin section of a control root of *Brassica oleracea*; *phi* cells are present in the innermost layer of the cortex (*Phi*). (**b**) Electron micrograph of a control root showing the endodermis and phi cell. (**c**) Semi-thin section of salt-treated root of *Brassica oleracea*; the *phi* cells are the two cortical cell layers adjacent to the endodermis. *Phi* thickenings are present in two cell layers in the radial cell walls. (**d**) Electron micrograph of salt-treated root showing the endodermis and *phi* cell (*arrows* indicate the cell wall ingrowths). (**e**) Electron micrograph of (**d**) showing a detail of *phi* thickening. (**f**) Electron micrograph of a tangential wall between a *phi* cell and endodermal cell showing a cell wall ingrowth. *CW* cell wall, *CWi* cell wall ingrowth, *E* endodermis, *P* pericycle cell, *Phi phi* cell, *PT phi* thickening. These images are a selection of Figs. 1, 2 and 6 from Fernandez-Garcia et al. (2009)

In the last decade, new studies have demonstrated that *phi* thickening can be altered by the environmental conditions. Different abiotic and biotic stresses may be modifying the distribution, structure and/or development of the *phi* thickening.

2.3.1 Solute Movement: Salt Stress

The solute movement in the roots can be via apoplast or symplast and is affected by the anatomy and the developmental stage of the roots (Enstone et al. 2003). The apoplast movement of solutes has been the most studied mechanism in roots. Therefore, one of the first interesting questions about the role of *phi* thickening was whether it was an apoplastic barrier for solute movement. Wilcox (1962) had shown that, in *Libocedrus decurrens*, the *phi* cell layer is not suberised, considering it unlikely that it would affect cell permeability. Peterson et al. (1981) have analysed the solute movement in the roots of *Pyrus* and *Pelargonium* using a brightener dye, Tinopal CBS-X. In these experiments, they showed that the apoplastic fluorescent dye was able to cross the *phi* thickenings but not the endodermis. But this experiment cannot exclude the possibility that *phi* thickenings could be reducing the rate of water and solute transport through the cell walls of the *phi* cell layers.

When *Brassica oleracea* is grown hydroponically, the *phi* cell layer is poorly developed, and *phi* thickenings are difficult to observe. However, when the plants were grown in NaCl, the *phi* thickenings were induced and lignified (Fernandez-Garcia et al. 2009; see Fig. 2.2). Interestingly, the authors demonstrated that the *phi* thickening may be acting at least as a partial barrier for ion movement from the cortex to the pericycle when *Brassica oleracea* plants were salt stressed. For this study, the authors followed a transmission electron microscopy technique and lanthanum ions that are known to be excellent apoplastic markers, and they have been used successfully to mark the pathway of ion movement across the apoplast of roots and leaves. In *Brassica oleracea* roots, La^{3+} can move freely in the apoplast of cortical cells, but in *phi* cells the La^{3+} movement was restricted to the periplasmatic apoplast of the *phi* radial cell wall thickenings. The authors suggested that the lignification of the *phi* thickening could prevent the movement of La^{3+} through the *phi* thickening. The greater deposition of La^{3+} in the apoplast between *phi* cells and endodermis of control roots, compared with salt-treated roots, could be due to the reduced presence of the *phi* thickenings in control roots. However, the deposition of La^{3+} in the apoplast between the *phi* cells and the endodermis of salt-treated roots indicates that the *phi* thickening is not a complete diffusion barrier for ions.

The symplastic movement of solutes is mediated by plasmodesmata between the different cell layers of the roots. The presence and distribution of plasmodesmata are critical for solute and ion movements via symplastic flux. Mackenzie (1979) observed a higher number of plasmodesmata in the outer tangential cell walls of the *phi* cells than between the *phi* layer and endodermis. He interpreted this as meaning that the *phi* cells can play a physiological role, in the accumulation of nutrients.

Fernandez-Garcia et al. (2009) have analysed the distribution and frequency of plasmodesmata in control and salt-treated plants of *Brassica oleracea*. The authors observed that plasmodesmata are scarce in the outer tangential walls of the *phi* cells or between the *phi* cell layer and endodermis. However, radial cell walls of *phi* cells showed several plasmodesmata connecting the two adjacent *phi* cells in control plants. But, when *phi* thickenings were observed in salt-treated plants, the majority of the plasmodesmata from the radial cell walls were occluded by the new cell wall formed. The authors suggested that the lack of plasmodesmata in the tangential cell walls of the *phi* cells clearly indicates that the symplastic flow via plasmodesmata is abolished. However, the radial flow between *phi* cells is possible in control plants but should be highly reduced in salt-treated plants due to the very low number of functional plasmodesmata observed. If *phi* cell layers are acting as a physiological barrier for ion movement, it can be expected retention of ions in these cell layers. Using X-ray microanalysis coupled to a scanning electron microscope, the authors demonstrated that the *phi* cell layer was a barrier when plants were grown with NaCl; the *phi* cell layer showed a significative build-up of Na^+ and Cl^- (Fernandez-Garcia et al. 2009, see Fig. 2.3).

2.3.2 Water Logging and Soil Compaction

Gerrath et al. (2005) have developed different experiments to demonstrate whether *phi* thickenings can be altered and/or induced by the environment. For these experiments, they used three different gymnosperms, *Cryptomeria japonica*, which presents *phi* thickenings, and two *Pinus* species, *P. aristata* Engel. and *P. rigida* Mill. whose *phi* thickenings were absents. Plants were subjected to water logging and soil compaction to observe the effect of the environment in the roots. The first conclusion of these experiments was that the presence or absence of *phi* thickening cannot be induced by the environment. The two *Pinus* species never developed *phi* thickenings in the different ambient conditions. For *C. japonica*, the area of *phi* thickenings relative to the total root area was reduced under waterlogged and compacted soil (see Fig. 2.4). However, there was no significant interaction between the effects of water logging and soil compaction, and they were independent of the maturity of the root. These authors suggest that the reduction of *phi* thickening density in waterlogged and compacted soil might correlate with a lower plant growth and a general reduction in plant vigour. The authors have also analysed the distribution of *phi* thickening and the phylogenetic tree for the gymnosperm families. Interestingly, they propose that the *phi* thickening presence appeared early on and/or repeatedly in gymnosperm evolution. They observed that the capacity to develop *phi* thickening disappeared twice: once in the Gnetales-Pinaceae clade and once in the Podocarpaceae.

Sibipiruna (*Caesalpinia peltophoroides* Benth.) is a tree of the Fabaceae family which presents a *phi* thickening of type I (Henrique et al. 2010). This plant is from Brazil and is typically used for gardening. Henrique et al. (2010) have observed that

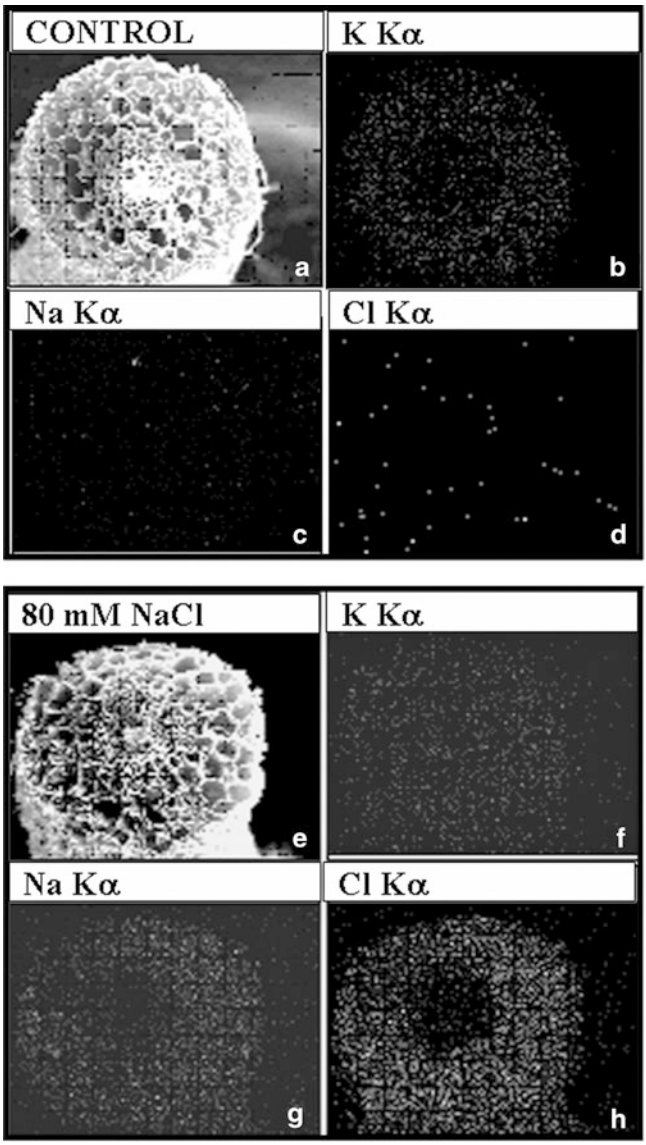

Fig. 2.3 Images of Na^+, Cl^- and K^+ distribution in control (**a**–**d**) and salt-treated (**e**–**h**) *Brassica oleracea* roots, from map scanning of an X-ray. (**a**, **e**) Scanning electron micrographs. (**b**, **f**) K^+ images; (**c**, **g**) Na^+ images; (**d**, **h**) Cl^- images. This figure is obtained from Fig. 9 of Fernandez-Garcia et al. (2009)

sibipiruna plants subjected to flooding showed a higher increment of *phi* thickening density. The authors suggest that the *phi* thickening may be acting as a physical barrier for water flux from the vascular cylinder to the cortical cells.

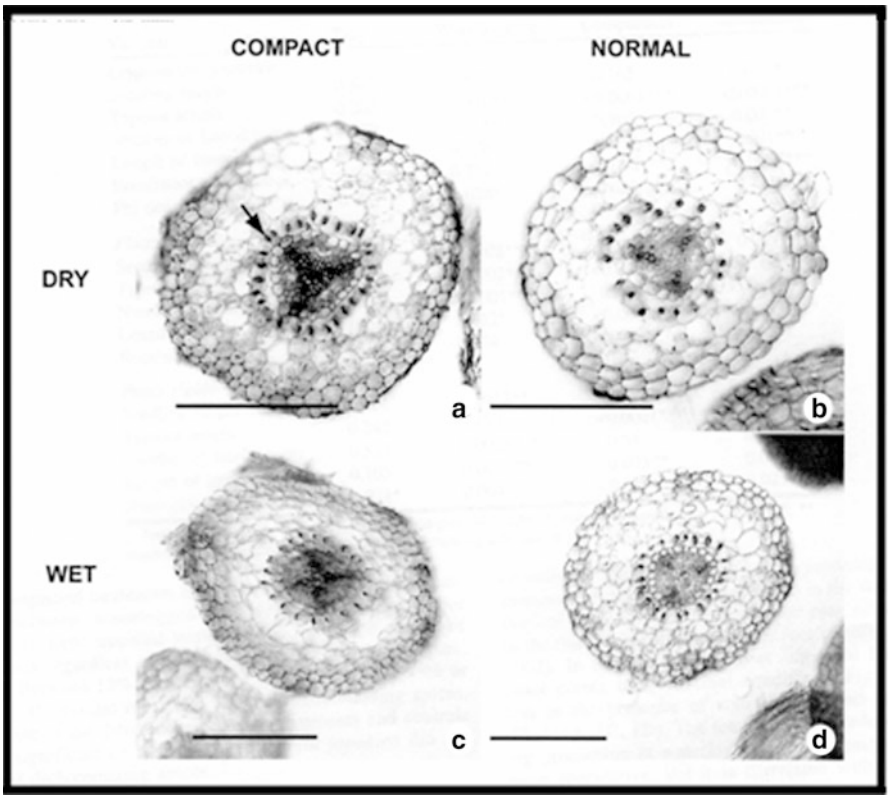

Fig. 2.4 Transverse sections of *Cryptomeria japonica* roots, under different treatments as indicated on the figure. (**a**) Compact and mesic (dry) conditions. (**b**) Uncompacted (normal) and mesic (dry) conditions. (**c**) Compact and waterlogged (wet) conditions. (**d**) Uncompacted (normal) and waterlogged (wet) conditions. The roots were stained with phloroglucinol. The *arrow points* to a phi thickening. Scale bars = 0.2 mm. Figure obtained from '© 2005 Canadian Science Publishing or its licensors. Reproduced with permission, Gerrath et al. (2005)'

Plant micropropagation under in vitro conditions is a technique frequently used for plant propagators. Rooting in agar media is an effective part of this technology. Soukup et al. (2004) have analysed the roots of wild cherry (*Prunus avium* L.) growing in perlite or in vitro conditions. When *P. avium* was grown in perlite, the roots develop *phi* thickenings in the radial and traverse wall of the *phi* cell layer directly in contact with the endodermis and within 5 mm from the root tip. However, *P. avium* grown in vitro culture did not show the *phi* thickenings in the roots, even after prolonged period of in vitro cultivation allowing formation of secondary tissues. The authors consider that *phi* thickening formation is not constitutive, but could be regulated by the environmental factors.

2.3.3 Drought Stress

Drought stress is a serious problem in the world and generates a progressive reduction in vegetation cover, coupled with rapid soil erosion. Drought stress is characterised by specific changes in plant anatomy and ultrastructure. Pan et al. (2006) studied the alteration of *phi* thickening in loquat (*Eriobotrya japonica* Lindl.) roots under drought stress. In control plants, phi thickenings were observed at 10 mm from the root tip, and these are formed by one or two phi cell layers. However, in trees grown under drought stress, *phi* thickening had developed dramatically compared with normal conditions. *Phi* thickening in drought-stressed plants extended to three *phi* cell layers and in some cases appeared beyond the fourth layers. The authors suggested that *phi* thickening of the cortical cells in loquat roots may be regarded as a defence mechanism against water stress under drought.

2.3.4 Heavy Metals and Solid-Waste Slag

An interesting case of *phi* thickening is the presence of cell wall thickenings in heavy metal hyperaccumulator species. *Thlaspi caerulescens* is a zinc hyperaccumulator, but *Thlaspi arvense* is a non-hyperaccumulator. *Thlaspi caerulescens* is characterised by the presence of a 'peri-endodermal' cell layer presenting thickened inner tangential walls but not in *T. arvense* (Broadley et al. 2007; Zelko et al. 2008). These thickenings develop close to the root tip, about 400–600 μm from the root cap boundary. The thickening extends to the radial walls where the cells are attached to each other. However, the corresponding cell layer in *T. arvense* is composed of thin-walled cells without thickening. In *T. caerulescens*, the cell wall thickening was lignified (Zelko et al. 2008). These authors prefer to use the term 'peri-endodermal' because they consider that *phi* thickening does not adequately describe the position of this cell layer.

What is the role of cell wall thickenings in Zn hyperaccumulators? Broadley et al. (2007) suggested that the presence or not of the cell wall thickenings might have an impact on apoplastic fluxes of Zn into the root stele. Interestingly, Zelko et al. (2008) have proposed the differences between the two *Thlaspi* species, in radial transport of Zn across the root. Its loading into the xylem and transport to the shoot may be determined structurally. These authors consider that the cell wall thickenings are increasing the surface of plasma membrane, as observed in *Brassica oleracea phi* cells (Fernandez-Garcia et al. 2009). These cells might function as transfer cells, and the increase of the plasma membrane could be intensifying the transport capacity of these cells.

Solid-waste slag from contaminated municipal soil has been used as substrate for roads, banks or parking lots as top layer. This kind of soil has a high amount of heavy metals and low nitrogen. Degenhardt and Gimmler (2000) have used this

substrate to investigate its effect on root growth of *Z. mays*. We mentioned above that the *phi* thickening is only present in dicotyledon species, except in the monocotyledon *Z. mays*, which can be induced by treatment with solid-waste slag. These authors observed that slag-grown plants showed *phi* thickenings in the radial cell walls of the rhizodermis but the distribution was incomplete around the whole root circumference. However, when plants were treated with other stresses, such as salinity, or were grown in hydroponic and aeroponic cultures, this did not induce *phi* thickening in the roots. The authors suggest that *phi* thickenings may be acting mainly as mechanical support for the roots and not as a physical barrier for solute movement.

2.3.5 Interaction of Mycorrhiza and Phi Thickening

Phi thickenings have also been suggested as a possible physical barrier for fungal movement through the cortical cells. Yellow birch (*Betula alleghaniensis* Britton) roots can be infected by the mycorrhiza *Chloridium paucisporum*. The fungal hypha is able to penetrate the radial cell walls of the epidermis and develop the Hartig net that can be well extended in the external cortical cells. However, the penetration in depth seems to be limited by the presence of the *phi* thickening in the second layer of cortical cells (Wilcox and Wang 1987). *Dryas integrifolia* is a boreal member of the Rosaceae family and establishes an ectomycorrhizal association with *Hebeloma cylindrosporum*. Melville et al. (1987) have shown that *D. integrifolia* roots develop *phi* thickenings in the inner cortical cells near the apex of first-order lateral roots when they interact with *H. cylindrosporum*. In some first-order lateral roots, the *phi* thickening is composed of two cortical cell layers, which then becomes impervious to hyphal penetration. Moreover, the *phi* thickening was not developed in the older portion of the root, where the Hartig net was poorly developed. These authors suggest that the Hartig net is inhibited beyond cortical cell layers with *phi* thickenings in their radial cell walls.

Elsewhere, other authors have reported that *phi* thickening is not a physical barrier for fungal penetration (Massicotte et al. 1988, 1999). Yellow birch is also able to form an ectomycorrhizal association with *Pisolithus tinctorius*, forming a Hartig net only in the epidermis. However, primary roots at the base of first-order mycorrhizal lateral roots develop *phi* thickenings in the second cortical layer or in the second and third layer of cortical cells. Therefore, the presence of the *phi* thickening seems to be not necessarily correlated with the fungal infection and penetration. Similarly, European black alder (*Alnus glutinosa*) may be associated with the ectomycorrhiza *Paxillus involutus*. The roots of *A. glutinosa* develop in the subapical regions *phi* thickenings in the second layer of cortical cells. However, in this region of the roots, the hyphae did not penetrate between epidermal cells. Moreover, if we advance to the apical region, the *phi* thickenings are thinner and fewer in number. Finally, close to the apical meristem, the *phi* thickenings are not

present, but the mantle and the Hartig net are well developed (Massicotte et al. 1999).

2.4 *Phi* Thickening as Taxonomic Tool

Gerrath et al. (2002) proposed that *phi* thickening can be a useful tool for taxonomic classification in gymnosperms. These authors analysed the presence or absence or *phi* thickening in roots of 22 species of gymnosperms representing all the major groups. This study demonstrated that *phi* thickenings were absent in the Cycadaceae, Gnetaceae, Pinaceae, Ephedraceae and Podocarpaceae families and were present in the Ginkgoaceae, Araucariaceae, Taxaceae and Cupressaceae. The results of this study were confirmed by a previous description of *phi* thickenings in gymnosperm species.

Pires et al. (2003) have also developed a study to separate taxonomically two genera, *Prosthechea* and *Encyclia*, using the anatomy of leaves and roots. These authors observed the presence of large *phi* thickenings in the cortical cells of both genera. These *phi* thickenings were placed concentrically, generally forming a continuous sheath in the cortical region of the root. However, the *phi* thickenings did not show any correlation that could help to discriminate between the two genera studied.

2.5 Conclusions

Phi thickening in cortical cells of the roots was discovered in the nineteenth century. *Phi* thickenings of cell walls can be present in the different cell layers of the cortical cells forming in many cases a continuous cell layer. We have found that more than 100 species present *phi* thickening in roots, including angiosperms and gymnosperms. A mechanical role was originally suggested for this structure, although it has never been demonstrated. In the last decade, new studies have demonstrated that *phi* thickening can be altered by the environmental conditions. Different abiotic and biotic stresses may be modifying the distribution, structure and/or development of *phi* thickening. Abiotic stresses such as salinity, heavy metals and flooding are altering *phi* thickening distribution and lignification. A physiological role for *phi* thickenings has also been proposed, acting as a barrier, altering cation movement through the apoplast and regulating the symplastic ion movement through the plasmodesmata system.

From the nineteenth century, the presence of *phi* thickenings has been described in many different species, but the controversy about its role is still open. It is evident that *phi* thickenings might present several roles, including different physiological and/or mechanical functions. If we consider the different studies, it is possible to extract some general conclusion:

1. *Phi* thickening cannot be induced in species that *phi* thickening is not present.
2. *Phi* thickening may be acting as a partial barrier in solute movement from the cortex to the vascular cylinder.
3. *Phi* thickening appeared early in the plant evolution.
4. *Phi* thickening development can be affected by environmental factors like culture, media, drought stress, salinity, soil contaminant, etc.

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Chapter 3
Root System Architecture

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3.1 Root System Architecture: Definition and Description

The root system architecture is a composite notion that encapsulates aspects of root structure and of root shape (Pagès 1989). On the one hand, the structure of root systems, which defines the assembly and properties of the different root segments, is bound to the developmental processes that lead to the expansion, direction, and senescence of roots and to the production of new roots (Hochholdinger et al. 2004; Waisel 2002). On the other hand, the shape of root systems describes the spatial distribution of roots and relates to major functions of the root system such as resource capture, anchorage, and plant hydraulic (Gregory et al. 2003).

The importance of RSA lies in the fact that major soil resources are heterogeneously distributed in the soil, so that the spatial deployment of roots substantially determines the ability of a plant to secure and transport edaphic resources (Lynch 1995). In addition, the structure and spatial configuration of the root system are important aspects of the mechanical soil-root system responsible for plant anchorage (Fitter 2002). Finally, RSA determines largely the extent of the contacts and interactions between the plant and the rhizosphere. These points are the focus of several chapters of this book.

Interestingly, RSA does not bear specific functions by itself. Instead, it carries the spatial and temporal dimensions that are needed to analyze many functions at the plant scale. Resource uptake, for example, is primarily realized at the root surface by multiple cellular or apoplastic transport mechanisms that have little connections with RSA. The placement of the roots, however, determines the maximum amount of resource that can be absorbed (King et al. 2003). Similarly, water transport through cells and in the apoplast is driven by local water potential gradients and regulated by local conductivities (i.e., aquaporins, cell wall

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decorations, and xylem diameter) which do not depend primarily on RSA. The complete structure of the root system is needed, however, to consider how the negative water potentials at the leaf surface propagate throughout the plant and the soil environment (Sperry et al. 2002).

As a consequence of its composite nature and of the multiplicity of functions where it is involved, RSA has become a topic on its own in many research communities (i.e., ecologists, geneticists, molecular biologists, crop physiologists, microbiologists) with contrasting interests, investigation scales, experimental strategies, observation techniques, or modes of description.

A major problem that results from this situation is the coexistence of very different ways to describe RSA. Beyond the fact that developmental biologists and breeders are looking at different aspects of RSA, different constraints apply to the RSA description of *Arabidopsis* seedlings in a Petri dish and of rice plants in soil columns or in the field. The ingeniosity of researchers has led to an arsenal of phenotyping methods, from 1D to 3D, static to dynamic, direct to indirect, partial (Fitter 1982) to exhaustive (Lobet and Draye 2013), with a variety of culture systems (Dhondt et al. 2013; de Dorlodot et al. 2007; Smit et al. 2000). This situation could be seen as an opportunity to generate complementary information on common genetic resources, especially given the amplitude of environmental influence on RSA. However, we have not been successful in developing a unified framework to exchange and discuss concepts and data on RSA.

A first step towards the development of such framework will come from phenotyping strategies where RSA is considered as the cumulative outcome of a limited set of developmental processes (i.e., root growth, formation, tropisms, and senescence) that are potentially influenced by root age, plant phenology, and the environment. In these strategies, the emphasis is shifted from the static description of RSA towards the quantification of underlying dynamic processes. This view, which is supported by the development of noninvasive methods allowing repetitive observation of growing plants (X-ray, gel chambers), paves the way for the construction of response curves of development as a function of age and environmental parameters. This type of analysis, first developed for leaf growth (Chenu et al. 2009), supports novel ways to address GxE interactions that can be very large in RSA.

The second step is being realized through an increasing number of mathematical models of RSA, which provide a link between developmental processes and RSA (Pagès 2011). Over the last years, a new generation of RSA models has arisen which integrate structure, function, and environment (Godin and Sinoquet 2005). These models have already been used for the analysis of water flow (Doussan et al. 2006; Draye et al. 2010; Javaux and Vanclooster 2006), nutrient uptake (Dunbabin et al. 2004; Ge et al. 2000), carbon allocation (Bidel et al. 2000; Nord et al. 2011), and anchorage (Dupuy et al. 2007; Stokes et al. 1996). The further incorporation of developmental response curves in these models should allow to simulate RSA in untested scenarios and perform in silico experiments to generate new hypotheses or predict RSA of novel genotypes. It can be hoped that a wider adoption of such models will support the development of RSA modeling platforms

and of standards of RSA description. Both will be needed to unleash the information generated by phenotyping experiments.

3.2 Motivations and Challenges for Engineering Root System Architecture

Changes in RSA have occurred as a consequence of domestication and breeding and have led to contrasting spatial arrangements of roots (de Dorlodot et al. 2007). Furthermore, there are several instances where the overlap of quantitative trait loci (QTL) for root features with those for productivity (yield, water use, or nutrient capture) has suggested the possible role of the former in determining the latter. Such considerations, combined with the fact that any genetic progress for resistance to abiotic stresses will be a lasting one, indicate that RSA engineering should have profound implications for improving water- and nutrient-use efficiency of crops and enhancing their productivity under abiotic stresses or in suboptimal conditions (de Dorlodot et al. 2007; Lynch 2007).

However, the identification of the genetic determinism of RSA and its exploitation in breeding is fraught with many difficulties. Firstly, it is now established that RSA is in most cases regulated by a suite of quantitative, small-effect loci (QTL, quantitative trait loci) that interact with the environment. In most cases, parameters of RSA have low heritabilities. This situation is largely due to the contingency effect that is inherent to the construction of root systems in soils and which causes extreme variability of RSA for the same genotype.

Secondly, environmental factors strongly influence the development of the root system and determine many aspects of its architecture (McCully 1999). In many instances, this plasticity leads to important gene-by-environment interactions, although there are some cases where these interactions were reassuringly weak (de Dorlodot et al. 2007; Price et al. 2002). This has several consequences, viz., that the beneficial effect of a QTL in a phenotyping screen or in a small number of field environments may vanish in other field conditions.

QTL validation in field conditions and in several genetic backgrounds is therefore crucial but relies on the expensive development and phenol typing of a series of introgression lines. The cloning of a QTL to validate its function is also extremely complex, and we are not aware of any successful root QTL cloning today (in crops). For this reason, the number of cases where promising QTL for RSA have been introgressed into elite germplasm remains extremely limited (Beebe et al. 2006; Clark et al. 2008).

The definition of breeding targets is another significant challenge prior to exploiting the genetic variability for RSA. There are few situations for which ideal architecture have been clearly established. Shallow soil exploration, for example, has been demonstrated to be a primary determinant of tolerance to low-P soils (Walk et al. 2006), while deep soil exploration is an interesting target

to reduce nitrate leaching (Dunbabin et al. 2004). The case of drought tolerance appears to be more complex. Indeed, deep roots increase the volume of soil explored and the global amount of transpirable water (Lynch 2013; Singh et al. 2011); however, they may cause a waste of subsoil water that is lost for the end of the crop cycle (Van Osterom, unpublished data). In addition, piling up root traits to support multiple targets may not always be possible. Finally, depending on the extent of GxE for particular architectural traits, breeders might have to look at constitutive or at environmentally responsive traits.

At least three steps will condition the success of RSA engineering: (i) via QTL cloning, build a repertoire of functional sequences; (ii) develop standard phenotyping screens: and (iii) integrate "omics" technologies with plant physiology, agronomy, breeding, and disciplines related to the rhizosphere (de Dorlodot et al. 2007). The latter will be especially important to validate QTL functions in a real context and to identify relevant combinations of breeding and management strategies targeting specific environmental scenarios.

3.3 Developmental Processes Underlying Root System Architecture

Dissecting the components of root structure into the functions of individual genes or gene networks is a preliminary step towards RSA engineering. In this section, we illustrate through several examples how our understanding of the major developmental processes is currently progressing. For a more complete information, we recommend recent reviews on molecular control mechanisms of root development in *Arabidopsis* (Lavenus et al. 2013; Petricka et al. 2012; Smith and De Smet 2012) and cereals (Coudert et al. 2010; Orman-Ligeza et al. 2013; Smith and De Smet 2012).

3.3.1 Root Growth and Gravitropism

Several mutants with a reduction in seminal root growth and gravitropism have been described in cereals (Gowda et al. 2011; Hochholdinger et al. 2004); however, most of these genes have not been identified. Genetic mechanisms regulating root growth rate as an output of meristem activity and cell elongation rate in *Arabidopsis* are just beginning to be revealed. Several plant hormones act in concert to govern root growth through complex interactions at multiple levels, including biosynthesis, metabolism, transport, and signaling. This includes auxin, cytokinin, ethylene, abscisic acid (ABA), gibberellin, and jasmonate (Brady et al. 2003; Monroe-Augustus et al. 2003; Werner et al. 2003). Cytokinin, for example, antagonizes auxin action in root growth and apical meristem (RAM) size determination.

Cytokinin is perceived by a family of AHK histidine kinase receptors, and the *ahk2 ahk3 ahk4* triple mutant shows inhibition in root and shoot growth (Nishimura et al. 2004). Its deficiency results in the increased growth of the primary root in *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) overexpressing plants (Werner et al. 2003). Mutations in genes responsible for cytokinin biosynthesis, e.g., *ATP/ADP ISOPENTENYL TRANSFERASES 3*, *5*, and *7* (*IPT3*, *IPT5*, *IPT7*), and signaling, e.g., *AHK3* and *ARABIDOPSISRESPONSE REGULATOR 1* and *12* (*ARR1 and ARR12*), decrease root-meristem cell number and root growth rate (Dello Ioio et al. 2007). Interestingly, the double mutant *arr1-3 arr12-1* shows reduced sensitivity to salt due to the increase in a high-affinity K(+) transporter responsible for sodium removal from the root xylem. Thus, cytokinin-regulated pathways may be used to improve root length and salinity tolerance (Mason et al. 2010).

The gravistimulus regulates the root growth angle through the concerted action of auxin efflux and influx carriers which modulate the distribution of auxin in the elongation zone. The efflux carrier PIN-FORMED 3 (PIN3) protein initiates the asymmetric auxin transport in the columella, driving more auxin to the concave side of the root, while the PIN2 and auxin influx carrier AUX1 transmits the signal through the lateral root cap to the epidermal cells in the elongation zone (Michniewicz et al. 2007). There, auxin receptors, including TRANSPORT NHIBITOR RESPONSE 1 (TIR1), induce auxin-signaling machinery (Dharmasiri et al. 2003) resulting in asymmetric cell elongation and, most likely, lateral root branching. Interestingly, few mutants were described that are impaired in lateral root gravitropism, but not in primary root gravitropism (Mullen and Hangarter 2003). On the opposite, both primary and lateral roots of the *long hypocotyl 5* (*hy5*) mutant show reduced gravitropism. Those results suggest that the lateral and or primary root angle could be potential engineering targets. Computer simulations showed the influence of root angle on inter-root competition and on the efficiency of nutrient acquisition (Guyomarc'h et al. 2012).

3.3.2 Root Formation

Root formation is an essential complement to root growth. It contributes to widen the volume of soil explored, increase the extension capacity of the root system, and compensate for root death. Postembryonic root formation includes the production of adventitious and lateral roots that have specific physical and physiological properties, uptake behaviors, construction costs, and survival rates. Hence, adventitious and lateral roots contribute in sensibly different ways to the multiple functions of root systems and to the efficiency of the root system (defined as a ratio of resource captured: carbon invested). Similarly, the processes of adventitious and lateral root formation are likely to be coordinated in order to achieve an optimal performance at the whole plant scale.

3.3.2.1 Adventitious Root Formation

The formation of adventitious roots seems to be strongly regulated by hormones (Gutierrez et al. 2012; Rasmussen et al. 2012; Steffens et al. 2006), external conditions (Takahashi et al. 2003), and genetic background (King and Stimart 1998). The molecular mechanism of adventitious root formation has been partially revealed in cereals and *Arabidopsis*. The term crown root is preferred in cereals, where the production of adventitious roots is a part of a normal developmental plan (Hochholdinger et al. 2004).

Auxin has a positive effect on crown roots formation in cereals, as proven by several cereal mutants impaired in auxin perception and response, of which three *CROWN ROOT LESS* genes (*CRL1*, *CRL4*, *CRL5*) have been cloned in rice. The mutants have no or fewer crown roots and lack lateral roots on their seminal roots (Inukai et al. 2005; Kitomi et al. 2011a, b). The *crl1* mutant is affected in a gene encoding a *LATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) protein, which is regulated by rice *AUXIN RESPONSE FACTOR 16* (*OsARF16*), an ortholog of *Arabidopsis ARF7/ARF19* (Inukai et al. 2005). *CRL4* encodes a protein homologous to *Arabidopsis GNOM* (*GN*) (Kitomi et al. 2008) that regulates polar auxin transport (Geldner et al. 2004). *CRL5* encodes auxin-responsive AP2/ERF transcription factor that regulates crown root formation through induction of rice type A *RESPONSE REGULATOR 1*(*OsRR1*), a repressor of cytokinin signaling (Kitomi et al. 2011a). Cytokinin is indeed known to act antagonistically to auxin during crown root formation. Mutation of the auxin and cytokinin-signaling gene called *WUSCHEL-RELATED HOMEBOX GENE 11* (*WOX11*) in rice also causes a reduced number of crown roots. WOX11 suppresses the expression of the type A *RESPONSE REGULATOR 2* (*RR2*) that in turn suppresses CK signaling (To et al. 2004). Thus, it has been hypothesized that *WOX11* integrates auxin and cytokinin signals to regulate crown root formation in rice (Coudert et al. 2010; Zhao et al. 2009). In Arabidopsis, the auxin-regulated *ARF17*, a target of miR160, is a negative regulator of adventitious root formation, whereas *ARF6* and *ARF8*, targets of miR167, are positive regulators of adventitious rooting (Gutierrez et al. 2009). These transcription factors regulate the expression of three auxin-inducible *GRETHEN HAGEN 3* (*GH3*) *genes*, GH3.3, GH3.5, and GH3.6, responsible for adventitious root initiation (Gutierrez et al. 2012).

3.3.2.2 Lateral Root Formation

Many genes involved in initiation and emergence of lateral roots have been identified in *Arabidopsis thaliana*, against only few in cereals (Gowda et al. 2011; Hochholdinger et al. 2004). In *Arabidopsis*, three auxin-signaling modules operating during lateral root initiation are known. In the basal meristem, the *INDOLACETIC ACID-INDUCED PROTEIN 28/SWIRM DOMAIN PAO PROTEIN 1* (*IAA28–SWP1*) module regulates the transcription of lateral root promoting

factors, such as *ARF5*, *6*, *7*, *8*, *and 19* and *GATA TRANSCRIPTION FACTOR 23* (*GATA23*) (Singh et al. 2012a), that control founder cell identity. The *SOLITARY ROOT* (*SLR*)–*ARF7–ARF19–LBD* module (Fukaki et al. 2002) regulates the division of xylem pole pericycle cells during lateral root initiation. Finally, the *BODENLOSS* (*BDL*)–*ARF5* module regulates later steps of lateral root organogenesis (De Smet et al. 2010). In cereals, crown root mutants are often impaired in the lateral root formation on seminal roots, suggesting that common mechanism regulates postembryonic root initiation in cereals (Inukai et al. 2005; Kitomi et al. 2008, 2011a). Interestingly, there are no known mutants in cereals that are impaired in lateral root formation on crown roots (Smith and De Smet 2012).

3.3.3 Root Morphology and Structure

Although the morphology and structure of individual root segments are not part of the notion of RSA, they are tightly coupled as they define important properties of root segments that can hardly be considered as independent of RSA. We discuss briefly the case of root hairs, vasculature, cell wall modifications, and parenchyma that are all under genetic control and constitute potential targets of root engineering.

3.3.3.1 Root Hairs

Water and nutrient uptake is often restricted by a limited contact area between the root surface and the soil particles, especially where roots clump within pores and cracks that are larger than the root diameter (White and Kirkegaard 2010). Both the length and the density of root hairs contribute to maintain the soil-root contact. Longer root hairs improve phosphorus acquisition (Bates and Lynch 2000; Gahoonia and Nielsen 2004; Lynch 2011) and likely water uptake (Segal et al. 2008), although their actual contribution has been recently revisited (Keyes et al. 2013).

Several root hair mutants have been described in cereals (Hochholdinger et al. 2008; Zuchi et al. 2011), yet the genetic background of root hair formation has been best described in *Arabidopsis*. Root hair mutants display variation in hair density (Bernhardt et al. 2003; Hauser et al. 1995; Ohashi et al. 2003; Schiefelbein and Somerville 1990; Walker et al. 1999), hair length (Cernac et al. 1997), and hair development (Grierson et al. 1997; Parker et al. 2000; Ryan et al. 1998). In *werewolf* (*wer*), *enhancer of glabra 3* (*egl3*), *transparent testa glabra 1* (*ttg1*), and *glabra 2* (*gl2*) mutants, root hairs are formed on nearly all root epidermal cells (Ohashi et al. 2003; Walker et al. 1999). In fact, the complex of WER, EGL3, and TTG1 proteins promotes *GL2* expression, which prevents root hair formation in non-hair cells and regulates the density of root hairs at the root surface. In this process, CAPRICE protein (CPC) competes with WER protein for binding to this transcriptional complex (Tominaga-Wada et al. 2011).

3.3.3.2 Vasculature

Early experiments indicated that xylem diameter in wheat influences grain yield in rain-fed environments (Richards and Passioura 1989). In fact, the number and size of xylem vessels are important determinants of the water flow capacity, the axial hydraulic conductivity, and the cavitation susceptibility of roots (Bramley et al. 2009; Sperry and Ikeda 1997) which, in combination with RSA, affect the spatial and temporal patterns of water uptake in the soil (Draye et al. 2010).

Mutagenesis in *Arabidopsis thaliana* revealed numerous regulators of radial tissue development, mutations of which disrupt the spatial organization of vascular tissue, endodermis, and cortex, such as *phloem intercalated with xylem* (*pxy*) (Fisher and Turner 2007), *woodenleg* (*wol*), *shortroot* (*shr*), *gollum* (*glm*), *fass* (*fs*), *scarecrow* (*scr*), *and pinocchio* (*pic*) (Scheres et al. 1995). Recently, several regulatory mechanisms that control the specification of xylem and phloem have been uncovered. These include class III HD-ZIP and KANADI gene family members that control the establishment of the spatial arrangement of phloem, cambium, and xylem (Ilegems et al. 2010) and *ALTERED PHLOEM DEVELOPMENT* (*APL*) that encodes an MYB coiled-coil transcription factor responsible for phloem differentiation. The phenotypes of the mutants indicate that small-scale changes in root anatomy may impact whole plant physiology and growth.

3.3.3.3 Cell Wall Modifications

Roots respond to different external stimuli by various cell wall modifications such as lignification of the sclerenchyma layer and suberization of the rhizodermis, exodermis, or endodermis (Hose et al. 2001). For example, the suberization of the sclerenchyma is thought to reduce water uptake in flooded conditions, while that of the endodermis seems to help the root to retain water under drought (Henry et al. 2012). In addition, suberization of endodermis and thickened cell walls restrict the water uptake to the distal region of the roots (Bramley et al. 2009), hence their obvious link with RSA.

Increased suberin content in *Arabidopsis enhanced suberin 1* (*esb1*) mutant causes enhanced hydraulic resistance to radial transport of water and results in decrease in transpiration and in shoot biomass accumulation. However, this reduction is smaller than the reduction in transpiration, leading to an overall increase in water use efficiency of *esb1* (Baxter et al. 2009). Increased root suberin content may therefore open new opportunities for enhancing drought resistance in crops.

3.3.3.4 Aerenchyma

Modeling studies indicate that the production of so-called “cheap roots” with extensive root cortical aerenchyma might increase root growth up to 70 % under

phosphorus-deficient conditions and thus tackle nutrient deficiencies (Lynch 2011; Postma and Lynch 2011). In addition, large intercellular spaces in cortex could also speed up an exchange of gases between aerobic shoot to the anaerobic root and serve as an important adaptation to flooding conditions (Jackson and Armstrong 1999). Therefore, this phenomenon driven by non-apoptotic programmed cell death (Joshi and Kumar 2012) has been extensively studied on cereals and is known to be stimulated by several stress factors, such as drought, transient nutrient and oxygen deficiencies, and mechanical impedance (Drew et al. 1989; Jackson and Armstrong 1999; Postma and Lynch 2011; Whalen 1988; Yang et al. 2012). Moreover, the process of aerenchyma formation is tightly regulated, where ethylene, calcium, H_2O_2, and abscisic acid orchestrate to produce it in a predictable pattern (Drew et al. 2000; Siyiannis et al. 2012) and can be therefore targeted in plant breeding programs. On the contrary, it has been shown that aerenchyma could impede the radial transport of water (Yang et al. 2012) or affect the mechanical strength of the roots and their susceptibility to root collapse (Mostajeran and Rahimi-Eichi 2008).

There is a limited data on this process in *Arabidopsis*. To date, studies have shown that *Arabidopsis thaliana* seedlings do not form root aerenchyma in response to hypoxia, yet *LESION SIMULATING DISEASE 1*(*LSD1*), *ENHANCED DEASEASE SUSCEPTIBILITY 1 (EDS1)* and *PHYTOALEXIN-DEFFICIENT 4* (*PAD4*) control aerenchyma formation in *Arabidopsis thaliana* leaves and hypocotyls (Muhlenbock et al. 2007).

3.4 Status of QTL Studies for Root System Architecture

Although there are a few examples of QTL that individually explain up to 30 % of phenotypic variation for root traits and for their response to environmental factors, it is now established that RSA is, in most cases, regulated by a suite of quantitative, small-effect loci that interact with the environment (de Dorlodot et al. 2007). More than 80 different studies have been realized today in cereals, largely dominated by rice work initiated in the 1990s and continued since then at a rate of 2.5 studies per year (Fig. 3.1). QTL studies in maize and wheat started between 2000 and 2010, with roughly similar annual rates as rice. QTL analysis therefore remains a favorite method to dissect the genetics of RSA in crops, despite the increasing availability of genomic resources supporting candidate gene approaches.

Given the available genotyping technologies and genetic resources, most of these studies relied on several mapping populations obtained from biparental crosses. QTL detected with independent mapping populations should preferably be validated in several genetic backgrounds, by introgression or at least by demonstrating their finding and co-localization in different populations. The development of large introgression libraries (Li et al. 2005) and of several association mapping panels (e.g., NAM panel of Buckler in maize) will offer novel possibilities that partly circumvent the limitations of segregating populations while providing

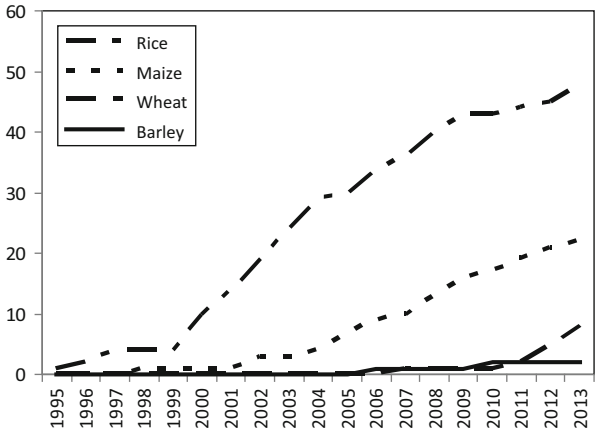

Fig. 3.1 Evolution of the number of QTL studies for RSA traits over the last 20 years

increased genetic resolution. RSA work with these new genetic resources is ongoing.

With more than 80 independent QTL experiments so far, *meta*-analysis should be encouraged to progress in the validation process of thousands of root QTL detected. Intraspecific consensus maps and interspecific comparative maps have already elaborated in cereals so that most root QTL have already been aligned and made accessible on web resources such as Gramene (http://www.gramene.org). The lack of standard in growing conditions and RSA descriptors remains therefore the major bottleneck for the *meta*-analysis of RSA datasets.

A very diverse set of RSA descriptors have been reported in the literature, illustrated in Table 3.1. Pioneering studies were generally conducted on mature plants in field conditions and described RSA by means of the vertical distribution of root mass (Champoux et al. 1995; Price and Tomos 1997) and, in few cases, number and diameter of crown roots. The scope has gradually moved towards descriptors seedling RSA in artificial conditions (de Dorlodot 2007; Price et al. 1997; Zhu et al. 2005). Most of these studies reported on maximum root length, while only few embarked in detailed measurements of lateral roots, root growth, or root formation dynamics. This variety of descriptors is further complicated by the lack of standard on plant age and growing conditions, so that it remains difficult to assemble QTL for putatively equivalent descriptors evaluated on plants of different ages, genetic backgrounds, and growing conditions. Over the last years, the evaluation of RSA in soil has become popular again, eased by the design of phenotyping techniques for field conditions like shovelomics (Trachsel et al. 2010) and DNA measurement (Borst et al. 1967), rhizotrons (Nagel et al. 2012; Singh et al. 2012b), and sophisticated 3D visualization systems based on computer tomography (Mooney et al. 2011).

In an early attempt to synthesize results of 27 QTL studies in rice, reporting more than 600 QTL for 53 different traits, de Dorlodot (2007) clustered all traits in a small number of functional categories (Table 3.1) that bind with root growth, crown root formation, and root branching. The alignment of these 600 QTL onto a

Table 3.1 Categories of root architecture descriptors reported in 27 QTL studies in rice

Trait	No. of independent studies
Maximum root axis length	20
Root dry mass vertical distribution	17
Root dry mass	13
Root axis length (vertical distribution)	8
Root axis thickness	8
Number of root axes	8
Root axis thickness (vertical distribution)	7
Root fresh mass or volume	5
Root growth rate	3
Root length density	3
Lateral root length	3
Lateral root number/density	3
Average root axis length	1
Lateral root growth rate	1
Lateral root thickness	1

Modified from de Dorlodot (2007)

consensus genetic map revealed several regions harboring QTL mostly related to either growth or formation. Beyond the cross-validation of QTL, the meta-analysis also allowed to dissect functionally several root mass QTL into root growth and root formation QTL or to reveal interesting co-localization of QTL for tillering and lateral root branching or crown root production and anthesis date. The study further stressed the usefulness of standard and fine descriptions of RSA for the functional validation of QTL.

Beyond the need to clarify the definition of traits, high genetic resolution is also required to ascertain accurately the role of linkage in the co-segregation of QTL effects for traits that are plausibly related on a functional basis (de Dorlodot et al. 2007). Positional cloning of a validated QTL and candidate gene identification are the most commonly used approaches to establish its function and narrow the gap between the genotype and the phenotype, which eases the exploitation of the allelic richness within germplasm collections. Advances in the understanding of the molecular nature of root growth afforded by studies in *Arabidopsis* are beginning to indicate good functional candidate genes for the regulation of root growth (see above). The number of root QTL that have been cloned so far in crops remains, however, very limited (Price 2006).

3.5 Concluding Perspectives

An increasing body of evidence indicates that the engineering of root system architecture has the potential to support a second green revolution targeting crop performance under suboptimal water and nutrient supply. This chapter summarizes

the recent evolution of this field and underlines important challenges to be addressed in the near future. Due to its importance for many plant functions, root system architecture has become a topic on its own in many research communities. Impressive progress has been achieved in our understanding of the developmental processes underlying root system architecture. In parallel, a large number of QTL studies have been reported for root architectural traits. We discussed several limitations that impede the exploitation of the genetic variability and available functional information on root system architecture in conventional breeding.

The engineering of RSA has attracted increasing attention over the last two decades, as a result of the awareness that root system improvement has the potential to support a second green revolution targeting crop performance under suboptimal water and nutrient supply. Along this chapter, we have reported the main trends in this field and underlined important challenges to be addressed in the near future.

Thanks to the impressive progress in our understanding of the molecular basis of RSA in *Arabidopsis* and, more recently, in rice, the repertoire of candidate functional sequences is continuously expanding. While this should ease the exploitation of QTL in conventional breeding, it will be crucial that this understanding embraces the mechanisms underpinning the quantitative variation of RSA. Indeed, most current work has been done with binary mutated phenotypes that have little agronomic significance. QTL analysis will therefore remain an important tool in the future.

As the panel of phenotyping techniques is expanding, it becomes increasingly important to adopt a core set of descriptors. It is now widely accepted that root phenotyping will benefit from a complementary set of techniques and is unlikely to be solved by a unique strategy. Data exchange and public data repositories are certainly one component of the future of RSA engineering. Common RSA descriptors are the first step in this direction.

With the development of nondestructive techniques allowing the visualization of roots in soil and the simultaneous characterization of the soil environment, it will become possible to address root plasticity and GxE interactions by modeling root growth response to local environment. This will open new opportunities to bind root development research and soil science. This might be an interesting avenue to analyze the strong residual variability of RSA in soils.

Finally, collaborations between breeders, geneticists, physiologists, crop physiologists, and soil scientists (among others) will be needed to develop new visions on how RSA engineering can be turned into improved crop performance. Multiscale simulations that connect the cell, tissue, organ, whole plant, and field scales may help to test in silico how given RSA features influence resource capture in a large array of conditions and to design critical scenarios in which experiments should be performed.

The field of RSA remains therefore in rapid evolution. Nowadays, new impulse can also be expected from new public–private consortia willing to develop strategies for the incorporation of RSA in conventional breeding.

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Chapter 4
Root Growth Model Based on Swarm Intelligence

Tomé Matos, Cristina Cruz, and Luís Correia

4.1 Introduction

In most agricultural systems, crop productivity is highly dependent on the supply of fertilizers. Traditionally, the use of fertilizers to restore nitrogen (N)-depleted soils has resulted in increased plant health and yields. However, in recent years, many regions with intensive agriculture have been seriously affected by fertilizer-based pollution. Thus, there is significant interest in developing technologies, at plant level, which could allow economically-viable production while applying less N. To complement recent reviews examining N-utilization efficiency in agricultural plants, this work will explore those strategies operating specifically at the root level, which may directly contribute to improved N-use efficiencies in agricultural crops such as cereals, where the majority of N-fertilizers used are lost to the environment. Root-specific phenotypes that will be addressed in the context of improvements to N-acquisition and N-assimilation efficiencies include root morphology, root-to-shoot ratios, root vigor and root length density, and root N transport and metabolism.

Various crucial questions about root growth remain unanswered, probably because the study of roots, in their natural environment, is extremely complicated.

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This is due to the fact that most of root growth and the interactions between them and their surroundings, namely, other roots, microorganisms, and their soil, occurs in opaque media. It is thus extremely difficult to arrive at a consensus on the processes that govern root growth in natural environments (Hutchings and John 2003).

Various predictive models of root growth have been developed, with various objectives, yet none has centered on the decisions a root has to take in exploring the soil, as considered in the present work.

With the model here presented, we hope to complement and contribute to the knowledge concerning the way the root makes the choices needed for what seems to be an optimal soil exploration, so that it might be applied to predictions of root architecture and efficiency of soil exploitation. Recent works suggest that each root apex operates almost independently, contributing to the global objective of soil exploration (Baluska et al. 2010). This emergent property is characteristic of swarm intelligence models and, until now, has only been attributed to animals (including human beings) (Bonabeau et al. 1999). It is known that the main factors behind root growth are plant genes, gravity, soil conditions (including nutrient distribution), and kin recognition (Biedrzycki et al. 2010). Based on this, and given that there is no anatomic evidence of a decision or information center in the root, it appears sensible to suppose that the decisions of growth direction, as well as most information retrieval from the environment, are made at apex level (Baluska et al. 2004; Hodge 2009). It is also interesting that the attribution of this type of intelligence to plants is not an entirely new idea, having already been suggested by Erasmus Darwin, who referred to the buds of the plant as separate individuals (Darwin 1800). It was with this theory in mind, and based on the property of swarm intelligence, that the computational model of root growth here presented was created. This model will not be able to prove or disprove whether this is truly the method the root uses to make decisions, but it will be at least able to verify its viability. Furthermore, if the simulated roots become similar to real ones, one might envisage the use of this model to infer the root architectures of plants growing in more heterogeneous soils, such as those created by more environmentally friendly agriculture.

A wide variety of root models have already been created and published. They differ not only in their objectives but also in their format, and can be classified into several groups: structural static (Gerwitz and Page 1974; Henderson et al. 1983) and dynamic models of root system growth and development (Lungley 1973; Porter et al. 1986; Rose 1983); water (Herkelrath et al. 1977; Lafolie et al. 1991; Taylor and Klepper 1975) and nutrient uptake models (Baldwin et al. 1973; Claassen et al. 1986; Habib et al. 1989; Passioura 1963): combined growth and uptake models (Barnes et al. 1976; Bland and Jones 1992; Protopapas and Bras 1987); and root architecture models (Diggle 1988; Fitter et al. 1991; Nielsen et al. 1994; Pages and Aries 1988; Pages et al. 1989).

Of these, only the last group explicitly references root architecture. Even though our model is more centered on soil exploration than root architecture, it is a root

architecture model. A brief presentation of the more relevant models in this group follows.

Hackett and Rose (1972) created the first computer model of root architecture, which simulated the patterns of root ramification, in two dimensions, according to a set of simple rules. They used it to describe the extension and branching of a seminal root of barley. However, the first models that simulated root architecture in three dimensions only appeared in 1988: ROOTMAP (Diggle 1988) and SARAH (Pages and Aries 1988).

ROOTMAP generates the age, position, and orientation of root segments over time in function of the root's growth rate and branching density. These two parameters are regulated by the soil temperature, which varies between each soil layer. The global reference temperature, the temperature of zero growth, and the temperature of each soil layer are all set by the user. The linear relationship specified by the temperature of zero growth and the reference temperature is used to regulate the growth parameters, according to the local soil temperature. With this information, the model generates the three-dimensional root architecture over discreet time steps during which all root apexes grow. The direction of growth is determined for each apex based on the angle of growth of the previous step and on a deflection angle (Diggle 1988). This model is still being developed and updated (Dunbabin et al. 2002; Dunbabin 2007).

Most of SARAH's development was dedicated to simulate the three-dimensional architecture of maize's root system. It describes the root as a collection of axes, characterized by their order and node of origin. The root's growth, as in the previous model, is calculated in discreet time steps. In each step, a new primary axis grows from the plant, and the axes already present grow and branch. The growth of each axis depends on its order, on its node of origin, and on the environmental conditions. Branches appear acropetally at a specified distance from the apex, with a branching angle specific to their order and node of origin (Pages et al. 1989).

Since then, various models have been created and used to recreate the root's architecture (Fitter 1991; Jourdan and Rey 1997; Lynch et al. 1997; Nielsen et al. 1994; Pages et al. 2004). Only in the most recent of these, a bi-dimensional model, named Root Typ, are the branching density, the growth intensity, and also the apex growth direction influenced by the soil, which is composed of horizontal layers of variable height. However, this influence is only due to abstract regulation factors that are attributed to each soil layer, never referencing nutrient exploration. Fitter et al. (1991) constructed a model that, although it does not model the soil, is relevant to the present model. Its purpose was to calculate the soil exploration efficiency of root architectures, that is, the ratio of the volume of soil explored by the volume of the root.

All of these models produce results that are largely akin to the architecture of roots observed in their natural environment. However, they are all based on mathematical methods, usually fractal, to generate the root's structure. This way, even though they reproduce the root's architecture, they do not offer a true reflection of decision processes that occur during root growth. It is precisely this

void that the present model tries to fill, focusing on the understanding of growth and on an attempt to explain it through a model based on distributed choice processes made by the apexes, on their internal state, and on the state of the soil section they occupy.

The work here presented is based on the SIMORG project (swarm intelligence modeling of root growth) of the European Space Agency, ESA (Simões et al. 2011), in which root growth was modeled to control a robotic swarm, with the objective of using the behavior to explore the surface of a planet. The project only modeled root exploration with the minimal level of realism required to implement this behavior in the robots. This work endeavors to add an extra level of complexity to the previous model, so that it might be closer to biological reality.

The most obvious and quickly detectable difference is the transition from two to three dimensions, which substantially increases the number of choices available to each apex. The increase is reflected not only in the choice of the soil section to explore but also in the possibility that each root module can generate a higher number of ramifications. As a result, it is possible to obtain a representation of the root's architecture closer to that normally found in nature. Another restriction of the original model was that only the apexes can grow or branch. In the present model, this option is given to all root modules, allowing a closer approximation to the real choices that occur during root growth. Both models share the same set of nutrients that are available in the soil; however, the model described here also considers carbon, which is supplied to the root by the aerial part of the plant. Finally, the model uses a wider array of parameters, with biological justification. The root growth model provides the user with access to all the parameters, which can be changed through a user-friendly graphical interface, and is available at http://labmag.di.fc.ul.pt/rootsim/sim.

4.2 Description of the Model

The explanation of the model will be subdivided in two sections. The first deals with the description of the medium the root will explore, that is, the soil model, and the second characterizes the root components and the root growth process.

4.2.1 Soil

The soil is modeled as a tridimensional grid of points that represent the center of cubic sections, arranged in a parallelepiped of given height, width, and length. Each of these sections possesses a certain quantity of water, phosphorus (P), and N. The concentration of each substance is obtained based on the soil's capacity, which is a configurable value that defines the maximum quantity of each nutrient that a soil cube can contain.

To create a soil nutrient distribution for each nutrient, supply points are defined from which a nutrient gradient is generated, reducing the concentration according to the 3D Manhattan distance to the supply point. These points can be generated randomly or at previously defined coordinates. Since P does not diffuse and since it is quite probable that no P supply point will be created in the vicinity of the seed, the lack of P would likely cause the premature death of the seed. To mitigate this problem, a given initial P concentration can be generated throughout the soil volume.

A layer of soil cubes is generated for each face of the parallelepiped to model boundary conditions, representing the water flux into and out of the model, as well as to confer a certain dynamic to the nutrient distribution. The trait of water sink or water source is randomly attributed to each cube of this layer. The sources will provide the neighboring cubes with water, while the sinks will absorb it. The probability of a certain cube becoming a water source or sink is a configurable value.

The diffusion of nutrients through the soil takes place in each step of the simulation. All soil cubes are updated in a random order, so as to avoid artificially influencing nutrient propagation. Water is diffused according to a set of rules based on the process of simple diffusion. N, being water soluble, is transported by it through the soil. Since P is almost immobile (Marschner 1995), it does not diffuse in the model.

The diffusion process obeys the following rules, repeated once for each of the six neighbors of a soil cube[1]:

$$
\begin{aligned}
&[H_2O]\ \text{dif} = \left([H_2O]\ \text{neighbor} - [H_2O]\right)/2 * \%\ \textit{Soil Diffusion}\\
&[H_2O]\ \text{final} = [H_2O] + [H_2O]\ \text{dif}\\
&[H_2O]\ \text{neighbor final} = [H_2O]\ \text{neighbor} - [H_2O]\ \text{dif}\\
&[N]\ \text{dif} = \left([N]\ \text{neighbor} - [N]\right)
\end{aligned}
$$

If both $[H_2O]$ dif and [N] are negative:

$$
\begin{aligned}
&[N]\ \text{final} = [N] - [N]\ \text{dif} * [H_2O]\ \text{dif}\\
&[N]\ \text{neighbor final} = [N]\ \text{neighbor} + [N]\ \text{dif} * [H_2O]\ \text{dif}
\end{aligned}
$$

If both $[H_2O]$ dif and [N] dif are positive:

$$
\begin{aligned}
&[N]\ \text{final} = [N] - [N]\ \text{dif} * [H_2O]\ \text{dif}\\
&[N]\ \text{neighbor final} = [N]\ \text{neighbor} + [N]\ \text{dif} * [H_2O]\ \text{dif}
\end{aligned}
$$

For the purpose of diffusion, the sinks and sources of the exterior plane operate as cubes with water concentration levels of 0 and 1, respectively.

[1] In the rule, the [x] denotes concentration of substance *x*.

4.2.2 Root

The root model is based on a modular conception of the root (Robinson et al. 2003) that it is composed of a set of modules, each being connected to the module from which it originated and to the modules that originate from it. A module occupies a soil cube in its totality, not allowing the existence of another module in the same cube. Therefore, all root growth is performed from cube to cube. This restriction implies that all root modules will have approximately the same length. Similarly to the soil, each module possesses a certain quantity of water, P, and N and a certain capacity. In contrast to the soil, the capacity of which remains constant throughout the computation, the capacity of each module can increase during the computation, so as to simulate root thickening. Besides that difference, each module also possesses a certain quantity of carbon.

The root begins its development from a seed (initial root module) placed in the middle of the superior plane of the soil parallelepiped. The seed contains certain quantities of each nutrient, and is in contact with another module that represents the aerial part of the plant. This module is located outside the soil and is not visible in the model.

Each module also has an associated level. The seed and the modules that depart from it are of level one. The first descendant of each module keeps the same level as its ascendant, but the other descendants go up a level. Thus, level one modules act like a structuring "spine" from where level two modules branch (see Fig. 4.1). Level two modules, in turn, will act as a "spine" for level three modules and so on. It is possible to configure the maximum level and thus control whether the root architecture will be more dichotomized or herringbone.

A module split can be inhibited, so that it cannot have more than one descendant. Since the descendant will share the level of its ascendant, it is considered its extension. The number of inhibited modules between each normal one can be configured so that, if it is raised along with the soil height (number of cubes), an increase in the model definition can be simulated.

Root growth takes place at discrete moments of time when the following steps occur, in order:

- Diffusion of nutrients through the root modules.
- Uptake of nutrients and water by the root.
- Every module is given the chance to grow or branch.
- Every module that possesses a high quantity of a certain nutrient raises its capacity.

The diffusion process in the root modules starts with the original module (the seed), proceeding to its descendants, and so on. The following rules are applied for water, N, P and carbon, and are repeated for each descendant:

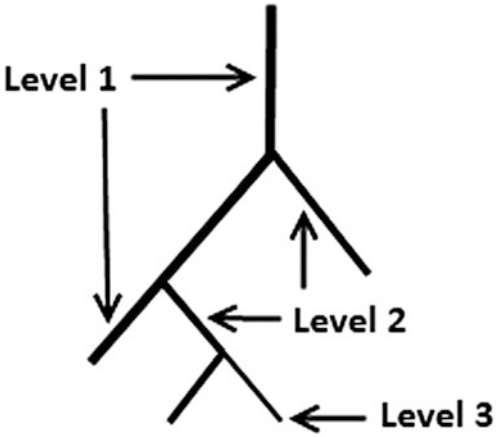

Fig. 4.1 Scheme of root levels

$$\text{Nut dif} = \left(\left[\text{Nut}\right]\ \text{desc}\right) - \left[\text{Nut}\right]\right) * \left(\text{Cap desc} * \text{Cap}\right) / \left(\text{Cap desc} + \text{Cap}\right)$$
$$\text{Nut final} = \text{Nut} + \text{Nut dif} * \text{“}\%\textit{Root Diffusion}\text{”}$$
$$\text{Nut desc final} = \text{Nut desc} - \text{Nut dif} * \%\textit{Root Diffusion}$$

Diffusion also occurs between the seed module and the one that represents the aerial part of the plant. This module acts as a sink for all nutrients, having a fixed low concentration of water, N, and P. It is through this module that the root receives carbon. At the start of the diffusion step, the aerial module produces a quantity of carbon equal to that necessary for the growth of all the apexes multiplied by a configurable parameter. Hence, it is possible to control the supply of carbon to the root. The sequential update order used for nutrient diffusion also facilitates carbon spread throughout the root.

After the diffusion step is complete, the model simulates uptake, by which all modules less than two modules from an apex are updated according to the following rules[2]:

$$\text{Nut dif} = \left(\left[\text{Nut}\right]\ \text{soil} - \left[\text{Nut}\right]\right) * \left(\text{Cap soil} * \text{Cap}\right) / \left(\text{Cap soil} + \text{Cap}\right) * \text{“}\%\textit{Root Diffusion}\text{”} * \text{distance}$$
$$\text{Nut final} = \text{Nut} + \text{Nut dif}$$
$$\text{Nut soil final} = \text{Nut soil} - \text{Nut dif}$$

The latter two rules only apply if “Nut dif” has a positive value, for it is assumed that the root cannot lose nutrients to the soil due to diffusion.

The value of *distance* is used to simulate the loss of absorption capabilities as the modules range further from the apex. The *“% Soil Diffusion”* and *“% Root*

[2] In the rule, *distance* equals 1 if it is an apex; 0.8, if it is one module from an apex; and 0.5, if it is two or more modules from an apex.

Diffusion" values define the maximum quantity of diffusible nutrients that are effectively diffused. They simulate the diffusion speed and reduce skewed results due to asynchronous actualization of the soil and root.

After the uptake step, all root modules "decide" whether they will grow or branch, according to the following rules:

- If the module possesses any nutrient with a quantity under a certain configurable value or all nutrients over other configurable values, it doesn't grow or branch, since it doesn't have enough nutrients to grow or it has excess and, as such, has no need to explore the soil**.** It also will not grow if the ratio of the soil concentration of the nutrient that it possesses in least quantity by the concentration of that nutrient in the module is below a specified value chosen by the user. Lastly, it should be noted that if the module is inhibited or if its level is equal to the maximum allowed, it will not be allowed to generate more than one descendant.
- If all nutrients are present in a quantity above defined values, the module grows, extending one unit. To calculate the direction of growth, the surrounding unoccupied soil cubes are compared by the concentration of the nutrient that the module possesses in least quantity, and the cube with the highest concentration is chosen as the direction of growth. However, it is assumed that the detection of nutrients occurs due to some form of nutrient diffusion; thus, the concentration perceived in each soil cube is divided by the distance from that cube to the module. There is no cutoff point beyond which the apex is no longer able to detect nutrients as the search range is defined solely by the user. If two or more soil cubes possess identical concentration values, one is randomly chosen. After the creation of the new module, the original passes onto the descendant a certain percentage of all of its nutrients.
- If the concentration of all nutrients is more than twice the value defined for growth, the module branches, forming two new modules, sequentially, as described above. When an apex branches, the first new module has the same level as the apex, and the other has the next level. Half of the time, the nutrients are distributed equally between the two new modules; the rest of the time, one is favored with a higher supply of nutrients.

4.3 Implementation

The entire model was programmed in Java SE 1.6, using the graphical library JavaView 3.95.001. It is constituted by five classes: *AppletRootSoil*, *RootSoil_IP*, *RootSoil*, *Root*, and *Soil* (Fig. 4.2).

The *Root* class represents the root and was created with a data structure of a generic tree, in which each node represents a root module and, consequently, the leaves represent the apexes. Each node can have any number of descendants and saves a reference to the ascendant. The *Soil* class represents the soil and was created using two arrays that store the data of all the soil cubes: an array of doubles, to store

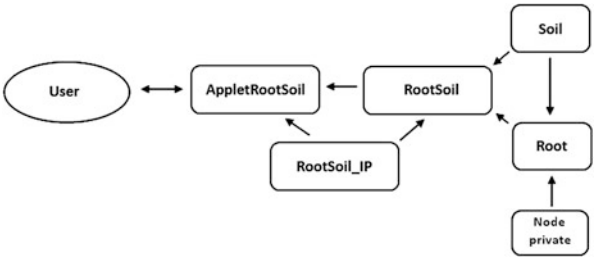

Fig. 4.2 Structure of the model classes

the nutrient quantity, and another of Booleans, to keep the state of occupation. The *RootSoil* class serves as the JavaView project and deals with the interactions between the *Root* and *Soil* classes, as well as with their graphical representation. *AppletRootSoil* is an applet that uses the JavaView project, in this case the *RootSoil* class, and its interface, the class *RootSoil_IP*.

The model was created as an applet with the aim of allowing easy operation by inexpert users. The use of a Java applet also allows the model to be run from any Internet browser that supports Java.

One of the focal points during the development of the model was the maximization of its flexibility, allowing the user to have maximum control over root development parameters. Accordingly, user interaction with the model takes place through a control panel that allows control over various parameters that might influence root growth (Fig. 4.3). All the parameters are presented with predefined functional values, which do not necessarily possess any associated biological meaning. Since the specific value of each parameter is set by the user, the choices made at the programming level focused on which parameters to introduce in the model and not on specific values which would be required to precisely simulate a certain type of root.

4.4 Interpretation and Inference

As initially mentioned, the purpose of this work was to verify whether the swarm intelligence property could be used to model the decisions that occur in the process of root growth and soil exploration.

For example, it is clear from the simulated root represented in Figs. 4.4 and 4.5 that at least two out of the three main ramifications explored soil areas that contained abundant quantities of both P and N, as expected. Plants generally increase root growth in nutrient-rich patches; however, there are exceptions (McNickle et al. 2009). Plants should only proliferate into patches if the potential for benefits outweighs the costs. In this context, it was interesting to note that the third ramification, in the foreground of the figures, did not explore an area of the soil particularly rich in neither P nor N. What seems to have occurred is a combined maximization of the concentrations of both nutrients, since the root explored a

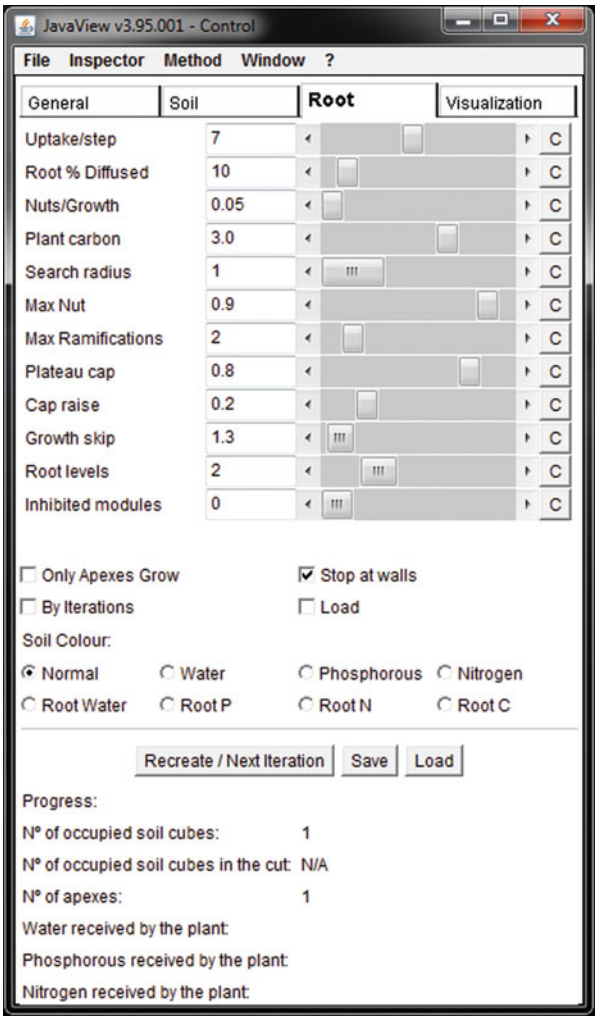

Fig. 4.3 Snapshot of the control panel

patch of soil where two deposits, one of each nutrient, crossed. Given that at any given moment a root module is only searching for a certain nutrient, the emergence of this type of behavior is interesting and is in agreement with observed root behavior (McNickle et al. 2009).

Root growth is highly asymmetric, reflecting the roots' ability to adapt to environmental factors such as the presence or absence of nutrients (López-Bucio et al. 2003). It has been documented that the roots of many species show localized bursts of growth when confronted with nutrient deposits. Thus, the root uses the resources necessary for growth in areas of the soil that are richest in nutrients (Forde and Lorenzo 2001). This behavior can also be observed in the simulations. It is clear

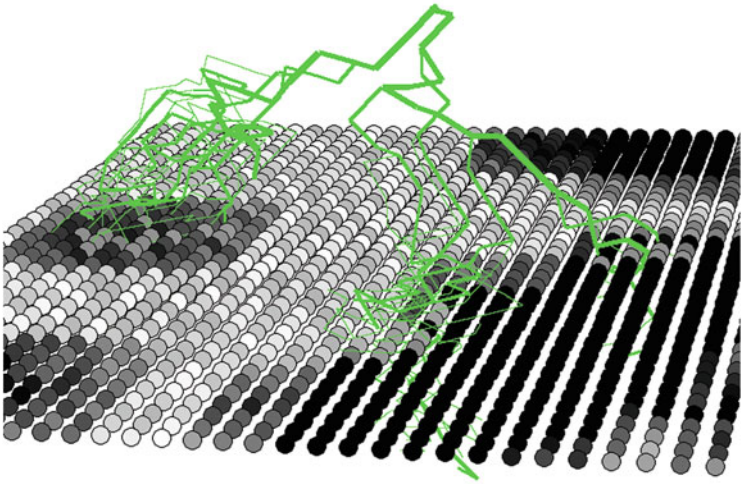

Fig. 4.4 Simulated root with soil section representing the P concentration (Each *point* represents a soil cube. *Darker color* denotes higher concentration)

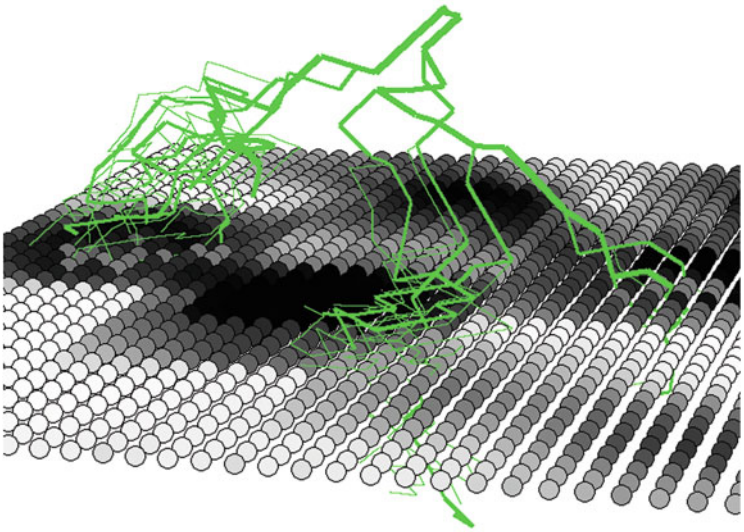

Fig. 4.5 Same simulated root as in Fig. 4.4 with soil section representing the N concentration (Each *point* represents a soil cube. *Darker color* denotes higher concentration)

Fig. 4.6 Simulated root in homogeneous soil

that roots initially expand without many ramifications, only starting to ramify when they encounter soil sections with high concentrations of nutrients.

It has also been reported that roots present a compacter, shorter, and denser architecture in nutrient-rich media than in less rich ones (Forde and Lorenzo 2001). For comparison's sake, a root was simulated using the same parameters as above but growing in a soil with highly homogeneous nutrient concentrations. The observed result seemed to match this response (Fig. 4.6).

Comparing the two previously simulated roots with those that might be found in a natural context, it is observed that the latter resembles what would be expected from a plant growing in a cultivated medium, while the former would be expected from a root that developed in a more heterogeneous medium (Hinsinger et al. 2005; Hodge 2004).

How is it then, that the apexes coordinate themselves to build an overall root architecture beneficial to the whole plant? The nutrient concentration is equivalent through all of the modules of a plant's roots, regardless of the concentration of that nutrient in the respective soil cube. With this knowledge, it can be theorized that each module makes up for its own lack of information of the global root state via the process of internal nutrient diffusion. With it, the module can, from only the concentration of nutrients present within itself, obtain a general perspective of the nutrient levels in the whole of the root. If a certain nutrient is quickly diffused from the module to its neighbors, this is because it is lacking in the rest of the root. This outward flow of the nutrient maintains its concentration in the module low, which

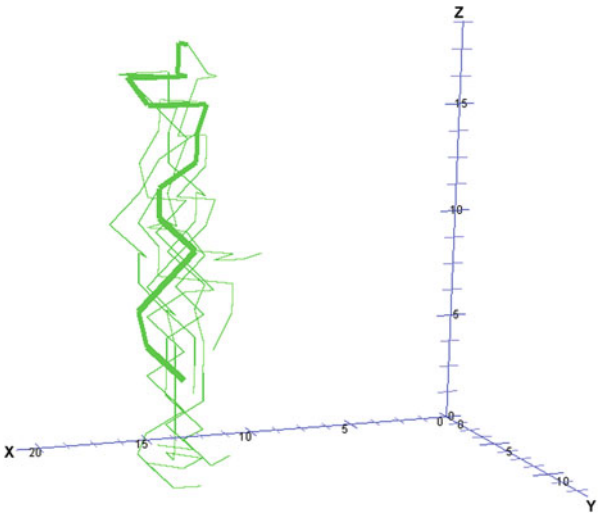

Fig. 4.7 Herringbone simulated root with deeper soil nutrient distribution

causes the module to keep searching for that nutrient, regardless how abundant it might appear in its vicinity.

Another reported phenomenon of root growth is the capability of the apexes to avoid one another, avoiding internal competition (Falik et al. 2006). Given that this occurrence has already been explained by communication between the apexes through the use of chemical compounds, it is possible to complement them through this model (Hodge 2009). During the exploration of the soil, each module captures nutrients from the soil cube it occupies, causing them to have a lower concentration of that nutrient than the neighboring soil cubes. By diffusion, these soil cubes will start to receive that nutrient from their neighbors. This process is then replicated by its neighbors, since they too suffer from a decrease in their nutrient concentration. Thus, the soil cube that the module occupies acts as a nutrient sink, provoking a nutrient gradient in the surrounding zone. If we consider that the majority of the modules will be searching for soil sections with a high concentration of the same nutrient, the creation of this gradient surrounding every module leads other modules to avoid this section due to its lower nutrient concentration. It should be noted that this explanation cannot be applied where the limiting nutrient is P, which cannot diffuse through the soil. However, this is logical: if there is no diffusion, there is also no competition between two neighboring modules.

By altering the parameters of the root, various types of roots can be obtained. For example, if the nutrient requirement for the growth of each module is increased, root density decreases. This change may also lead the root to explore the soil differently, as nutrients that were not scarce under the previous growth requirement might become so. By simulating two roots in a similar soil environment, but with different parameters, it is possible to obtain two very different architectures, one herringbone (Fig. 4.7) and the other dichotomic (Fig. 4.8). In order to simulate these

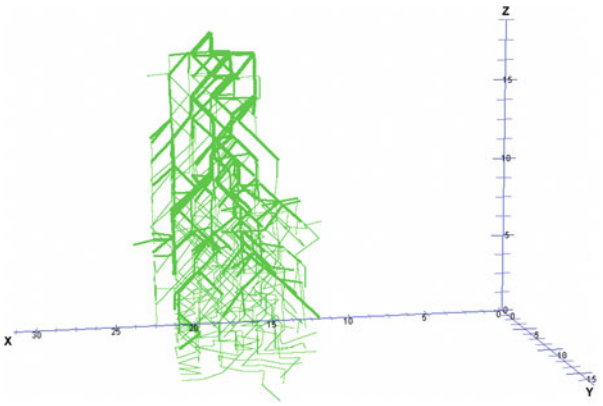

Fig. 4.8 Dichotomic simulated root with deeper soil nutrient distribution

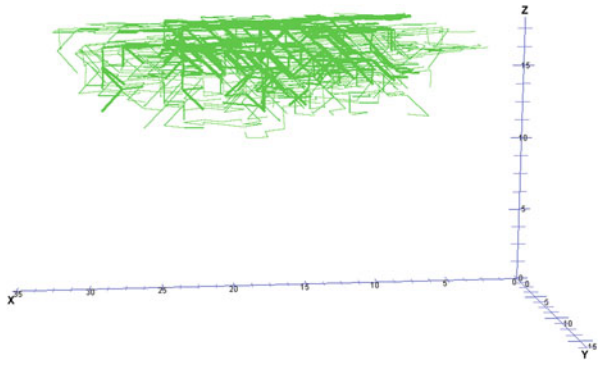

Fig. 4.9 Simulated root with superficial soil nutrient distribution

two different root architectures, only the limit of levels and ramifications allowed to the root was modified. All other parameters, as well as the soil nutrient distribution, remained the same.

By controlling the nutrient distribution in the soil, instead of the root parameters, it's possible to cause the same root parameters to give rise to a variety of root architectures, for example, the above comparison of roots grown in homogenous and heterogeneous soils. As a further example, another root was generated, using the same root parameters, but differing as to the nutrient distribution in the soil. Figure 4.8 shows the root architecture where nutrients were distributed at depth as in a natural nutrient-rich soil, while Fig. 4.9 shows the roots in a soil where nutrients were distributed superficially, as in an irrigated soil. In both cases, the root responds appropriately, by either exploring the soil deeper or growing close to the surface.

4.5 Conclusion

From the above, it is clear that the described model provides good simulations of soil exploration by roots, presenting architectures comparable to those found in natural roots. The model replicates the more general responses to the various nutrient distributions attributed to root's soil exploration strategy, demonstrating the viability of the interpretation that root growth is a form of swarm intelligence, emerging from the simple local behaviors of the various modules. In fact, the observations made reinforce this hypothesis and are in agreement with the regulatory effects of N on root architecture (Ruffel et al. 2008). Further studies will undoubtedly be needed to understand the mechanism integrating the intrinsic processes associated with root growth and the swarm intelligence behavior associated with each of the active agents of root growth, the apexes.

By introducing a swarm intelligence approach in root growth models, it should be possible to engineer the root architecture that best fits a certain practical situation, for example, optimization of root architecture to stabilize steep soils against slippage or root architectures that would be effective to fix trees in shallow soils, such as those found in most urban landscapes.

By further simulating root growth in different soils, with differing nutrient concentrations, we should be able to gain information about the decisions that occur in the process of root growth and soil exploration. With this knowledge, we can hope to engineer root architecture, by distributing the nutrients in the soil, so that the root growth promotes nutrient-use efficiency. In modern plantations, fertilizers need to be used in great quantities to provide the necessary nutrients to the plants. Since the roots cannot capture all of the nutrients provided, much of this fertilizer is wasted and pollutes surface and groundwater. Consequently, by raising the soil exploration efficiency of the roots, it is possible not only to cause the roots to capture the same amount of nutrients with less fertilizer application but also to reduce the impact of nutrient leaching on the environment.

The model presented is not without limitations. The fact that the soil is represented by cubes forces the model's roots to always grow modules of approximately the same size and in a discrete manner. Other limitations, which more directly pertain to root soil exploration, are that the model considers neither the mutualistic association between certain roots and fungi, the mycorrhizae, nor root hairs. However, it is known that both improve nutrient acquisition (Smith and Read 2008). Lastly, the relations between the root and the aerial part of the plant were also extremely simplified. Notwithstanding, it is believed that these limitations do not disprove the obtained results.

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Chapter 5
The Development of the Maize Root System: Role of Auxin and Ethylene

María Victoria Alarcón, Pedro G. Lloret, and Julio Salguero

5.1 Introduction

Maize (*Zea mays*) is an herbaceous monocotyledon with an annual cycle. When the seed germinates, a primary or seminal root arises from the embryonic radicle. This root elongates rapidly and, as it grows, forms very many new lateral roots (LRs). The primary root may persist throughout the plant's life, although frequently it ceases growing and branching (Feldman 1994). Consequently, it has relatively little functional importance after the first stages of seedling development (Hochholdinger et al. 2004). The mature plant has an extensive fibrous root system consisting of several whorls of adventitious roots, some of which become widely spread, horizontally growing roots, while others become deeply penetrating vertical roots. Because they originate from shoot nodal regions, these adventitious roots are often categorized as nodal roots. Post-embryonic nodal maize roots have been subdivided into crown and brace roots according to whether they initiated from underground or aboveground nodes, respectively (Hochholdinger et al. 2004).

Despite its relatively poor functional importance, the primary root is a wonderful system in which to analyse basic processes of development such as (i) organ polarity establishment, (ii) cell proliferation regulation, (iii) cell fate decisions,

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(iv) cell signalling, (v) cell growth coordination, etc. Together, these processes occur in a beautiful and highly ordered histological context and are affected by multiple physicochemical factors, some of which have long been well known, while others are the object of current research.

In order to draw rigorous conclusions, the study of the physiology and molecular biology which regulates root development requires an adequate analysis of the temporal evolution of the histological structure of the root. In this sense, the present study is an attempt to contribute to understanding how the primary root system proceeds in maize and what is the presumptive role played in this process by two specific phytohormones: auxin and ethylene.

The development of the maize root system has two main aspects which occur simultaneously although they are conceptually different. Firstly, it is necessary to understand how each individual root develops by a continuous contribution of new cells at the root apex and the subsequent growth of these cells. Secondly, one must consider how new root meristems destined to originate lateral and adventitious roots are formed endogenously in mature root or shoot tissues.

The present work is mainly concerned with the description of the development and branching of the primary roots. Adventitious roots follow paths of development which have been appropriately described in maize and other related species elsewhere (Erdelska and Vidovencova 1993; Hetz et al. 1996; Mergemann and Sauter 2000).

5.2 Morphology and Structure of the Primary Maize Root

5.2.1 General Morphology and Growth Zones

The maize primary root is a cylindrical structure, finished at the tip of its distal end by the presence of the root apex (Fig. 5.1a). It is generally considered to consist of a series of consecutive root zones. The root apex comprises approximately the distal-most 2 mm of the root extension (Fig. 5.1a). It contains the root apical meristem, consisting of undifferentiated meristematic cells that are in continuous cell division. Its function is to produce the cells destined to constitute the various tissues of the root.

Just above the root apex is located the elongation zone that extends approximately from 2 to 10 mm behind the tip (Fig. 5.1a). In this region, the cells stop dividing and begin to grow intensely and predominantly in a direction parallel to the longitudinal root axis in a process known as cell elongation. Initially, in the apical-most part of this zone, cell growth is both longitudinal and transverse, whereas subsequently, in the rapid elongation zone, it is only longitudinal (Ivanov 1997).

In the root zones located at more than 10 mm from the tip, one may consider the cells to have reached their definitive length, and cell differentiation begins. This region is defined as a maturation zone. In it, the vascular tissues progressively

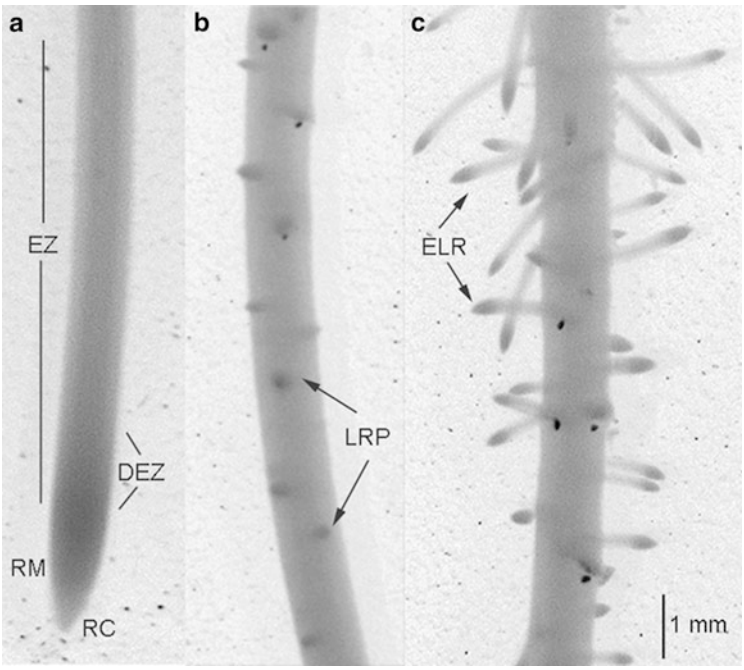

Fig. 5.1 Segments of the primary root of *Zea mays* showing the morphology of root apex and the development of lateral roots. (**a**) Root apex and elongation zone. (**b**) Maturation zone (distal). (**c**) Maturation zone (proximal). *RC* root cap, *RM* root meristem, *DEZ* distal elongation zone, *EZ* elongation zone, *LRP* lateral root primordia, *ELR* emerged lateral roots. Bar: 1 mm

acquire maturity. The root hairs are formed and begin their development at this level of the root, and the LRs continue their growth (Fig. 5.1b, c).

5.2.2 Histology of the Primary Root

The arrangement of tissues that form the body of the primary root can be studied by means of transverse and longitudinal sections. In the root, as in the rest of the plant organs, three main systems of tissues can be distinguished: the epidermis, the cortex, and the vascular cylinder.

The outermost layer of cells of the root is the epidermis (Fig. 5.2a). The epidermis is uniseriate in maize, and the cell walls are asymmetrically thickened. Epidermal cells are elongated parallel to the long axis of the main root, and their internal content is very clear because almost all of the cell volume is occupied by a large vacuole. The cell nucleus is usually located away from the centre in a position adjacent to the cell wall (Fig. 5.2a).

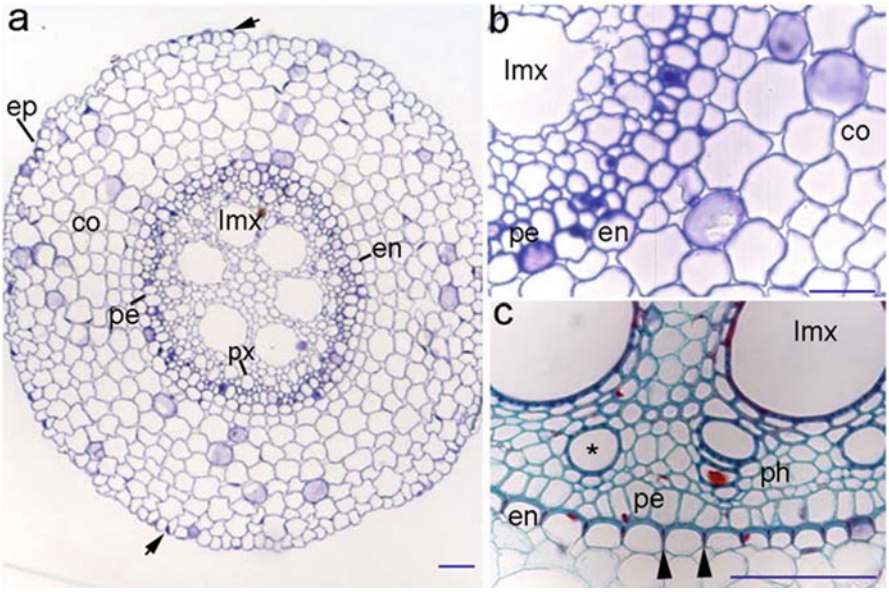

Fig. 5.2 Cross section of maize roots. (**a**) General view at the end of the elongation zone (toluidine blue staining, TBS). (**b**) Detail of the vascular cylinder and inner cortex at the end of the elongation zone (TBS). (**c**) Detail of the vascular cylinder and inner cortex at the proximal part of the maturation zone (safranin-fast green staining procedure). Note in (**a**) that the nucleus of the epidermal cells are displaced to the periphery of the cell (*arrows*) and in (**c**), the U-shaped wall of endodermal cells (*arrowheads*) and the early metaxylem elements (*asterisk*). *ep* epidermis, *co* cortex, *en* endodermis, *pe* pericycle, *px* protoxylem pole, *lmx* late metaxylem, *ph* phloem. Bars: 50 μm

The cortex consists of six to ten layers of parenchymatous tissue with small intercellular spaces at the angles of the cells (Fig. 5.2a). The outermost and innermost cortex layers have special characteristics and are called the exodermis and endodermis, respectively. The cells of these two layers are highly specialized, and in most higher plants, their cell wall development passes through three stages (Clarkson and Robards 1975). In Stage I, they deposit Casparian strips on their radial and transverse cell walls. Later in ontogeny (Stage II), a suberin lamella is deposited between the primary wall and the plasmalemma. Thanks to the Casparian strips and the suberin lamellae, the system of cortical extracellular spaces remains sealed, preventing the access of ions to the vascular cylinder via the apoplast (Enstone et al. 2002). In some species, these cells progress to a third stage of development (Stage III) with the deposition of a tertiary cellulose wall. This can be observed very clearly in the maize endodermis which, near the apex, presents a slightly thickened cell wall (Fig. 5.2b), while further away it presents the characteristic U-shaped tertiary cell wall with clearly discernible asymmetric thickening (Fig. 5.2c). The transition from Stage II to Stage III is not always sharply demarcated (Clarkson and Robards 1975).

The first layers of the vascular cylinder form the pericycle. The maize root pericycle is initially single layered, consisting of thin-walled parenchymatous cells (Fig. 5.2a–c) which may become thick-walled later in ontogeny (Sutherland and McCully 1976). Pericycle cells are capable of recovering their meristematic activity and of initiating the process of LR production.

The vascular cylinder has an alternating organization of xylem and phloem poles, which is typical of most roots. The number of protoxylem poles is variable in maize roots, ranging from 20 to 40. There are commonly two or three protoxylem strands to each large metaxylem vessel (Fig. 5.2c). The primary phloem poles alternate with the protoxylem poles and are in contact with pericycle cells with a particularly large cross-sectional area (Fig. 5.2c). Often, parenchymatous cells that are located between the xylem and phloem poles eventually present thickened and lignified cell walls. The centre of the root is occupied by a parenchymatous tissue called pith, which extends between the vascular tissues until it reaches the pericycle (Fig. 5.2a).

The LRs originate as endogenous anlages named lateral root primordia (LRP). The LRP primarily consist of an arc of pericycle which more or less extends over three phloem and two intervening xylem poles (Fig. 5.3a). Although most of the cells that constitute the LRP derive from the pericycle, there is an endodermal cover which is clearly incorporated into the primordium (Fig. 5.3a). Longitudinal sections show that the early LRP consist of two short pericycle cells lying end to end in the same column, flanked above and below by two longer pericycle derivatives (Fig. 5.3b). More developed LRP gradually make their way through the cortex of the parent root (Fig. 5.3c, d) until they reach the level of the epidermis and break through it to emerge as young LRs (Fig. 5.1c).

5.3 Root Development

In general terms, biological development can be defined as a series of gradual changes in size, structure, and function affecting individuals, organs, or cells. The development of a root basically consists of the processes of growth and differentiation that represent, respectively, quantitative and qualitative changes (Segura 2000). In the study of the development of the root system of any plant, it is necessary to consider two specific aspects: the growth and differentiation of each individual root and how new LRs are formed.

The classical concept of the development of an individual root considers successive zones of division, elongation, and maturation. The source of cells for the body of the growing root is the root apex. Cells produced in the root apex are usually regarded as members of a larger unit, undergoing more or less abrupt transitions between different developmental stages at the same time as they transit specific root zones.

In most angiosperms, root branching occurs far from the apical meristem when specific founder cells initiate a sequence of events that leads them to create a new

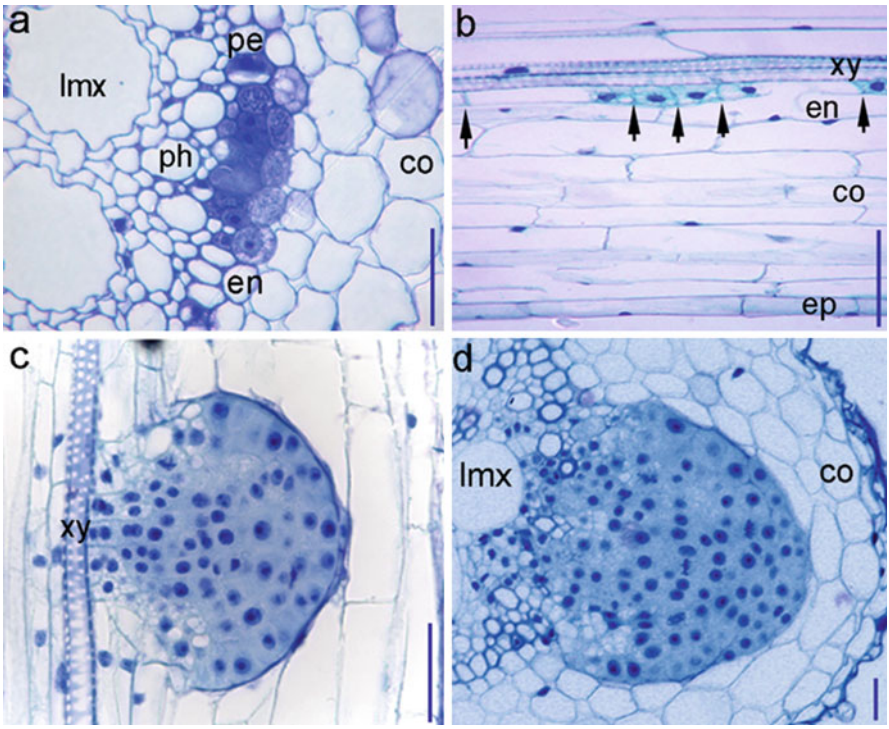

Fig. 5.3 Development of lateral root primordia (LRP) in *Zea mays*. (**a**) Transverse section of an arc of basophilic cells from the pericycle and the endodermis that are initiating an LRP (toluidine blue staining technique, TBS). (**b**) Longitudinal section of a column of pericycle where two founder cells have undergone the first transverse divisions during LRP initiation (Feulgen staining technique). Note that four cells, two short central and two long peripheral, can be distinguished. *Arrows* indicate the transverse walls of each cell. (**c**) Longitudinal section of a maize root segment along a xylem pole (TBS). Note that the LRP of the image has a perfectly organized apical meristem and has crossed more than half of its way towards the external surface of the parent root. (**d**) Transverse section of maize root showing an LRP in a more advanced stage of development (just emerging) which extends covering an arc of pericycle of approximately three phloem poles and two intervening xylem poles (TBS). *co* cortex, *en* endodermis, *pe* pericycle, *xy* xylem strand, *lmx* late metaxylem, *ph* phloem. Bars: 50 μm

apical meristem inside the parent root. Because founder cells belong to the outer cell layer of the vascular cylinder, i.e., the pericycle, LRP are initially endogenous structures (Lloret and Casero 2002). Nevertheless, they quickly grow through the parent root cortex (Fig. 5.3c, d) and break the epidermis to emerge as young LRs (Fig. 5.1c).

In the following, we shall discuss some basic aspects of the development of the root system of plants, with special emphasis on the situation in maize.

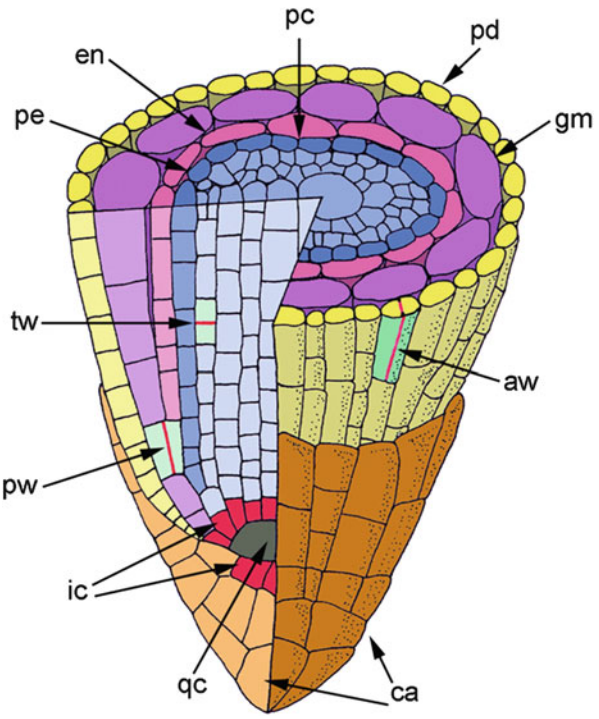

Fig. 5.4 Schematic representation of the maize root apex including an explanation of the different orientation planes of cell divisions in meristematic cells. *ca* root cap, *qc* quiescent centre, *ic* initial cells, *pw* periclinal wall, *aw* anticlinal wall, *tw* transverse wall, *pe* pericycle, *en* endodermis, *pc* procambium, *pr* protodermis, *gm* ground meristem

5.3.1 The Root Apex

The structure and developmental dynamics of the apex of both the main root and the LRs are similar. There is always a terminal root cap and a sub-terminal root meristem (Figs. 5.4 and 5.5).

The root cap forms a cover at the tip of the apex and consists of cells that differentiate quickly, secrete mucilaginous compounds, and slough off as they reach the organ's surface. In addition to its protective function, the root cap also serves to sense the direction of gravity and other environmental signals (Sievers et al. 2002).

Interposed between the root cap and the meristem, there is a special region which is very important for both the organization and the physiology of roots: the quiescent centre (QC). The QC is discoid in shape (Fig. 5.4) and consists of cells which, on average, divide relatively infrequently (Fig. 5.5c). Clowes (1958) showed that, in the root apex, the cells undergoing DNA synthesis and mitoses are located around the QC. This means that the functional initials of root tissues are really two groups of cells located above and below the QC. The initial cells located below the QC will, by repeated cell divisions, form the root cap, whereas those located above the QC will form the rest of the root tissues. Often this latter cell population is considered to constitute the promeristem, from which originate the true primary

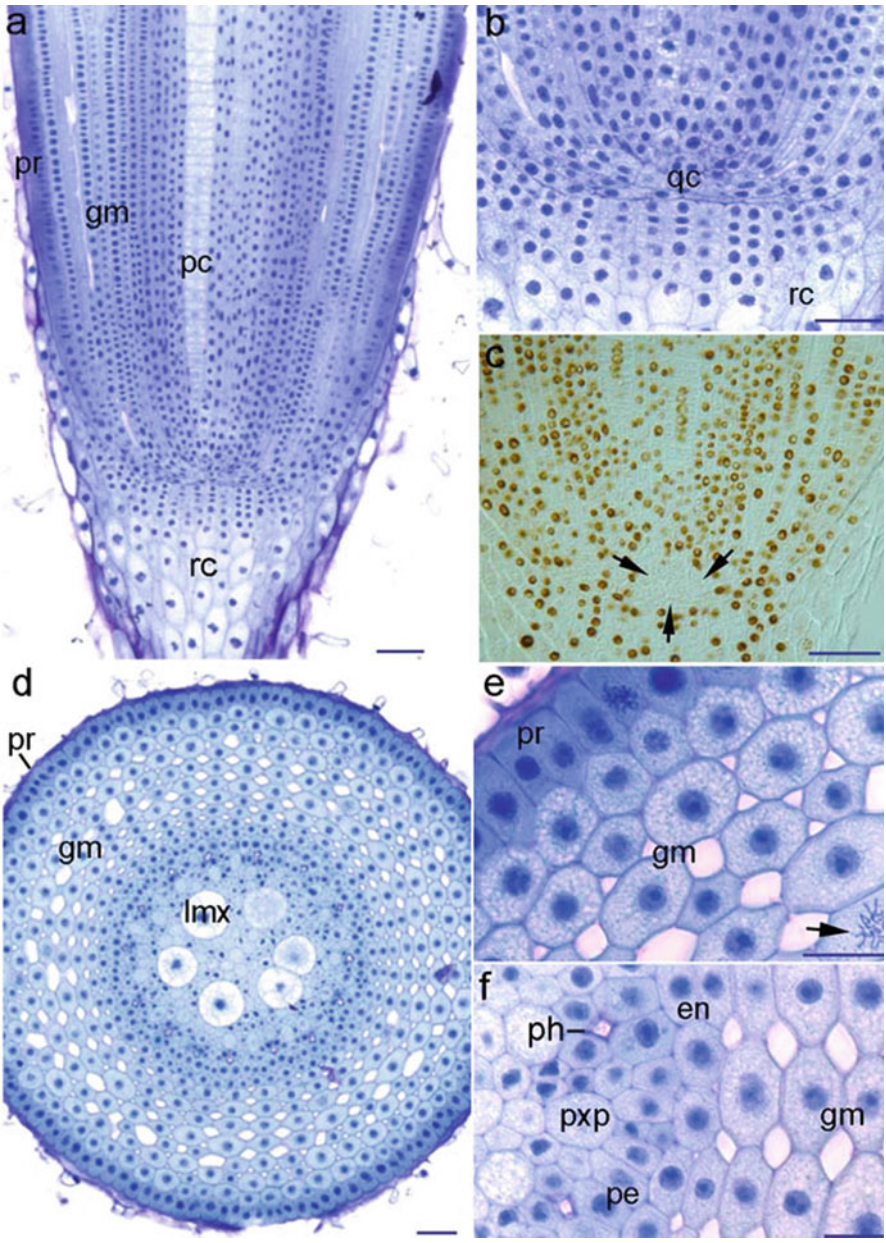

Fig. 5.5 (**a**) Longitudinal section of a maize root apex showing the overall organization (toluidine blue staining technique, TBS). (**b**) Detail of the promeristem and the quiescent centre as well as the base of the root cap. The cell columns of the ground meristem can be followed through the quiescent centre, leaving the initial cells of the root cap at a lower typical position of closed meristems (TBS). (**c**) The application of an immunocytochemical technique to detect proliferative cells (BrdU labelling technique) in the promeristem region of the corn root apex delimits clearly

meristematic tissues of the root apex: protodermis, ground meristem, and procambium (Fig. 5.4). To prevent depletion of the initials, after every division of each initial cell, the distal daughter retains its status of initial cell. Therefore, the initial cells are in theory capable of self-perpetuating indefinitely.

It has been found that there is a very high rate of auxin release in the QC by the mechanism of polar auxin transport (Kerk et al. 2000). Also, high auxin concentrations in the QC have been correlated with high ascorbic acid oxidase activity, which in turn results in low ascorbic acid concentrations and the establishment of the characteristic quiescence of this region (Kerk et al. 2000). Functionally, there is growing evidence that the QC is an organizing structure, and it has been shown to maintain the stem cell's niche necessary for continuous root growth via the expression of the homeobox gene WOX5 (Sarkar et al. 2007).

Most cell divisions in the root meristem occur with the new cell wall in a plane perpendicular to the root's long axis (transversal divisions), and the cells are arranged in long columns (Fig. 5.4). The outer columns of the meristem constitute the protodermis which, when it matures, forms the epidermal covering of the root. Further inwards is the ground meristem which gives rise to the cortex in the mature root, and in the centre is the procambium which gives rise to the vascular cylinder (Fig. 5.4).

The development of the primary root includes the processes of cell division, elongation, and differentiation. In principle, cell divisions are restricted to the promeristem and the meristem in the proximal part of the root apex and to the initial cells of the root cap and their immediate derivatives in the distal face of the QC. All the cells which will eventually form the root body are generated through the proliferation of the procambium, ground meristem, and protodermis. In maize roots, longitudinal growth occurs within the region extending 12 mm from the tip (Silk 2002). Although the maximum growth rate is attained at a distance of 4–6 mm from the apex (Silk 2002), cells in the meristematic region also elongate along the apical-basal axis at a slower growth rate. Consequently, one may assume that the processes of cell division and elongation overlap at the root apex.

Within the apical meristem, two types of cell division take place: formative and proliferative (Barlow 1984). The formative divisions follow an anticlinal (i.e., perpendicular to the root surface) or periclinal (parallel to the root surface) plane and have the function of producing new columns of cells (Fig. 5.4). New tissues can be established by means of this kind of cell division. For example, in most angiosperms the epidermis and cortex share a common ontogenetic origin in the root apex initial cell zone (Clowes 1994). The proliferative divisions have the equatorial plane oriented transversely with respect to the root's long axis, thereby

Fig. 5.5 (continued) the location of the quiescent centre (*arrows*). (**d**–**f**) Transversal sections of maize root apex taken at the middle of the meristematic region (TBS). Note in (**e**) a mitotic figure in a cell of the ground meristem (*arrow*). *qc* quiescent centre, *rc* root cap, *pe* pericycle, *en* endodermis, *lmx* late metaxylem, *ph* phloem, *pc* procambium, *pr* protodermis, *gm* ground meristem, *pxp* protoxylem pole. Bars: 50 μm

giving rise to daughter cells that belong to the same column. Consequently, it becomes possible for the number of cells constituting a given column to increase while the total number of columns remains unaltered (Fig. 5.4).

Anatomically, the apical meristem of the maize root is considered to be "closed" because it is possible to continue along all cell columns up to the groups of initial cells located around the QC (Fig. 5.5b). Closed root apices are also characterized by having a clear separation between the root cap and the rest of the root body. In other species, the meristem is considered as "open" because there is no such clear continuity of columns to the QC region, and the boundaries between cortex, epidermis, and cap are unstable (Clowes 1994).

The meristematic cells of the maize root apex are very small isodiametric cells, with there being scarcely any intercellular spaces observable between them (Fig. 5.5a). They present a very thin primary cell wall and middle lamella. Their cytoplasm is dense, with many small vacuoles, and the nucleus is located centrally (Fig. 5.5d–f).

The maize root apex reaches a distance of about 2,000 μm from the distal end of the QC (root cap junction, RCJ). Most of the root apex is occupied by the meristematic region where cell divisions occur (Barlow 1987). The behaviour of cells in this region is affected by environmental changes monitored in the aerial part of the plant (Muller et al. 1998). Cell division by itself does not increase the length of the root. However, the cell division rate, the fraction of proliferating cells, and how long these cells remain mitotically active determine the rate at which cells are incorporated from the apex into the elongation zone, and therefore the root elongation rate (Beemster and Baskin 1998, 2000; Baskin 2000).

Beemster et al. (2003) divide the root apical meristem into two halves—the apical and the basal. In the apical half, cell divisions and root growth are correlated in such a way that the average cell length remains constant. Along the basal meristem, cells divide roughly at the same rate as in the apical meristem, but the elongation rate increases progressively. As a consequence, cell size also increases. The basal meristem cells share some common features with cells that are located at the beginning of the elongation zone (De Smet and Jürgens 2007). For example, it is assumed that these two areas are particularly important for integrating environmental and/or hormonal stimuli and that they modulate root growth (Ishikawa and Evans 1995; Beemster et al. 2003).

5.3.2 The Elongation Zone

In maize roots, the elongation zone (EZ) reaches to a distance of about 10–12 mm from the RCJ. It is characterized basically by the cessation of cell division, with the cells continuing their elongation at a variable rate that depends on their position. Ishikawa and Evans (1993) subdivide the EZ into five segments depending on the growth rate of the cells at each point. In the distal-most segment, the elongation growth rate is fairly slow (less than 0.3 times the maximum rate), and the cells seem

to be particularly sensitive to auxin and to environmental changes. This root segment has been termed the transition zone (Baluška et al. 1996) or the distal elongation zone (Ishikawa and Evans 1993) and is located between the apical meristem and the region where rapid elongation takes place (Ivanov 1997). Some authors argue that the distal EZ, the transition zone, and the basal meristem are terms referring to the same region of the root (Beemster et al. 2003). The key to choosing which term to use would be whether or not cell division has ceased at this level of the root.

In the segment termed the rapid elongation zone, which lies at between 3,000 and 6,000 μm from the RCJ, major growth takes place. The beginning of this segment coincides with the cessation of transverse growth (García-Sánchez et al. 1991). Further away from the RCJ, the elongation gradually diminishes, until the expansion of the wall completely ceases at about 10 mm from the RCJ (Ishikawa and Evans 1993).

5.3.3 The Maturation Zone

In their transition through the three zones, as a result of polarized growth, the morphology of the root cells changes, and they gradually acquire traits of maturity through cell differentiation. From 10 mm from the RCJ onwards, the cell length remains constant, and those cells that acquire specific features of cellular differentiation gradually mature. This is particularly noticeable in cells such as those that constitute the hypodermis and endodermis that acquire Casparian strips and suberin lamellae (Zeier et al. 1999). In this zone, the conductive elements of the xylem, and even pericycle and stellar parenchyma cells, increase the thickness of their walls and deposit lignin (Sutherland and McCully 1976). The root hairs are also formed and eventually wither (Wen and Schnable 1994). Another remarkable phenomenon that occurs in this region is the beginning of the formation of the LRP. This is initiated by a series of cell divisions in some cells of the parent root pericycle. The first divisions are transversal and significantly reduce the length of the LRP's founder cells (Fig. 5.3b) (Dubrovsky et al. 2000; Casimiro et al. 2003).

5.3.4 Lateral Root Ontogeny

The initiation of LRs is a fascinating developmental process because it involves the production of an entire organ from a small number of differentiated cells in response to intrinsic and environmental cues (Malamy 2005). In *Zea mays*, LR initiation occurs endogenously and far from the root apex, in particular, at a distance of approximately 12–15 mm behind the tip (Casero et al. 1995) when a few pericycle cells are stimulated to dedifferentiate and proliferate to form an LR primordium. The first sign of this process that is visible under light microscopy is

that, by means of coordinated asymmetrical transverse divisions, one column of pericycle cells produces two very short cells (about 25 μm long, Fig. 5.3b) lying end to end and flanked above and below by two longer cells (Fig. 5.3b). The initiation of LRs by this procedure has been described in many species, and it can be assumed that this type of LR initiation is the commonest case among angiosperms (Lloret and Casero 2002).

In the circumferential plane, the columns which start the development of LR primordia are always located just in front of the protophloem poles of the parent root (Fig. 5.3a). Later, successive rounds of transversal root divisions extend longitudinally along the same column and transversally to adjacent columns, and a small plate of short cells is formed. This plate has been termed the rhizogenous plate (Van Tieghem and Douliot 1888) and extends transversally to an arc of approximately 12 columns of pericycle cells which cover three phloem poles and the two intervening xylem poles (Fig. 5.3a). In the longitudinal plane, the long founder cells of the LR primordium undergo a series of transverse divisions which give rise to many new short cells [see Fig. 5.6 in Lloret and Casero (2002)].

The pairs of cells of adjacent columns that give rise to the primordial rhizogenous plate are known as founder cells (Laskowski et al. 1995). Once these founder cells have undergone the first asymmetric transverse divisions, the process of LR development continues quickly. The following events are a radial enlargement of cells located at the centre of the rhizogenous plate, a reduction in the size of vacuoles, and, finally, periclinal divisions. In maize LR primordia, these cell divisions are asymmetrical, giving rise to an inner cell larger than the outer cell (Casero et al. 1995). The ulterior development of LRs in *Raphanus sativus* and *Arabidopsis thaliana* has been subdivided into seven and six developmental stages, respectively (Blakely et al. 1982; Malamy and Benfey 1997).

As the LRP pass through successive stages of development, they become ever more complex. During this process, the number of cells and the size of the LRP increase (Malamy and Benfey 1997), and the LRP acquire the capacity for autonomous growth, i.e., their growth becomes self-sufficient, no longer requiring growth regulators from the parent root. This phenomenon apparently occurs when the primordium organizes its own root apex (Laskowski et al. 1995).

The LRP must break through the parent root cortex and epidermis to emerge as young LRs. As the primordia cross these parental tissues, the primordial cells of pericyclic origin are covered by a cap of cells deriving from the endodermis termed the endodermal covering (Seago 1973). From the beginning of LR development, the endodermal covering cells present morphological features like those of pericyclic-derived cells, including increased protoplast stainability and transverse divisions which produce very short cells, etc. (Bell and McCully 1970; Seago 1973). Periclinal divisions are scarce, however, so that this layer does not constitute a permanent covering of the primordia, but must instead be regarded as a temporary structure to be replaced by a true root cap formed from cells of pericyclic origin (Seago 1973; Clowes 1978).

The outgrowth of the LRP through parent root tissues requires the “dissolution” of cells located in the way of the young developing primordium. In the case of the

parent root cortex, it has been proposed that cells are pushed out by a combination of pressure (Pond 1908) and the secretion of hydrolases by the young primordium (Bonnett and Torrey 1966; Sutcliffe and Sexton 1968; Keller and Lamb 1989; Peretto et al. 1992). Also, it has been demonstrated recently that auxin promotes LR emergence through the regulation of the spatial and temporal distribution of aquaporin channels (Péret et al. 2012).

The final stage of LR development is emergence (Fig. 5.1c). In order to be fully functional, the vascular systems of the young LR and the parent root must be connected. This vascular connection matures either at about the time of LR emergence or later in freshly emerged LRs. Phloem and xylem connector elements are formed from the pericycle derivatives and vascular parenchyma cells of the parent root vascular cylinder (Peterson and Peterson 1986). It is generally accepted that maturation of the vascular connectors proceeds acropetally into the LRs (Peterson and Peterson 1986). These maturing vascular elements connect with newly formed vascular elements that have originated from the root apex of the young LR which is already active at this time.

5.4 Role of Auxin, Ethylene, and Other Phytohormones in the Development of Root Systems

An important challenge for plant developmental biologists is to understand the mechanisms that control the patterned development of more or less complex systems. The focus of the present section will be on the hormonal mechanisms that shape the growth, differentiation, and architecture of the maize root system. In particular, we shall analyse the effects on maize's primary root development of treatments with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and with the synthetic auxin 1-naphthaleneacetic acid (NAA), alone or in combination. Experiments have also been carried out using an ethylene antagonist and inhibitors.

Plant development sciences have evolved from being mostly descriptive and comparative to a field dominated by genetic and molecular approaches. This change has been favoured by the systematization of experimental procedures and the emergence of ever more sophisticated research tools. Nevertheless, classical studies of root development using such simple techniques as the exogenous application of growth regulators are still useful in this context, while recent physiological studies using new tools such as auxin transport inhibitors, ethylene biosynthesis inhibitors, or ethylene action inhibitors, for example, continue to improve our knowledge of the process (Casimiro et al. 2001; Alarcón et al. 2009; Strader et al. 2009; Steinitz et al. 2010).

Usually, the exogenous application of auxin promotes three main alterations in root system development: inhibition of elongation, increased transversal expansion, and enhanced lateral root formation. Physiologically, these changes occur in

association with increased ethylene production due to increased transcription of ACC synthase genes (Abel et al. 1995). This leads to the accumulation of the ethylene precursor ACC and its product, ethylene, in tissues as a result of the auxin treatment.

The morphological changes observed in dark-grown seedlings treated with ethylene or its metabolic precursor ACC have been termed the triple response syndrome: exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and shortening of the hypocotyl and root (Ecker 1995).

Since the effects of auxin and ethylene on root development share some common features, it is difficult to assign the causality of these effects to one or the other of these growth regulators or to the interaction between the two. As auxin stimulates ethylene production by increasing the biosynthesis rate of ACC synthase, it has been posited that ethylene is the mediator of, and directly responsible for, the effects of auxin on root growth (Eliasson and Bollmark 1988; Jackson 1991). Hansen and Grossmann (2000) report that auxin-induced ethylene triggers the production of abscisic acid (ABA) and inhibits root growth through this growth regulator. The fact that there is some crosstalk between auxin- and ethylene-resistant mutations suggests at least that these two phytohormones interact in the regulation of root development (Stepanova et al. 2007). Consequently, there is a need for a precise dissection of the particular effects of each growth regulator on different aspects of root development.

5.4.1 Phytohormones and Root Elongation

The most obvious generalization about the effect of exogenous auxin on root elongation is that its inhibition of growth is dose dependent (Blakely et al. 1982; Lloret and Pulgarín 1992). Indeed, when it is applied at very low doses, it can even stimulate root growth (Díez et al. 1971). Such stimulation by exogenous auxin at very low concentrations has also been observed in maize roots pretreated with ethylene biosynthesis inhibitor (Mulkey et al. 1982). Moreover a negative correlation has been found between root growth and endogenous indole-3-acetic acid (IAA) content (Pilet and Barlow 1987). These results support the idea that the endogenous IAA level should be optimal or supraoptimal for elongation, and consequently any increment in the auxin level would result in root growth inhibition (Evans et al. 1994).

Auxin is mostly synthesized in the shoot, whence it is transported to the root. Inhibition of this transport from shoot to root reduces root elongation (Rashotte et al. 2003). In the root, IAA moves acropetally towards the root apex through the central cylinder and basipetally from the root apex towards the base through the epidermal and cortical cells (Ruzicka et al. 2009), controlling root elongation (Rashotte et al. 2000). In *Arabidopsis*, basipetal auxin transport is sufficient to control root elongation, and inhibition of this transport by *N*-1-naphthylphthalamic acid (NPA) reduces root elongation (Casimiro et al. 2001).

Auxin can regulate the elongation of root cells by modifying different aspects of their physiology. Elongation requires both a driving force and a modification of the cell wall elasticity by loosening cross-links between cell wall components. In coleoptiles, auxin has been demonstrated to promote a rapid increase in cell wall elasticity (Cleland 1992; Taiz 1994; Cosgrove 2000). The acid growth hypothesis (Rayle and Cleland 1970) is that hydrogen ions are the agents responsible for cell wall loosening during *Avena* coleoptile growth, and auxin-induced stimulation of growth in coleoptiles is driven by proton extrusion into the apoplastic space. The pH falls to below 5.5, and this acidification enhances cell wall extensibility and increases the activity of enzymes involved in cell growth. In roots, exogenous auxin usually inhibits proton extrusion and hence cell elongation (Evans et al. 1980).

However, as was noted above, root elongation is stimulated by very low auxin concentrations if ethylene biosynthesis has been inhibited (Mulkey et al. 1981). In maize, exogenous NAA at concentrations greater than 0.001 μM inhibit root elongation, and complete inhibition is observed at 1 μM, with the inhibition correlating with the logarithm of the NAA concentration (Alarcón et al. 2012). This inhibitory effect is probably due to the greater sensitivity of roots than of stem tissues such as coleoptiles, in which these concentrations are stimulatory (Thimann 1936).

Similarly, ethylene has long been recognized as a growth inhibitor, but there has recently been reported evidence of its effects in promoting plant growth. In particular, the inhibitory effect of ethylene on root growth was described in 1901 by Neljubov (Dugardeyn and Van Der Straeten 2008), but today it is understood that while high concentrations inhibit root elongation and increase swelling, very low concentrations may promote root elongation (Chang et al. 2004; De Cnodder et al. 2005; Dugardeyn and Van Der Straeten 2008).

ACC reduces cell length in the rapid elongation zone, and ethylene also controls cell elongation (De Cnodder et al. 2005). However, a certain threshold of ethylene is also known to be necessary to maintain root growth in rice (Yin et al. 2011), and other evidence for ethylene's stimulation of growth has been reported (Pierik et al. 2006). The stimulatory effects occur mostly at very low concentrations and are more pronounced in situations of stress. In P-sufficient white clover, low ACC concentrations stimulate root growth, while higher concentrations have no such effect (Dinh et al. 2012). In P-deprived seedlings, however, not only is root elongation enhanced, but ACC at higher concentrations super-stimulates growth, suggestive of an increased ethylene sensitivity in response to the low availability of P (Dinh et al. 2012). *Arabidopsis* root growth is promoted by ethylene under phosphorus stress (Ma et al. 2003). Also, ethylene is required for root development under non-stressful growth conditions. In particular, in *Oryza sativa*, the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) reduces primary root elongation (Yin et al. 2011). In coherence with this result, AVG or cobaltous ions also cause a strong reduction in ethylene levels and inhibited root elongation in maize (Alarcón et al. 2009).

Pierik et al. (2006) propose a biphasic ethylene response model which integrates growth inhibition and stimulation, with low ethylene levels promoting, and high

levels inhibiting, growth. The levels required to stimulate or inhibit growth depend on the species, plant organ, environmental conditions, and endogenous hormone levels. Four types of plant have been distinguished according to their ethylene dose–response relationship. In terrestrial plants, aerial organ growth can be stimulated by ethylene at low concentrations, but ethylene always has an inhibitory effect at high levels. In semi-aquatic plants, ethylene stimulates petiole elongation at high concentrations.

Ethylene is needed for the development of the root cap, the organ which facilitates the penetration of the roots into the growth medium (Zacarias and Reid 1992). In particular, high impedance triggers an increase in ethylene biosynthesis which then serves to facilitate the penetration of the root into the soil (Sarquis et al. 1992). Recently, it has been reported that a coaction between auxin and ethylene is required for root penetration during tomato seed germination (Santisree et al. 2011).

The inhibitory effect of aluminium on root elongation is well known, and it has recently been shown that ethylene mediates this effect in *Arabidopsis* (Sun et al. 2010). The inhibitory effects of aluminium are stronger in the wild type than in ethylene signalling-defective (*etr1-3* and *ein2-1*) and auxin polar transport-defective (*aux1-7* and *pin2*) mutants. Inhibitors of ethylene and auxin polar transport partially prevent the aluminium-induced inhibition. Aluminium and ACC increase the transcripts of *aux-1* and *pin-2*, but this increase is not observed in the presence of ethylene inhibitors. It has therefore been suggested that aluminium-induced ethylene alters the auxin distribution by disrupting AUX1- and PIN2-mediated auxin polar transport, inhibiting root growth elongation (Sun et al. 2010).

Root development is not only regulated by auxin and ethylene. Gibberellins, cytokinins, and other phytohormones play a significant role (Hansen and Grossmann 2000). Also, ethylene biosynthesis is stimulated by other growth regulators: ACC synthase is up-regulated by cytokinin (Rodrigues-Pousada et al. 1999), abscisic acid (Wang et al. 2011), and brassinosteroids (Joo et al. 2006).

5.4.2 Root Transversal Expansion

Generally, the inhibition of root elongation produced by auxin is associated with an increased rate of transverse expansion, altering the predominant direction of root growth from longitudinal to transversal (Burström and Svensson 1974).

This change in growth polarity may be related to changes in the orientation of the cortical microtubules, which pass from an essentially transversal orientation to one which is predominantly longitudinal. Such a relationship is proposed by the alignment hypothesis that there is a causal link between the orientation of cortical microtubules and the orientation of nascent cell wall microfibrils (Baskin 2001), which would in turn regulate cell expansion (Green 1984; Baskin and Williamson 1992). With respect to this hypothesis, it has been demonstrated that treatment with auxin affects the direction of cell expansion and the orientation of the cortical

microtubules in the epidermal cells of maize coleoptiles (Bergfeld et al. 1988). It seems, however, that auxin treatment does not affect the orientation of microtubules in the cells of the root vascular cylinder in the same way (Blancaflor and Hasenstein 1997), indicating that the response to auxin treatment could be tissue dependent.

As with auxin, ethylene also increases the root's radial expansion (Smalle and Van der Straeten 1997; Buer et al. 2003), which change in the growth pattern has also been related to a change in the disposition of the cortical microtubules (Baskin and Williamson 1992).

Treatments of maize roots with the synthetic auxin NAA or with the ethylene precursor ACC inhibit root elongation and increase radial growth, although the responses to the two treatments differ in degree (Alarcón et al. 2012). In particular, NAA enhances radial expansion by nearly 200 %, whereas ACC only increases it by about 40 %, so that NAA seems to be more effective than ACC as a root swelling promoter (Alarcón et al. 2012).

5.4.3 Auxin–Ethylene Interaction

Since auxin specifically stimulates ethylene biosynthesis, it is often unclear whether the effects observed are due to auxin alone, to ethylene alone, or to an interaction between the two. In maize roots, both hormones inhibit elongation and promote swelling in the root tips (Alarcón et al. 2012). Two hypotheses have been proposed to explain auxin's regulation of root growth. One is that auxin's action is mediated by ethylene, as indeed has been demonstrated in the case of light-promoted inhibition of root growth (Eliasson and Bollmark 1988; Jackson 1991). The other is that auxin directly affects root growth, as seems to be the case in *Pisum sativum* for which it has been demonstrated that auxin inhibits elongation without the mediation of ethylene (Eliasson et al. 1989). Recent studies in *Z. mays* and *Oryza sativa* are consistent with this concept of auxin exerting its effect directly (Yin et al. 2011; Alarcón et al. 2012). Nevertheless, the fact that auxin has a direct effect on root elongation and transversal growth does not imply that it cannot interact with other growth regulators (including ethylene). Indeed, our results show that auxin and ethylene can inhibit root elongation and increase radial growth cooperatively. The effect of combined treatments is synergistic when NAA or ACC are applied at very low concentrations and additive at slightly higher concentrations. Cooperation between the two hormones is absent, however, at high concentrations of either (Alarcón et al. unpublished results).

The synergy between auxin and ethylene has been described in processes affecting seedling development (Muday et al. 2012), root hair growth and differentiation (Pitts et al. 1998), root gravitropism (Buer et al. 2006), and root growth (Swarup et al. 2007). Particularly interesting for the present context is the work of Santisree et al. (2011) on the coactions between auxin and ethylene required for roots to penetrate into the soil during tomato seed germination.

Another possible mechanism of interaction could be related to the role of ethylene in the regulation of auxin transport and/or biosynthesis. It is known that ethylene stimulates the biosynthesis of auxin and its basipetal transport to the elongation zone (Ruzicka et al. 2007). This would increase the concentration of auxin in this zone and hence decrease the root elongation rate (Lee et al. 1990).

Molecular studies have demonstrated an interaction between auxin and ethylene at the genetic level. Auxin response factors ARF19 and ARF7 participate in auxin signalling and play a critical role in ethylene responses in the *Arabidopsis* root, indicating that ARFs serve as a point of crosstalk between the two hormones (Li et al. 2006a).

5.4.4 Lateral Roots

The generation of LRs has a decisive influence on the root system's capacity to anchor the plant to the soil and to acquire water and nutrients. It is unsurprising therefore that the regulation of the formation of LRs is under redundant control mechanisms performed through both endogenous and exogenous agents (Lloret and Casero 2002).

It is generally accepted that, among the endogenous factors that regulate LR formation, the most important is the phytohormone auxin (Casimiro et al. 2003). Many lines of experimental evidence relate auxin to diverse aspects of LR development. In particular, it appears to be involved in LR initiation, in the organization of the apical meristem, and in the emergence and ulterior growth of LRs.

Increased production of LRs has been demonstrated in most species after treatment with exogenous auxins (Torrey 1962; Blakely et al. 1972, 1982; Webster and Radin 1972; Wightman et al. 1980; Zeadan and MacLeod 1984; Hinchee and Rost 1986; MacIsaac et al. 1989; Lloret and Pulgarín 1992; Baum et al. 1998; Vuylsteker et al. 1998; Zhang and Hasenstein 1999). Nevertheless, the experimental procedures involved in these exogenous auxin treatments have been subject to criticism (Lloret and Casero 2002). The results of more recent studies with mutants and transgenic plants strongly relate LR formation to the biosynthesis or metabolism of auxin (Casimiro et al. 2003). For example, the *sur1* mutants of *A. thaliana* that overproduce auxins also show increased LR formation (Boerjan et al. 1995). Similarly, transgenic plants that overexpress bacterial *iaa* genes, and consequently have high auxin levels, also tend to form many LRs (Klee et al. 1987). On the contrary, the *diageotropica* (*dgt*) tomato mutant and the combination of two auxin resistance mutations (*axr4* and *axr1*) both reduce the number of LRs (Hobbie and Estelle 1995; Muday et al. 1995). The common feature of these two kinds of mutation is their reduced sensitivity to auxins.

Auxin overproducers and auxin-resistant mutants show striking physiological alterations in addition to changes in LR production. For example, there are specific effects on LR formation in *alf1-1*, *alf3-1*, and *alf4-1* mutants of *A. thaliana* (Celenza et al. 1995). The *alf1-1* mutation promotes the formation of LRs, *alf4-1* inhibits

their initiation, and *alf3-1* is defective in their maturation. The *alf1-1* mutants are likely to have a defect in auxin catabolism that favours the accumulation of the hormone in the root, resulting in an increased number of LRs. The *alf4-1* mutant seems to be affected in the perception or the response to auxin and does not produce LRs, and *alf3-1* initiates the LRP but soon aborts its development. The treatment with auxin reverts *alf-3* but not *alf4-1* (Celenza et al. 1995). These findings suggest a model for LR formation in which IAA is required for at least two stages in LR development: (i) to initiate cell division in the pericycle and (ii) to promote cell division and maintain cell viability in the developing LR.

The regulation of the development of LRs is not just a matter of the concentration of auxin but is also related to the transport of this hormone. Auxin transport is a directional process. Applied to the stem, auxin is transported to the root (McDavid et al. 1972), where it moves acropetally through the vascular cylinder (Kerk and Feldman 1995) to accumulate in the LRP and in the apex of the main root (Rowntree and Morris 1979; Sabatini et al. 1999). When the hormone reaches the root apex, its transport is inverse, i.e., basipetally from the apex to the elongation zone (Muday and DeLong 2001). This transport seems to occur through the root epidermis or the external layers of the cortex (Tsurumi and Ohwaki 1978; Yang et al. 1990). The acropetal movement of auxin has been associated with the regulation of LRP growth (Reed et al. 1998), while the basipetal movement would be associated with the initiation of the LRs (Casimiro et al. 2001).

There are essentially three lines of experimental evidence concerning the transport of auxin in regulating the LR formation process. First, elimination of the plant's aerial organs (the source of auxin) reduces the LR frequency, and then application of exogenous auxin to the aerial part of the mutilated plant reverses this effect (Hinchee and Rost 1986). Second, mutants with defects in the auxin transport system (*tir3*) present fewer LRs (Ruegger et al. 1997). And third, NPA, an inhibitor of auxin transport, suppresses the formation of LRs in tomato (Muday and Haworth 1994) and *Arabidopsis* (Reed et al. 1998; Casimiro et al. 1999, 2001). Together, these three lines of evidence clearly reflect a fundamental role for auxin transport in the formation of LRs.

Unlike auxin, ethylene is a negative regulator of LR formation in both *A. thaliana* and *Solanum lycopersicum* (Ivanchenko et al. 2008; Negi et al. 2008, 2010), with enhanced ethylene synthesis resulting in reduced LR initiation independently of whether the enhancement is due to ACC treatment or to *eto1* or *ctr1* mutations. Similarly, Prasad et al. (2010) report that the protein XBAT32 negatively regulates ethylene biosynthesis by modulating the abundance of the enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS), and, as expected, *xbat32* mutants produce an excess of ethylene and have a limited number of LRs.

Since auxins promote ethylene biosynthesis, it is difficult to evaluate any possible direct influence of ethylene on LR formation. The enzyme ACS is involved in the ethylene biosynthesis pathway. It is well known that, during the initiation of LRs, ACS activity increases in response to auxin (Rodrigues-Pousada et al. 1993). This is a perfect example of how these two hormones work together in regulating a biological process.

Another form of interaction between auxin and ethylene could be by the effect of ethylene on the regulation of auxin transport. It has been suggested that the increase in the concentration of ethylene in the root affects the distribution of proteins involved in auxin transport, causing two effects: accumulation of auxin at the root apex and depletion in the zone where LRs are initiating (Muday et al. 2012).

In contrast to LR initiation, the development of LRs is stimulated by treatments that raise ethylene production in the root (Ivanchenko et al. 2008). In the maturation and abscission processes, ethylene increases the hydrolytic activity of the enzymes that degrade the cell wall (Roberts et al. 2000). It has therefore been proposed that ethylene also favours the degradation of the cortical cells located just in front of the LRP during its development. Obviously, this would facilitate the emergence of the LRP (Bonfante and Peretto 1993).

LR development is regulated antagonistically by the plant hormones auxin and cytokinin. Indeed, cytokinins are considered to be good inhibitors of the formation of LRs because their exogenous application frequently reduces this formation (MacIsaac et al. 1989). However, very low concentrations of these hormones can stimulate auxin-promoted LR formation (Torrey 1962). Furthermore, the application of cytokinins not only inhibits the initiation of LRs but also their emergence (Van Staden and Ntingane 1996). The root apex is the main site in the plant at which cytokinins are synthesized (Van Staden and Davey 1979). From there, they are transported by the xylem to the rest of the root, inhibiting elongation and LR formation (MacIsaac et al. 1989).

Cytokinins inhibit LR formation both in wild *A. thaliana* plants and in mutants defective in auxin transport or response mechanisms (Li et al. 2006b). The application of exogenous auxin to these mutants does not reverse this inhibition. It is therefore posited that auxin and cytokinin act in this process via different signalling pathways, with the cytokinins exerting their inhibitory effect by blocking pericycle founder cells from progress in the cell cycle from G2 to mitosis (Li et al. 2006b). This does not mean, however, that there is no crosstalk between auxin and cytokinin in regulating the formation of LRs. Xylem-pole pericycle cells have been described as being sensitive to cytokinins, whereas young LRP are not (Laplaze et al. 2007). It appears that cytokinins perturb the expression of PIN genes in LR founder cells and inhibit the accumulation of auxin in them, which in turn promotes LR initiation and regulates cell spacing (Laplaze et al. 2007).

ABA is also considered to be an LR inhibitor. Recent studies by Guo et al. (2009) in *Arachis hypogaea* report that exogenous ABA treatments decrease root branching in a dose-dependent manner. In *Arabidopsis*, mutation in the Abscisic Acid Insensitive 4 (ABI4) transcription factor promotes an increase in the number of LRs. It has been demonstrated that the expression of ABI4 is enhanced by ABA and cytokinin but repressed by auxin. The production of LRs in *abi4* mutants is not affected by cytokinin or ABA (Shkolnik-Inbar and Bar-Zvi 2010). As the expression of the auxin efflux carrier protein PIN1 is reduced in ABI4 over expressors and enhanced in *abi4* mutants, it has been suggested that ABA and cytokinin counteract the effect of auxin in the regulation of LR formation by reducing polar auxin transport (Shkolnik-Inbar and Bar-Zvi 2010).

Gibberellins are reported to interact with auxin in the regulation of LR development in *Populus*. GA-deficient and GA-insensitive transgenic plants show increased LR proliferation and elongation which is reversed by exogenous GA. Microarray analyses suggest a crosstalk of GA with auxin and that GA modulates LR development by modifying polar auxin transport (Gou et al. 2010).

In sunflowers, the lipid-derived hormone jasmonic acid and its derivates (collectively named jasmonates), applied exogenously, inhibit primary root elongation and reduce LR growth and number. Treatment with ibuprofen, an inhibitor of jasmonate synthesis, enhances primary root and LR lengths, but auxin elicits its typical response even in the presence of ibuprofen. Jasmonates, therefore, may induce primary root and LR growth inhibition via an auxin-independent pathway (Corti-Monzón et al. 2012).

In contrast, recent studies on *A. thaliana* indicate that jasmonates are promoters of both the initiation and emergence of LRs (Raya-González et al. 2012). In addition, regulation of the development of the root system seems to operate through mechanisms of two types, some dependent on auxin and others independent of this hormone. These results indicate that it is still unclear how jasmonates contribute to many aspects of root development and that there may be differences between species.

In sum, while auxins seem to constitute the main regulator of LR formation, other growth regulators appear to collaborate with it in fine-tuning the process and accommodating it to environmental influences.

5.4.5 Root Hairs

Auxin and ethylene are required for the normal development of root hairs (Pitts et al. 1998; Rahman et al. 2002). Ethylene increases their growth in various species including pea, fava bean, and lupin (Abeles et al. 1992). Analysis of *Arabidopsis* mutants has shown that root hair elongation in plants with enhanced ethylene production (*eto1-1*) was greater than in wild-type plants, and the elongation was negatively affected in the ethylene-insensitive mutant *etr1* (Pitts et al. 1998).

The constitutive ethylene mutant of *Arabidopsis ctr1* has more root hairs than the wild-type phenotype, and the application of ethylene to the wild type produces root hairs with a morphology similar to that of the mutant. In addition, application of an inhibitor of ethylene biosynthesis (AVG) or action (silver thiosulfate, STS) to the wild-type phenotype reduces the production of root hairs (Tanimoto et al. 1995).

The root hair in *Arabidopsis* mutants insensitive to ethylene (*etr1*) or auxin (*aux1*) exhibits normal morphology, whereas double mutants show reductions in root hair formation (Smalle and Van der Straeten 1997). These reductions can be reverted by the application of IAA but not ACC, suggesting that ethylene may act via more than one route (Masucci et al. 1996).

5.5 Conclusions

The organs of higher plants are complex multicellular organizations which grow by means of coordinated activity from their constituent cell types. It has long been recognized that hormones play a crucial role in this coordination. The root is no exception in this respect. It has only been recently that researchers have begun to understand how different growth regulators interact to properly regulate the development of the root system and adapt it to changing environmental conditions. It is now clear that the root cells exchange signals and that the signalling network they form is far more intricate than was previously assumed. Over the past few decades, particular attention has been paid to analysing how individual tissues may contribute to the regulation of overall root growth, and there is a need to continue on this line of inquiry in even greater depth.

In genetic studies of higher plants, the material of choice has been *A. thaliana*. While such a concentration of research effort on this plant model has yielded impressive results, it may now be advisable to expand our vision somewhat to look at other species. In this sense, it would be very interesting to compare the situation in monocots and dicots. Even if it were only for being one of humanity's largest volume crops, the maize plant merits particular attention in the immediate future.

The maize primary root is a cylindrical structure formed by consecutive zones: (a) the root apex contains the apical meristem, where cell divisions occur; (b) the elongation zone in which cells stop dividing and start to elongate; and (c) the maturation zone, where cells reach their definitive lengths, cell differentiation begins, and lateral roots initiate. In the root, three main tissue systems can be distinguished: the epidermis, the cortex, and the vascular cylinder. The first layer of the vascular cylinder is the pericycle. Cell cycle activation in pericycle cells is clearly connected with lateral root initiation.

Root grows basically by the elongation of its cells and branches through proliferation of pericycle founder cells. Auxin is the main hormone in regulating these both processes. Exogenous auxin inhibits root growth, increases transversal expansion, and enhances lateral root formation. As auxin also enhances ethylene production, it is difficult to know whether certain auxin effects are mediated by ethylene or not. Based on own results and on the specialized literature, we discussed on regulation by auxin and ethylene of the development of the maize root system. The emerging model is that auxin and ethylene regulate root elongation depending on concentration and that both regulators interact to regulate root growth. The role of auxin in regulating lateral root formation is clearly established. However, ethylene does not seem to have such a direct role in this process.

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Part II
Rhizosphere and Microorganisms

Chapter 6
Mycorrhizosphere: The Role of PGPR

Rosario Azcón

6.1 Introduction

Microorganisms interact among themselves, and with plant roots, to develop the multifunctional plant mycorrhizosphere, a scenario of diverse activities relevant for plant productivity either in sustainable agriculture or in the maintenance of natural plant communities (Fig. 6.1). Here, we established strategic and applied research which has allowed a comprehensive understanding of the formation and functioning of the plant mycorrhizosphere. Manipulation of the microbial activities allows tailoring efficient mycorrhizosphere systems for improving plant establishment and productivity.

Mycorrhizal symbioses, found in almost all ecosystems, are fundamental to improve plant fitness and soil quality through key ecological processes (Smith and Read 2008). The mycorrhizal fungi colonise the root cortex and develop an extraradical mycelium, in the surrounding plant roots and soil. This hyphal net is the fungal biomass specialised for the acquisition of mineral nutrients from the soil, particularly those having poor mobility or present in low concentration in the soil solution, as is the case of P (Barea 1991). This mycorrhizal function in soils with low P availability provides the plant with an adaptive strategy for P-acquisition.

Mycorrhizae are symbiotic associations established between specific soil fungi and most vascular plants where both partners exchange nutrients and energy (Brundrett 2002). Mineral nutrients via the fungal mycelium are transported to the host plant, while the heterotrophic fungus obtains carbon compounds from the host's photosynthates.

Symbionts and soil saprophytic microorganisms inhabiting root zone (Barea et al. 2005b) both interact in the rhizosphere (Adesemoye and Kloepper 2009; Finlay 2008; Jaderlund et al. 2008; Kiers and Denison 2008), and some of the

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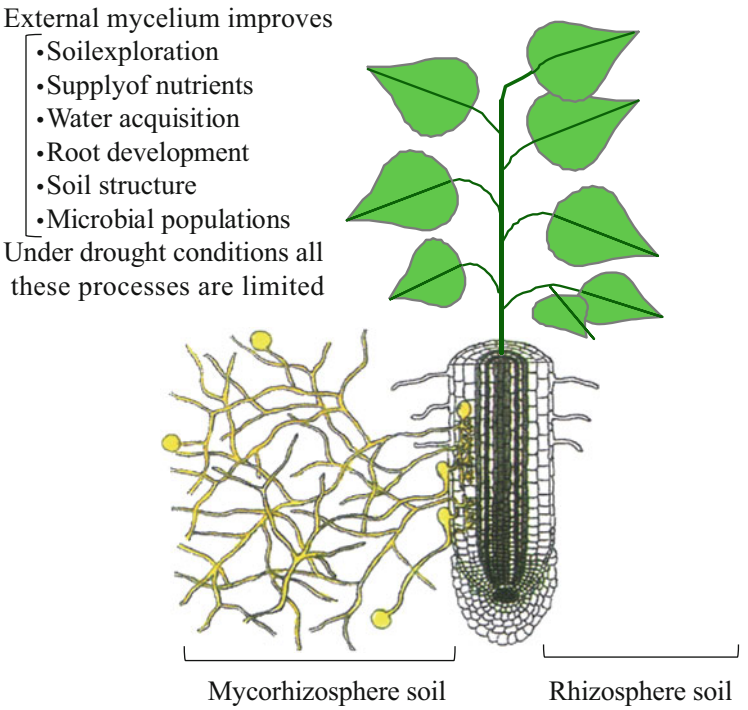

Fig. 6.1 Changes in the rhizosphere as affected by the extraradical mycelium development (mycorrhizosphere)

resultant interactions are fundamental for sustainable plant developments (Barea et al. 2008). Among other functions, soil microorganisms are involved in the biogeochemical cycling of nutrients and organic matter and the maintenance of plant health and soil quality (Barea et al. 2005a; Jeffries and Barea 2001). These activities are particularly relevant at the root–soil interface microhabitats, known as the rhizosphere, where microorganisms interact with plant roots and soil constituents. The major groups of mutualistic microbial symbionts are the fungi, which establish the arbuscular mycorrhizal associations with the roots of most plant species (Smith and Read 2008). The AM fungi are obligate microbial symbionts, which are not able to complete their life cycle without colonising a host plant.

Particularly, after mycorrhiza establishment, rhizosphere microorganisms interact with mycorrhizal structures to generate the so-called mycorrhizosphere, a key issue for plant productivity improvement (Azcón and Barea 2010; Barea et al. 2005b).

The widespread and ubiquitous AM symbiosis is characterised by the tree-like symbiotic structures, termed "arbuscules", which the fungus develops within the root cortical cells and where most of the nutrient exchange between the fungus and the plant is thought to occur (Azcón-Aguilar et al. 2009).

The AM fungi were formerly included in the order Glomales, Zygomycota (Redecker et al. 2000), but they have recently been moved to a new phylum Glomeromycota (Schüßler et al. 2001). Earlier studies on diversity of AM fungal communities were based largely on the morphological characterisation of their large multinucleate spores. However, more recently, the ribosomal DNA sequence analysis has been used to determine the diversity of natural AM populations (Alguacil et al. 2009b; Hempel et al. 2007; Öpik et al. 2008a, b; Rosendahl et al. 2009; Santos-González et al. 2007; Toljander et al. 2008). Nevertheless, despite the advancement in molecular techniques, the identification approaches employed for AM fungi based on morphological characteristics are still valid and are considered complementary to the molecular methods (Morton 2009; Oehl et al. 2009).

There are two main types of mycorrhiza, ecto- and endomycorrhiza, which have considerable differences in their structure and physiological relationships with symbionts (Smith and Read 2008).

Rhizosphere microorganisms can either interfere with or benefit mycorrhiza establishment (Gryndler 2000; Pivato et al. 2009). A particular interest has been expected about the so-called mycorrhiza helper bacteria (MHB), a term that was coined by Garbaye (1994) and later updated by Frey-Klett et al. (2007) for those bacteria which stimulate mycorrhizal mycelial growth and/or enhance mycorrhizal formation or activity. In the same way the establishment of plant growth-promoting rhizobacteria (PGPR) inoculants in the rhizosphere can also be affected by arbuscular mycorrhizal (AM) fungal coinoculation (Artursson et al. 2006; Barea et al. 2005b; Jaderlund et al. 2008; Mallik and Williams 2008). The establishment of the AM fungus in the root cortex is known to change many key aspects of plant physiology such as the mineral nutrient composition in plant tissues, the hormonal balance and the patterns of C allocation.

The extraradical mycelium generated after root infection by AM fungi is profusely branched and provides a very efficient nutrient-absorbing system beyond the nutrient depletion zone surrounding the plant roots, thereby reducing the distance that nutrients must diffuse through the soil prior to reach the root surface (Fig. 6.2). Actually, the AM fungal mycelium can spread through the soil over considerably longer distances (usually several cm) than root hairs (Finlay 2008).

AM colonisation induced changes in plant physiology affecting the microbial populations, both quantitatively and qualitatively, in either the rhizosphere or the rhizoplane. Thus, there are specific modifications in the environment surrounding the AM extraradical mycelium itself, the mycorrhizosphere (Andrade et al. 1997; Gryndler 2000; Linderman 1988). Therefore, the rhizosphere of a mycorrhizal plant, generically termed as the mycorrhizosphere, can have features that differ from those of a nonmycorrhizal plant (Finlay 2008).

The obligate character of the AM fungi is such that specific methodological approaches are needed to investigate the processes involved in the formation and functioning of the symbiosis (García-Garrido et al. 2009; Gianinazzi-Pearson et al. 2009; Gryndler et al. 2009; Smith et al. 2009). The most important role of AM fungi is to contribute, acquire and supply nutrients particularly P to colonised

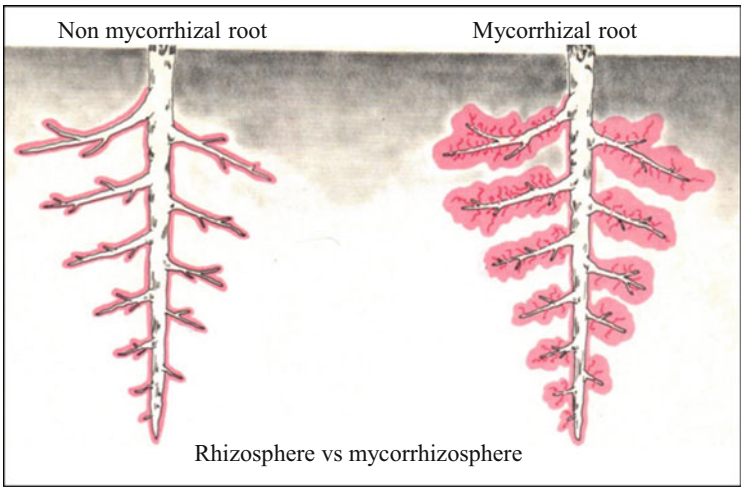

Fig. 6.2 Mycorrhizal and non-mycorrhizal roots abilities to water and nutrients uptake particularly under stress conditions

plants (Jeffries and Barea 2001). But also the AM fungi are able to tap other nutrients (Barea et al. 2005a), especially N, from either inorganic (Tobar et al. 1994a, b) or organic (Leigh et al. 2009) sources.

Important aspects in order of the bacterial intimacy with the associated plant are the niche that ranged from almost casual to extremely regulated and housed in specialised structures. There are bacteria living in the soil near roots, bacteria colonising the rhizoplane (root surface) and bacteria residing in root tissue (inside cortical cells) (Gray and Smith 2005). In all these niches, PGPR can interact with AM fungi.

In this chapter the cooperative plant–microbial interactions have focused their attention only on bacteria and arbuscular mycorrhizal fungi (Barea et al. 2004; de Boer et al. 2005). In addition the importance of microorganisms and processes involved in the establishment and functioning of the mycorrhizosphere, the impact of the mycorrhizosphere activities on plant growth and productivity particularly in environmental stresses and the possibilities to manipulate an efficient mycorrhizosphere to improve plant in either agrosystems or natural ecosystems here are reported. All these aspects are here revised.

The exact mechanisms of plant growth stimulation by rhizosphere bacteria remain largely speculative. It is known that they differ between bacterial strains according to the various compounds released by the different microorganisms (Dimkpa et al. 2008, 2009c).

The action mechanisms of PGPR can be direct or indirect ones. Direct mechanisms include activities as N_2 fixation, soil mineral solubilisation, production of plant growth-promoting substances (auxins, cytokinins or gibberellins) and reduction of ethylene levels, as the most important. But indirect mechanisms and activities include enhancing colonisation by other beneficial soil microorganisms,

as mycorrhizal fungi, and depressing the growth of plant pathogenic microorganisms (Lugtenberg and Kamilova 2009). The fact that some bacteria only showed differences on plant growth when associated with AM fungi suggests that they produced some substances responsible for AM functioning (Frey-Klett et al. 2007; Garbaye 1994; Souchie et al. 2007). Bacteria called "mycorrhiza helper bacteria" stimulate AM root colonisation (Vivas et al. 2006a) and mycelial growth from *G. mosseae* spores in vitro culture (Azcón 1987).

In their interaction with plants, the bacterial motility is important (Lugtenberg et al. 1996). At the molecular level, signal-based communications take place through plant perception of eubacterial flagellins (Gómez-Gómez and Boller 2002; Navarro et al. 2006). Flagella synthesis is an energy-consuming event leading to the downregulation of genes involved in auxin signalling, thereby restricting bacterial growth in the plant when bacteria reach the root epidermis (Navarro et al. 2006).

Mycorrhizosphere-associated bacteria that have beneficial effects on plant growth are able to produce IAA, and as a result, the inoculation of plant with such bacteria increased root growth and/or enhanced formation of lateral roots and root hairs (Chakraborty et al. 2006; Long et al. 2008; Rajkumar et al. 2005). A larger root surface has positive effects on water acquisition and nutrient uptake. Common adaptation mechanisms of plants exposed to environmental stresses (water and nutrient deficiency or heavy metal toxicity) include changes in root morphology, a process in which phytohormones are known to play a key role (Potters et al. 2007).

The aim of numerous studies is to elucidate the plant beneficial effects of microorganisms under different abiotic stress conditions. It is known that the beneficial effects of PGPR inoculation are most significant under unfavourable conditions such as nutrient deficiency, drought or metal toxicity. As consequence, it has been reported that microbial inoculants from the selected root-colonising microorganisms increase tolerance against abiotic stresses such as drought, salinity and metal toxicity and also can provide "bioprotection" against biotic stresses.

6.2 Relevance of Microbial Activities in the Mycorrhizosphere for Nutrients Cycling

The beneficial saprophyte microbes promote plant growth and health acting as plant growth-promoting rhizobacteria (PGPR) or antagonists of plant pathogens. Beneficial plant mutualistic symbionts are the N_2-fixing bacteria and the multifunctional AM fungi (Barea et al. 2005b). The processes involved in nutrient cycling by PGPR include nitrogen fixation and phosphate solubilisation besides releasing other nutrients in soil (Marschner 2008; Richardson et al. 2009; Zaidi et al. 2009).

Microbial N_2-fixation by specific bacteria is the first step in N-cycling to the biosphere from the atmosphere, a key input of N to plant productivity (Vance

2001). Prokaryotic bacteria are the only organisms able to fix N_2 as they are the only organisms possessing the key enzyme nitrogenase, which specifically reduces atmospheric N to ammonia in the symbiotic root nodules (Markmann and Parniske 2009). Other bacteria (actinomycetes), belonging to the genus Frankia, form nodules on the root of the so-called "actinorhizal" species, plants having a great ecological importance (Vessey et al. 2004). The bacteria able to fix N_2 in symbiosis with legume plants belonging to diverse genera (Willems 2007) are collectively termed as "rhizobia".

The widespread presence of the AM symbiosis in nodulated legumes and the impact of AM fungi in improving nodulation and N_2-fixation were recognised (Azcón and Barea 2010; Barea and Azcón-Aguilar 1983). In fact, rhizobial bacteria and AM fungi are known to interact among themselves and with their common legume host roots, either at the colonisation stages or at the symbiotic functional level (Azcón 1987; Barea and Azcón-Aguilar 1983; Barea et al. 2005c). Asai (1944) concluded that nodulation by rhizobial bacteria appears to be dependent on mycorrhiza formation by the common host legume.

The main cause of such beneficial interactions is the supply of P by the AM fungi to satisfy the high P demand of nodule formation. The AM fungi have also been shown to have a general influence on plant nutrition, but more localised effects of AM fungi are reported either at the root, nodule, or bacteroid levels (Azcón and Barea 2010).

Once AM colonisation was established, and the efficiency of P nutrition increased, nodule development and host growth were improved, and concomitantly, N_2-fixation enhanced. The AM fungi were the dominant symbiont for host C in the tripartite symbiosis, due to its rapid development. The subsequent AM role in supplying P benefited both host legume and nodule performance. Moreover, the CO_2 fixation rate expressed as g C g^{-1} shoot dry matter h^{-1} increased in symbiotic plants. This is in fact a mechanism that enhances photosynthesis to compensate for the C cost of the dual symbioses (Mortimer et al. 2008; Barea et al. 2005c).

The tripartite symbiosis has been investigated both at physiological and structural levels, with results indicating that the effects depend on the particular endophyte combination (Ruíz-Lozano and Azcón 1993) and/or the legume genotype (Monzón and Azcón 1996). Under natural conditions, AM fungi and rhizobia symbiosis do not seem to compete for infection sites and colonise the root almost simultaneously (Bethlenfalvay et al. 1985).

The use of Myc^- legumes contributed to a better understanding of the signalling processes involved in the formation of microbe–legume symbioses. It has been suggested that both AM formation and nodulation share a common signal transduction pathway (Parniske 2004). The information generated from molecular tools suggests that some plant genes can modulate both types of legume symbiosis (Gianinazzi-Pearson et al. 2009; Parniske 2004).

Multitrophic interactions involving other microorganism, such as PGPR, have been analysed in different studies, which describe how these bacteria enhance the beneficial effects of the legume microsymbionts and enhanced dry matter yield, N

concentration and total N yield (Azcón 1993; Bisht et al. 2009; Rinu and Pandey 2009).

The use of ^{15} N allowed us to distinguish the effect of AM fungi on N-acquisition where mycorrhizal fungi enhanced the amount of N derived both from soil and from fixation, as compared with phosphate-added or control plants (Tobar et al. 1994a, b). This indicated that AM fungi acted both by a P-mediated mechanism to improve N_2 fixation and by enhancing N uptake by the legume from soil (Azcón et al. 2001).

When the available P and N contents in the test soils were low, the appropriate management of plant by consortia of microbial symbionts improved soil fertility/productivity and consequently the overall performance of legumes. This has also been reported for forage legumes in Spain (Azcón 1993) and soybean plants in Nigeria (Babajide et al. 2009), with an agroforestry system, including tropical tree legumes, in Brazil (Pagano et al. 2008) or with common bean-based production system in Turkey (Uyanoz et al. 2007).

The tree legume *Medicago arborea* grown under drought conditions was positively benefited by coinoculation with AM fungi, *Rhizobium meliloti* strains and PGPR (Galleguillos et al. 2000), suggesting that the mixtures of microbial symbionts and their coordinated activities in the mycorrhizosphere could serve as a successful biotechnological alternative to aid the recovery of desertified ecosystems in semi-arid areas.

The role of the AM fungi, and the cooperation with other microbes, in the formation of water-stable soil aggregates (Rillig and Mummey 2006) is relevant in the natural ecosystems (Caravaca et al. 2006; Medina et al. 2004). The improvement in the physico-chemical properties in the soil around the *Anthyllis* plants was shown by the increased levels of N, organic matter and number of hydrostable soil aggregates.

The tailored mycorrhizosphere not only enhanced establishment of the target legume but also increased soil fertility and quality (Caravaca et al. 2004). This included enhanced seedling survival rates, growth, P-acquisition, N-fixation and N-transfer from N-fixing to associated nonfixing species in the natural succession (Barea et al. 1987, 1989).

Another important PGPR activity is carried out by phosphate-solubilising bacteria (PSB). But their effectiveness in the soil–plant system is variable (Barea et al. 2007; Zaidi et al. 2009). One of the reasons is that phosphate from P-solubilising activity of PSB could be newly fixed by the soil's constituents before they reach to the root surface. However, if the phosphate ions, as released by the PSB, are taken up by a mycorrhizal mycelium, this would result in a synergistic microbial interaction that in turn improves the amount of P acquired by the plant (Azcón et al. 1978).

The use of ^{15} N allowed the corroboration of a positive effect of P-supply from the tailored microbial treatments on N_2-fixation by the test inoculated legume (Azcón et al. 1988, 1991). Results from the field trial suggested that interactions between AM fungi, rhizobia and PSB can have a cooperative fundamental role in P- and N-cycling in a tailored legume mycorrhizosphere (Azcón-Aguilar et al. 1979).

The interactions between AM fungi and phosphate-solubilising microorganisms (PSM) are important for P-acquisition by the plants (Barea et al. 2002). Solubilisation of unavailable P-sources in soils and the uptake of solubilised Pi by mycorrhizal plants (Kucey et al. 1989; Richardson 2001) are processes involved in P-cycling in the mycorrhizosphere that have been described for increasing the Pi availability in soils. The solubilisation/mineralisation of unavailable P compounds is carried out by diverse saprophytic bacteria and fungi (Barea et al. 2007).

The Pi made available by PSB acting on sparingly soluble P-sources, however, may not reach to the root surface due to limited diffusion of this ion in soil solution. However, it was proposed that if P is solubilised by PSB, AM fungi can tap these phosphatic ions and translocate it to plants suggesting an interaction in the mycorrhizosphere, which could improve P-supply to the host plants synergistically or additively, as reported by Barea (1991) and Toro et al. (1997, 1998).

The AM symbiosis not only influences nutrient cycling in soil–plant systems but also improves plant health through increased protection against environmental stresses including biotic (e.g. pathogen attack) or abiotic (e.g. drought, salinity, heavy metals, organic pollutants), as now will be reported as beneficial microbial activities in the mycorrhizosphere.

6.3 Importance of Microbial Activities in the Mycorrhizosphere in Alleviation of Drought Stress

Plant growth is limited in arid sites due to the adverse conditions coming from water stress. Moreover, arid soils are generally characterised by nutrient deficiency, poor soil structure, low water-holding capacity and lack of organic matter. Thus, it is important in arid areas to improve soil quality and the ability of plant species to resist the detrimental conditions. In these semi-arid environments, the low fertility of the soil and the severe water deficits seriously limit plant development. Under such situation the inoculation of plants with beneficial microorganisms such as AM fungi and other PGPR may increase drought tolerance of plants growing under limit water conditions. Nevertheless, microbial populations and/or activities are reduced under stress conditions, and this reduction becomes critical for plant establishment and further development. In fact, in drought-stressed soils, plants are highly dependent on microbial activity to tolerate this environmental stress (Augé 2001; Medina and Azcón 2010; Porcel et al. 2003). The most prominent beneficial effects of inoculation with a potential PGPR are to be expected in poor soils (Ramos-Solano et al. 2006), but the main problem is that under detrimental conditions the development of the indigenous microbial community is nearly inhibited.

Plants possess natural protection systems against stresses, but their interaction with soil microorganisms can alleviate in the highest extent detrimental symptoms, which is important to survive and to establish for drought stress protection

(Goicoechea et al. 1998; Marulanda et al. 2008). The relevance of AM symbiosis in plant drought tolerance under nutritional and water stress conditions is based on a range of physiological and cellular mechanisms (Porcel et al. 2003).

In the mycorrhizosphere AM fungi interact with several soil microorganisms, including PGPR that make the host plant more tolerant to these stresses (Azcón et al. 2009a, b; Barea et al. 2004, 2005b; Vessey 2003). Beneficial effects are usually enhanced when both microorganisms are coinoculated, although it depends on the bacterium–fungus pair (Galleguillos et al. 2000; Valdenegro et al. 2001; Vivas et al. 2003c). The inoculation of selected microbial groups may be decisive for the plant establishment and development under limiting soil conditions (Vivas et al. 2003c).

Studies show that salt inhibits germination of spores or other AM fungal propagules. However, AM fungi are found in saline soils, and they are important in increasing the plant salt tolerance and in decreasing plant yield losses in these environments (Al-Karaki et al. 2001; Cantrell and Linderman 2001; Hajiboland et al. 2010; Ruíz-Lozano et al. 1996). Under osmotic stress conditions, plants must respond by decreasing their water potential to maintain a gradient for water flow from soil into roots and to avoid cell dehydration, and AM colonisation can facilitate such physiological plant balance.

Some mechanisms have been suggested to explain the greatest osmotic tolerance of AM plants as an increased nutrient and water uptake due to the fungal mycelium, that extended explored soil by roots, enhanced K^+/Na^+ ratios, root hydraulic conductivity and osmotic adjustment (Medina and Azcón 2012; Ruíz-Lozano et al. 2012).

Production of proline and/or betaines under osmotic stress increased when the plant was colonised by AM fungi (Al-Garni 2006). AM plants accumulate more proline to cope with the low water potential of drying soil and to maintain a water potential gradient favourable to water entrance into the roots (Porcel and Ruíz-Lozano 2004). Betaines maintain the integrity of membranes against the damaging effects of osmotic stress and also can stabilise protein complexes and the structures and activities of enzymes (Evelin et al. 2009). The accumulation of sugars in mycorrhizal plants is an important defence mechanism against drought stress (Porcel and Ruíz-Lozano 2004) and against salinity (Sheng et al. 2011; Talaat and Shawky 2011). The high levels of sugars in AM plants may be the consequence of an increase in photosynthetic capacity, and these sugars contributed to the osmotic adjustment of the plants (Sheng et al. 2011).

Mycorrhizal colonisation is able to prevent Na^+ translocation to shoot tissues and on the other hand to increase K^+ absorption under saline conditions (Alguacil et al. 2003; Talaat and Shawky 2011; Zuccarini and Okurowska 2008). AM plants have developed these strategies to limit the entrance of Na^+ into the root (Na^+ can be sequestrated into the vacuole or limited its transport and distribution to the leaf) and, at the same time, the AM plants enhanced K^+. The nutrient K^+ has a great importance in opening and closing of stomata, it is required for maintaining the osmotic balance, it is a cofactor for many enzymes, and it is involved in the biosynthesis of proteins, as K^+ participates in the binding of tRNA to the ribosomes

(Blaha et al. 2000). Thus, AM plants have the ability to maintain a higher K^+:Na^+ ratio, preventing the disruption of cellular enzymatic processes and inhibition of protein synthesis. High cytosolic K^+:Na^+ ratio is very important in osmotic stress tolerance.

The mechanisms by which osmotic stresses reduce the hydraulic conductance in cells and roots are not totally known. But it could be due to changes either in the aquaporin function or in the amount of this protein present in the membrane.

Aquaporins are water channels and they serve as a major way for uptake of water by roots. Aquaporins are a group of water channel proteins that facilitate and regulate the passive movement of water molecules following a water potential gradient (Kruse et al. 2006). Early experiments show that root water transport can be inhibited by the general aquaporin blockers mercury ions (Maggio and Joly 1995).

In plants, aquaporins are subdivided into five evolutionarily subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the small basic intrinsic proteins (SIPs), the nodulin26-like intrinsic proteins (NIPs) (Chaumont et al. 2001; Johanson et al. 2001) and the uncharacterised X intrinsic proteins (XIPs) (Gupta and Sankararamakrishnan 2009), which have been recently shown to transport a variety of uncharged substrates (Bienert et al. 2011) [see Knipfer and Fricke (2014)].

Both PIPs and TIPs aquaporin have important role in osmoregulation of cell cytoplasm and in the regulation of root hydraulic conductivity (Luu and Maurel 2005).

As it is known, a low water potential in saline or drought soils requires from the plants adaptation to acquire enough water from soil (Ouziad et al. 2006), and as response to salt, the plants are able to reduce their root water uptake capacity (i.e. root hydraulic conductivity). Fortunately, AM symbiosis regulates root hydraulic properties, including root hydraulic conductivity, and these effects have been linked to regulation of plant aquaporins (Ruíz-Lozano and Aroca 2010). Mycorrhizal plants subjected to osmotic stress enhanced water flux through their roots, and this effect was concomitant with the enhanced expression of three out of four aquaporin genes analysed in these plants, with PIP2 protein amount and phosphorylation state (Aroca et al. 2007). In addition, under stress conditions, AM plants increased mechanisms evolved in efficient systems for ROS removal, which include the accumulation of non-enzymatic molecules that act as ROS scavenger such as ascorbate, glutathione, α-tocopherol, flavonoids, anthocyanins and carotenoids, as well as the synthesis of specific ROS-scavenging antioxidative enzymes acting in synchrony. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidase (POX) or the accumulation of antioxidant compounds such as ascorbate and glutathione was enhanced by AM symbiosis helping the plants to alleviate salt- or water-deficit stresses (Alguacil et al. 2003; He et al. 2007; Porcel and Ruíz-Lozano 2004; Ruíz-Sánchez et al. 2010). Ascorbate was shown to play an important role in the protection of photosynthesis during salt stress (Noctor and Foyer 1998; Shao et al. 2008).

The response of the individual enzymes is varied with respect to the fungal species and the host plant. Moreover, the effect of the AM symbiosis inducing antioxidant activities may also be the result of the mycorrhizal effects on plant nutrients (P, N) acquisition (Alguacil et al. 2003; Evelin et al. 2009).

Physiological values as photosynthetic activity and water use efficiency have been also improved in mycorrhizal plants growing under osmotic stress conditions (Hajiboland et al. 2010; Sheng et al. 2008; Zuccarini and Okurowska 2008). Nevertheless, Sheng et al. (2008) suggested that this AM effect on photosynthetic capacity of plants was mainly due to an enhancement of water status, but not to a mycorrhizae-mediated enhancement of chlorophyll concentration.

Mycorrhisation improved the net assimilation values by increasing stomatal conductance and by protecting photochemical processes of PSII against salinity (Hajiboland et al. 2010). This parameter (ΦPSII) can be used as a quick index to know if the AM symbiosis alters the photosynthetic activity during stressful conditions such as drought.

Results support that the AM symbiosis regulates synthesis and accumulation of compatible solutes and also may control the ion homeostasis, regulates water uptake and its distribution to plant tissues and reduces oxidative damage and improved photosynthesis. But many physiological aspects and the molecular bases of such regulation are almost completely unknown.

Since AM colonisation can help plants to cope with drought and salinity stresses (Augé 2001; Ruíz-Lozano 2003), the role and the mechanisms involved in the AM symbiosis to help plant performance under osmotic stress conditions have been the subject of many studies (Ruíz-Lozano and Aroca 2008; Ruíz-Lozano et al. 2008). In this context, the pioneering work of Ruíz-Lozano and Azcón (1993) and Azcón et al. (1988) showed that AM inoculation improved plant performance at low levels of water potential, and the negative effects of osmotic-stressed plants in general could be compensated by AM inoculation.

Both AM fungi and PGPR can adapt to specific conditions and develop tolerance to stressful environments (Ruíz-Lozano and Azcón 2000). Autochthonous fungal isolates from arid soils are more resistant to drought than those isolated from non-stressed sites. It is hypothesised that they have undergone selection of adaptation to these stressed environments (Marulanda et al. 2007, 2009). The inoculation of these autochthonous drought-tolerant AM fungal strains showed the greatest water stress tolerance and benefited the growth of lavender plants more than those allochthonous AM fungal strains. Similarly, the effectiveness of a native drought-adapted bacteria was greater in improving water transport and root development of *Retama sphaerocarpa* (Marulanda et al. 2006).

Bacteria seem to have developed mechanisms to cope with drought stress (Dimkpa et al. 2009c). We tested the increased IAA production in axenic medium by *Bacillus megaterium* at the highest PEG concentration used of 60 %. This is an indication of bacterial resistance to drought. The bacterial IAA production under such stressed conditions may explain their effectiveness in promoting plant growth and shoot water content and in increasing plant drought tolerance.

In fact it is well documented that inoculation with PGPR induces plant tolerance to abiotic stresses like drought (Marulanda et al. 2008). The beneficial effects of PGPR inoculation under osmotic stress conditions tested are an improvement in water status (Nadeem et al. 2007).

To maintain plant water status under osmotic stress conditions, plant tissues need to reach a balance between water lost by leaf transpiration and water gained by root uptake. The effect of PGPR inoculation on leaf transpiration has been largely studied with contrasting results (Alguacil et al. 2009a; Bashan et al. 2009; Rincón et al. 2008). But the role of PGPR influencing root water uptake capacity remains almost unexplored. The increased root hydraulic conductance (L) found in sorghum plants inoculated with *Azospirillum brasilense* under control and osmotic stress conditions may be explained by a higher leaf dehydration caused by osmotic stress in non-inoculated plants having a higher leaf transpiration rates and/or lower root water uptake capacity (Sarig et al. 1992). Plant root and shoot biomass are limited by the osmotic stress, but the microbial inoculation attenuates these negative effects.

A strain of *Bacillus megaterium* isolated from arid soil caused in inoculated plants higher root hydraulic conductance (L) values under both unstressed and osmotic-stressed conditions, which evidenced the ability of this bacterium to increase water use efficiency in colonised plants. These higher L values in inoculated plants correlated with higher plasma membrane type two (PIP2) aquaporin amount in their roots under osmotic-stressed conditions. Also, ZmPIP1;1 protein amount was higher in inoculated leaves than in non-inoculated ones under osmotic-stressed conditions. Hence, the different regulations of *PIP* aquaporin expression and abundance by the inoculation with the *B. megaterium* strain could be one of the causes of the different osmotic responses in terms of root growth, necrotic leaf area, leaf relative water content and L by the inoculation. The *B. megaterium* strain—used in this study—also produced the auxin indole acetic acid (Marulanda et al. 2009), which can up- or downregulate plant aquaporin expression (Lin et al. 2007; Mut et al. 2008; Werner et al. 2001). Anyway, the higher amount of PIP2 proteins in inoculated roots under osmotic stress could be the cause of the higher L of those plants (Sade et al. 2010). Thus, the modification of PIP aquaporin expression and abundance by inoculation with *B. megaterium* could be one of the causes of the different plant osmotic responses. The higher accumulation of ZmPIP1;2 protein in leaves of inoculated plants under osmotic stress could increase their water use efficiency (Sade et al. 2010). However, which signal mechanisms (hormonal or not) are behind this different aquaporin regulation in inoculated plants will be the matter of future studies.

A well-developed and persistent bacterial community needs to be established to be efficient. The activity of such microbial communities may be essential in the establishment and nutrition of plants in such environments. Thus a good survival in osmotic-stressed environments is required. We tested that this *B. megaterium* strain colonised not only rhizosphere but also endorhizosphere zone. These good colonisers and adapted strains have an important role on plant development under drought environment (Dimkpa et al. 2009c).

Rhizosphere bacteria can have ACC deaminase activity, and regulation of ACC is one of the mechanisms involved in the beneficial effect of PGPR on abiotically stressed plants (Dimkpa et al. 2009c). At the molecular level, gene expression changes related to ethylene production have been reported (Timmusk and Wagner 1999) in abiotically stressed plants inoculated with PGPR. As it is known, the ethylene synthesis increases when the plant is exposed to different types of stress, and it plays a key role in stress-related signal transduction pathways. Thus, low level of ethylene changes the general stress status of the plant.

Bacteria possessing the ACC enzymatic activity can use the immediate ethylene precursor ACC as a source of nitrogen, and the bacterial hydrolysis of ACC leads to a decreased plant ethylene level. This results in increasing root development (Belimov et al. 2007, 2009).

In addition, bacteria occurring on root surfaces containing ACC deaminase have been shown to modify the sensitivity of root and leaf biomass to soil drying, apparently by changing ethylene signalling. In fact, ethylene production was decreased in PGPR-inoculated plants, and this was related with improved recovery from water deficiency. The importance of decreased endogenous ethylene levels in PGPR-mediated tolerance to osmotic stress has been reported by Mayak et al. (2004) and Saravanakumar and Samiyappan (2007). The inoculation with *Pseudomonas fluorescens* TDK1 having ACC deaminase resulted the most efficient on groundnut grown under osmotic stress conditions, compared to the strains lacking the ACC enzyme (Saravanakumar and Samiyappan 2007).

In plants of pepper inoculated with *Bacillus* sp. TW4 under osmotic stress, genes linked with ethylene metabolism such as *caACCO* (encoding ACC oxidase) and *caLTPI* (an abiotic stress-inducible gene encoding a lipid transfer protein) (Jung et al. 2003) were downregulated (Sziderics et al. 2007). Enzyme ACC deaminase may be involved in the lower expression of these genes because *Bacillus* sp. TW4 showed this enzyme. Similarly, droughted pea inoculated with ACC deaminase activity-containing *Variovorax paradoxus*, as against an ACC deaminase mutant strain, showed hormone signalling-mediated plant growth improvement, biomass yield and water use efficiency (Belimov et al. 2009).

Salt-stressed maize inoculated with *Azospirillum* increased K^+/Na^+ ratios (Hamdia et al. 2004). In the same way, salt-stressed maize inoculated with *Pseudomonas syringae*, *Enterobacter aerogenes* and *P. fluorescens* having ACC deaminase activity resulted in higher K^+/Na^+ ratios concomitantly with high relative water and chlorophyll and low proline contents as reported by Nadeem et al. (2007). As all these results showed, osmotic stress tolerance appears to be dependent on different direct or indirect mechanisms.

Maize inoculated with *Azospirillum brasilense*, under water deficiency, improved relative and absolute water contents. This bacterial treatment also prevents a significant drop in water potential and in parallel increases root growth, total aerial biomass and foliar area with a concomitant proline accumulation in leaves and roots. These changes, including root morphology, by the inoculation of *Azospirillum* increased the maize tolerance to drought (Cassan et al. 2001; Dobbelaere et al. 1999). Bacterial production of hormone-like substances and

their ability to stimulate endogenous hormone levels were believed to play the key role in this process.

It is known that proline is often synthesised by plants in response to various abiotic stresses mediating osmotic adjustment, free radical scavenging and subcellular structure stabilisation (Hare and Cress 1997). Moreover, proline synthesis has been increased in abiotically stressed plants inoculated with PGPR such as *Burkholderia* and also with *Arthrobacter* and *Bacillus* (Marulanda et al. 2008; Sziderics et al. 2007).

In the same way, the greatest proline accumulation in axenic culture of the native bacteria *B. megaterium* under increasing stress conditions shows that it could induce the adjustment of cell osmotic potential, indicative of osmotic cellular adaptation. Proline may act as osmolyte, stabilising proteins, scavenging hydroxyl radicals and regulating NAD/NADH ratio. This is a mechanism by which cells can cope with drought stress (Paleg et al. 1984). In fact these both compounds (proline and IAA) resulted as useful markers of bacterial performance in plants growing in water-stressed soils (Boiero et al. 2007).

Water and phosphorus deficit led to changes in phospholipid composition in the root, and PGPR produced changes in the elasticity/plasticity of the root cell membranes. Changes in phospholipid content in the cell membranes have been observed upon inoculation with *Azospirillum* (Bashan et al. 1992). This could be one of the PGPR mechanisms towards an enhanced tolerance to water deficiency. Cell membranes are the most important interfaces within a complex system regulating a plant's physiological status. The electrolyte leakage under water deficiency has been shown to be correlated with membrane damage and lipid composition (Moran et al. 1994; Ruíz-Sánchez et al. 2010).

Inoculation with native AM fungi produced the greatest improvement in nutrient and water status as well as in long-term growth for *O. europaea* and *R. lycioides* plants. The $\delta^{13}C$ data showed that intrinsic water use efficiency in *Olea* was stimulated by native AM fungi, and foliar δ^{18} values indicated that native AM fungi enhanced stomatal conductance to a greater extent than the non-native AM fungus in these plants. These results indicated that modulation of leaf gas exchange by native, drought-adapted, AM fungi is critical to the long-term performance of host plants in semi-arid environments. In fact, the enhanced transpiration and plant water status are considered key mechanisms involved in plant growth stimulation by native AM fungi in semi-arid soils (Querejeta et al. 2007).

The most efficient AM fungus in drought soils exhibited greater extraradical mycelium (glomalin) and concomitantly the highest plant water content (Marulanda et al. 2007), probably due to an improvement of root conductivity to water flow and/or via mycelium able to transport water to the AM-colonised root system (Bryla and Duniway 1997; Marulanda et al. 2007; Ruíz-Lozano and Azcón 1995). As result, this increased plant water content was accompanied by decreasing antioxidant compounds as glutathione, ascorbate and H_2O_2 that have an important role in plant protection and metabolic functions under water deficit.

Marulanda et al. (2006) reported the effect of drought-tolerant AM fungus and *Bacillus* sp. in improving plant tolerance to drought stress and water transport in

Retama sphaerocarpa. Moreover, coinoculation with the native AM fungi resulted in the most effective interaction.

In fact *Azospirillum* inoculation reduced significantly the synthesis and accumulation of glutathione, proline and lipid peroxides in AM plants, which seem to be related to the plant protection from dehydration through drought avoidance mechanisms (Ruíz-Sánchez et al. 2010).

The performance of photosystem II indicates a better performance of photosynthetic apparatus, and it was increased in singly AM-colonised plants or in combination with *Azospirillum*. A positive correlation exists between tolerance to drought stress and maintenance of efficiency of photosystem II, which also keep plant productivity (Loggini et al. 1999; Saccardy et al. 1998). AM colonisation increased shoot biomass by 50 %, and this effect was also ascribed to an enhancement of the plant photosynthetic efficiency (Ruíz-Sánchez et al. 2010).

Combined inoculation of bacterial strains with AM fungi that compatibilised at a physiological level produced growth-stimulating effects that surpassed those of individual inoculations (Galleguillos et al. 2000). These results suggest that dually inoculated plants were better protected against the drought stress imposed (Ruíz-Sánchez et al. 2010).

6.4 Significance of Microbial Activities in the Mycorrhizosphere for Remediation of Heavy Metal-Contaminated Soils

Increasing industrial and anthropogenic activities have raised the concentrations of toxic metals and have caused environmental pollution in agricultural soils, water and atmosphere (Amoozegar et al. 2005). The remediation of heavy metal (HM)-contaminated soils is a challenging task because metals are not easily degraded. Thus, they are dangerous and pose indefinite persistence in the environment since, unfortunately, metals cannot be biodegraded. Nevertheless, soil microorganisms play a major role in bioremediation or biotransformation processes (Amoozegar et al. 2005). Microorganisms can transform these heavy metals by changing their oxidation state through the addition of (reduction) or removing of (oxidation) electrons (Tabak et al. 2005).

Prokaryotes are usually the agents responsible for most bioremediation strategies, but also eukaryotes, such as fungi, can transform and degrade contaminants (Tabak et al. 2005). It is well known that microorganisms already living in contaminated environments are often well adapted to survival in the presence of existing heavy metals. The adaptative potential is the battery of specific stress responses which allow these organisms to respond to the environmental signals by changing their pattern of gene expression (Aertsen and Michiels 2005).

Phytoremediation uses plants as a way to extract heavy metals from soil (phytoextraction) or to stabilise the metals in the soil (phytostabilisation), and

these both processes are low cost and have a high efficiency (Mulligan et al. 2001). For the success of these processes, soil microorganisms are very important. They include plant root associated free-living as well as symbiotic rhizobacteria and mycorrhizal fungi in particular that are integral part of these rhizosphere biota. The overall results of plant–rhizosphere–microbe interactions evidence a higher microbial density and metabolic activity in the mycorrhizosphere, even in metal-contaminated soil (Rajkumar et al. 2008).

Rhizoremediation is defined as a specific type of phytoremediation that involves both plants, and their associated rhizosphere microbes which can occur naturally or by introducing specific microbes.

Different strategies might be involved in preventing toxicity damage in inoculated plants. Changes in metal uptake and/or internal transportation storage can confer metal tolerance to the host plant (Scheloske et al. 2004). In addition, changes in root exudates, pH and physico-chemical properties of the soil (Grichko et al. 2000) may be involved, and such changes could reduce metal root uptake or translocation from root to shoot tissue.

Many mycorrhizosphere-colonising bacteria typically produce metabolites, such as siderophores, biosurfactants or organic acids that not only stimulate plant growth (Glick 1995) but also may reduce heavy metal availability in the medium. The fact that AM fungi and bacteria have been found in heavy metal-contaminated soils is an indication of fungal and bacterial tolerance, and functioning in polluted environments (Azcón et al. 2009a, b).

Most commonly the mechanism of resistance in prokaryotes is an efflux of the toxic metals by the action of P-type ATPases or secondary efflux systems (Nies and Silver 1995; Paulsen and Saier 1997). An important mechanism on this respect is the synthesis of extracellular polymeric substances, a mixture of polysaccharides, mucopolysaccharides and proteins which can bind significant amounts of potentially toxic metals and entrap precipitated metal sulphides and oxides. In bacteria, peptidoglycan carboxyl groups are main cationic binding sites in Gram-positive species. Chitin, phenolic polymers and melanins are important structural components of fungal walls, and these are also effective biosorbents for metals. These microorganisms can interact with heavy metals by many mechanisms, some of which may be used as the basis of potential bioremediation strategies (Valls and de Lorenzo 2002).

The tolerance mechanisms in AM plants must be ascribed to immobilisation of heavy metals by compounds secreted by the fungus or precipitation in polyphosphate granules, HMs adsorption to chitin in the cell wall or chelation of metals inside the fungus, changes in rhizosphere pH and/or the regulation of gene expression under stress conditions (Malekzadeh et al. 2011; Paszkowski 2006).

Vivas et al. (2003a) reported that the amount of Pb absorbed per root weight unit decreased considerably in plants inoculated with *Brevibacillus* sp. or with AM fungi plus *Brevibacillus* sp. (both native strains isolated from Pb-contaminated soil). These results also show an important ability of *Brevibacillus* sp. for Pb biosorption (26 % of the biomass weight) that may have contributed to Pb removal from soil and to alleviated Pb toxicity for plants. This *Brevibacillus* sp. was able to

produce in vitro 3.8 mg L^{-1} IAA, and this might have contributed to the beneficial effects observed (Pishchik et al. 2002). In addition, it is known that the AM mycelium has a high metal sorption capacity compared to other soil microorganisms (Joner et al. 2000). It is also likely the bacterial stimulation of extraradical mycelium production by AM colonised roots. Thus, the interaction between these microorganisms could have contributed to protect host plants against Pb toxicity (Vivas et al. 2003a).

Based on the chemical properties of Cd, the possibility of conversion to a less toxic form is likely to be very low. Nevertheless, the interaction between autochthonous *Brevibacillus brevis* and *Glomus mosseae* both isolated from Cd-polluted soil increased plant growth and nutrition and decreased Cd concentration in plant tissues (Vivas et al. 2005b). This fact was particularly relevant at the highest Cd level tested. Inoculated plants have reduced Cd concentration and lower the proportion of Cd soil/plant transfer. Both native microorganisms showed the highest functional compatibility (tested as the AM colonisation rate and physiological characteristics of this AM infection) and benefit to the plant as it was tested when compared with a reference *G. mosseae* strain (Vivas et al. 2005b).

Results also provide evidence that the inoculation of *G. mosseae* and *B. brevis* (both adapted to Cd) decreased by 1.5-, 3.3- or 2.8-folds shoot Cd concentration depending on available Cd in soil. But due to the plant growth stimulation, these treatments increased by two- or threefolds Cd uptake by *Trifolium* plants at 13.6 and 33 mg kg^{-1} of Cd, respectively (Vivas et al. 2003a). Thus, the inocula increased the Cd extraction in this Cd-contaminated soil.

Sorption to cell components followed by intracellular sequestration or precipitation as insoluble organic or inorganic compounds reduces heavy metal mobility (Gadd 2004). Microorganisms also affect metal bioavailability by acidification of the microenvironment. Cd ions can be bound by bacteria into complex forms that cannot be taken up by the plant (Pishchik et al. 2002). Bacteria with cell wall components having metal-binding properties are important in their ability for the accumulation of metals. This bacterial activity can contribute in the reduction of heavy metal uptake by plants associated with these bacteria (Vivas et al. 2005a). The important Cd biosorption by *B. brevis* seems also to contribute to the effects described in these studies. Bacteria with this ability may protect plants against either cadmium, iron, copper, nickel, lead or zinc toxicity. This PGPR activity has previously been shown to be related to the production of siderophores (Dimkpa et al. 2008, 2009b). Microbial siderophores were also shown to alleviate metal-induced oxidative stress in plants and exerted a bioprotective effect by lowering the formation of cell-damaging free radicals. The antioxidant activities of *Bacillus* cereus and *Candida parapsilosis* (from multicontaminated soil) were also determined when growing in a multicontaminated medium and used as index values of heavy metals cells tolerance (Azcón et al. 2010).

In addition, microbial-inoculated plants showed an increased root system that improved plant nutrition and, in turn, increased carbon and nutrient leakage to the rhizosphere zone that could also account for the enhancement of plant Cd tolerance by the coinoculation of these microorganisms (Vivas et al. 2005b). In this

Cd-contaminated soil, rhizosphere from non-inoculated plants showed reduced enzymatic activities which is an indicator of the perturbations caused to the ecosystem functioning under polluted conditions (Medina et al. 2004; Naseby and Lynch 1997). Cd reduced the uptake of Mg needed for chlorophyll biosynthesis (Kapoor and Bhatnagar 2007), and Cd reduced the uptake of P which contributes to pigment biosynthesis in its role as an energy carrier. In addition, Cd induced lipid peroxidation which causes degradation of photosynthetic pigments (Somashekaraiah et al. 1992); and Cd decreased the synthesis of chlorophyll enzyme, consequently reducing the photosynthesis and inhibiting the growth of plants (Liu et al. 2011). The increase of enzymatic phosphatase, ß-glucosidase and dehydrogenase activities in the mycorrhizosphere of inoculated plants could be due to the effect of changes in nutrient leakage from roots (quantitative and/or qualitative changes in root exudates) (Vivas et al. 2005b) and mycorrhizosphere activity (Medina et al. 2003).

Nickel was among the most toxic metals to *R. leguminosaru*m bv trifolii according to Charudhry et al. (1998) and Vörös et al. (1998). The reductions in nodule size and nitrogenase activity were also determined in white clover grown in metal-polluted soils (Märtensson 1992). These evidences suggest that microorganisms are far more sensitive to heavy metal stress than plants growing on the same soils.

Results from Vivas et al. (2006a) showed that the formation of symbiotic associations (mainly the nodules) decreased as available Ni in the soil increased. Nevertheless, results provided in this study showed that the detrimental effect of Ni could be reduced by the interactive effect of selected beneficial microbes in the soil–plant system. When roots of legume plants growing in Ni-polluted soils were coinoculated with PGPR and AM fungi, plants reduced considerably Ni uptake per mg of root (SAR), and this decreased SAR for Ni seems to be the main effect involved in the improved nodulation and shoot plant biomass in inoculated plants. The microbial associations here assayed may potentially affect the root Ni uptake in different ways involving a combination of factors such as via a hyphal quelation or sequestration in the vacuolar membrane vesicles (Nishimura et al. 1998) and bacterial sorption or precipitation as insoluble compounds reducing Ni mobility (Gadd 2004) or siderophore production (Dimkpa et al. 2008).

Zinc is an essential metal for normal plant growth and development since it is a constituent of many enzymes and proteins. However, excessive concentrations of this metal are well known to be toxic to most living organisms.

Microbially inoculated plants with adapted Zn-tolerant microorganisms also reduced shoot Zn concentration in plants growing in Zn-polluted soil. The proportion of soil/plant Zn transfer was decreased by the inoculants, applied particularly in association. The mycorrhizal and bacterial plant growth stimulations caused in part by their sequestration or precipitation of metals are activities that may be involved in these beneficial effects found (Vivas et al. 2006a).

In AM fungal-colonised plants, the expression of genes encoding plasma membrane transporters affecting element accumulation by plants has been reported (Burleigh and Bechmann 2002). The expression of a Zn transporter gene

(*MtZ1P2*) was decreased in root of mycorrhizal plants at a high Zn concentration of 100 mg g^{-1} as described by Burleigh et al. (2003). Also, González-Guerrero et al. (2005) suggested the role of *GintZnT1*, encoding a putative Zn transporter in Zn compartmentalisation and in the protection of *Glomus intraradices* against Zn stress.

The AM fungi/bacteria interaction in the mycorrhizosphere can be also explained by the fact that the bacterial inoculation was a critical factor for reaching maximum growth rates of AM spore germination and further mycelial development under nonpolluted and, particularly, under polluted conditions (Vivas et al. 2005a). Reduction or even inhibition of mycorrhizal colonisation by heavy metals has been reported by several authors (Liu et al. 2011; Weissenhorn and Leyval 1995). This reduction in mycorrhizal colonisation may be due to the deleterious effects of heavy metals on fungal spore germination leading to a loss in the fungal infective capacity or the high heavy metal accumulation in roots that may affect the AMF hyphal growth inside the cell. Spores of *G. mosseae* demonstrated increased mycelial growth by 56 % (without Zn) and by 133 % (with 200 μg Zn mL^{-1}), when inoculated with the bacterium as compared with uninoculated spores (Vivas et al. 2005a). In a subsequent study, the same bacterium not only stimulated presymbiotic AM fungal development but also the quantity and quality (metabolic characteristics) of mycorrhizal colonisation, with the highest improvement for arbuscular vitality and activity (Vivas et al. 2005b).

The antioxidant activities involved in detoxifying the toxicity of heavy metals to plants were found to be increased by microorganisms (Azcón et al. 2009b; Dimkpa et al. 2009a). The antioxidant enzymes such as SOD, POD and APX are important components in preventing the oxidative stress in plant. It is based on the fact that the activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions (Abdel Latef 2010). The physiological/biochemical mechanisms by which the microbial isolates enhanced phytoremediation activity in AM plants include the following (1) improved rooting and AM formation and functioning; (2) enhanced microbial activity in the mycorrhizosphere; (3) accumulation of metals in the root–soil environment, thus avoiding their transfer to plant and to the trophic chain or to aquifers; and (4) regulation of antioxidant activities.

The use of selected HM-adapted microorganisms can apparently be engineered to improve plant tolerance to HMs and to benefit bioremediation of HM-contaminated soils. The molecular mechanisms involved in HM tolerance in AM-inoculated plants have been recently discussed (González-Guerrero et al. 2009).

Regarding microbial HM tolerance, the activities of antioxidant enzymes are known to play an important role in cell protection by alleviating cellular oxidative damage. Antioxidants seem to be directly involved in the adaptative microbial response and survival in HM-polluted sites (Azcón et al. 2010).

Most of the reports on this topic concluded that AM-colonised plants translocate less HMs to their shoots than the corresponding nonmycorrhizal plants, as shown for herbaceous (Díaz et al. 1996; Redon et al. 2009) or tree legumes (Lin et al. 2007). These findings suggest that the role of AM fungi in phytoremediation

is mainly based on the immobilisation (phytostabilisation) of HMs in soil (Leyval et al. 2002; Turnau et al. 2006). Furthermore, both rhizobacteria and AM fungi have been found to interact synergistically to benefit phytoremediation.

The most adapted rhizosphere microbial strains (fungi and/or bacteria) tend to cope and to prevail in heavy metal-contaminated soil, and microbial interactions seem to be crucial for plant survival in heavy metal-polluted soil Vivas et al. (2003b and 2006b). Hyphae of metal-adapted AM fungi may have the capacity to bind metals present in roots or in the mycorrhizosphere, and this activity would decrease metal translocation from the root to the shoot, which has been proposed as the main mechanism for enhancing plant tolerance (Díaz et al. 1996; Lin et al. 2007; Redon et al. 2009). AMF may immobilise metals in several ways including the secretion of special compounds and the precipitation of heavy metals in polyphosphate granules. Moreover, glomalin is an insoluble glycoprotein that can immobilise heavy metals by binding them in the soil; fungal vesicles may be involved in storing toxic metals and thereby avoiding their translocation to shoot; the binding of heavy metals to chitin in the fungal cell walls causes a reduction in the translocation of heavy metals to the shoot of the AM-colonised plants (Göhre and Paszkowski 2006).

On the other hand, metal-adapted *Brevibacillus* strains have demonstrated metal biosorption ability and plant growth-promoting (PGP) and mycorrhizae helper (MH) activities. Thus, dual inoculation of plants with native *Brevibacillus* strains and AM fungi seems to be a strategy which can be recommended for promoting plant growth in heavy metal-polluted soils, inferring a phytostabilisation-based activity; however, as the total HM content in plant shoots was higher in dually inoculated plants, due to the effect on biomass accumulation, a possible phytoextraction activity was suggested.

6.5 Microbial Activities in the Mycorrhizosphere Involved in Plant Protection Against Biotic Stress

Biological control of soil-borne diseases is known to result from the reduction in the saprophytic growth of the pathogens followed by reduction in the frequency of the root infections through microbial antagonism and/or from the stimulation of "induced systemic resistance (ISR)" in the host plants (van Loon et al. 1998). Some mycorrhizosphere microorganisms are able to benefit plants by more than one mechanism. In fact, developmental genetics and evolution timing analysis of microbe–plant symbioses, including both mutualistic, either N_2-fixing or mycorrhizal, and pathogenic associations, have revealed a common developmental programme for all of these compatible microbe–plant associations (Markmann et al. 2008; Provorov and Vorobyov 2009).

Different mechanisms have been suggested for the biocontrol activity of AM fungi (Barea et al. 2005b). One mechanism involves microbial changes that result

as the mycorrhizosphere develops, which is based on the shifts and resulting microbial equilibria that could help plant health. Activation of plant defence mechanisms, which can develop systemic resistance reactions, including protection against foliar pathogens, has been also reported (Pozo and Azcón-Aguilar 2007; Pozo et al. 2009).

A great interest will be to select bacterial species that are useful antagonists to biotic stressors and to investigate their ability to mitigate concomitant stresses that have a great importance. Rhizosphere bacteria have demonstrated to enhance resistance to biotic stressors, as well as increase tolerance to abiotic stresses in host plants.

Siderophore production by PGPR, which protects plants against pathogenic bacteria through better competition for iron, has also been shown to be able to protect plants from induced oxidative stress (Dimkpa et al. 2009c). Not only siderophores but also organic acids and antibiotics may be responsible for indirect plant stimulation which includes the suppression of pathogens (Kloepper et al. 1999).

Anthracnose, caused by the fungus *Colletotrichum acutatum*, is one of the most important diseases in strawberry crop. But in strawberry plants, previously inoculated with *A. brasilense*, it was observed that siderophore production showed in vitro antifungal activity against *C. acutatum* M11 and results showed the reduction of anthracnose symptoms on strawberry plants (Tortora et al. 2011).

The isolation of siderophores-producing strains of *A. brasilense* from strawberry plants with plant growth-promoting effects over this crop has been reported (Pedraza et al. 2007, 2010). The plant growth-promoting bacterium *A. brasilense* REC3 participates actively in the induction of systemic protection on strawberry plants against anthracnose disease caused by *C. acutatum* M11. Biochemical and transcriptional studies revealed a transient accumulation of SA and the induction of defence-related genes, suggesting further that this response is related to structural cell wall modifications as consequence of the observed increase in phenolic compounds and callose deposition (Tortora et al. 2011).

Some studies have been conducted on *Azospirillum* as biocontrol agent to promote plant growth indirectly, e.g. by limiting the proliferation of phytopathogens such as the agent of crown gall disease (Bakanchikova et al. 1993), bacterial leaf blight of mulberry (Sudhakar et al. 2000) and soil-borne plant pathogens that attack *Cucumis sativus* (Hassouna et al. 1998).

Imposing energy stress on *Rhizoctonia solani* and promoting growth of tomato plants also controlled bacterial leaf tomato diseases caused by *Pseudomonas syringae* pv. *tomato* (Bashan and de-Bashan 2002a, b) and also inhibited development of bacterial diseases on fresh-market and cherry tomato (Romero et al. 2003) and on *Prunus cerasifera* (Russo et al. 2008) or promoted disease resistance on rice crops (Yasuda et al. 2009).

Siderophores can improve vegetal growth by increasing plant nutrient availability through iron uptake and preventing the growth of soil-borne pathogens due to iron limitation (Chaiharn et al. 2009; Miethke and Marahiel 2007; Sayyed and Chincholkar 2009). Although siderophores vary greatly in chemical structure, they

were classified in two main groups: catechol and hydroxamate, according to the chemical group involved in iron (III) chelation. Bacterial siderophore production is a biocontrol mechanism.

Salicylic acid (SA) besides being a compound with siderophore activity (Meyer et al. 1992; Visca et al. 1993) is a precursor in the biosynthesis of microbial catechol-type siderophores, such as yersiniabactin, pyoverdin and pyochelin (Cox et al. 1981; Jones et al. 2007; Serino et al. 1995).

Therefore, SA-producing microbial strains may increase defence mechanisms in plants. However, bacterial SA participation on plants' induced systemic resistance (ISR) is still controversial (Cornelis and Matthisj 2007).

Profound physiological changes take place in the host plant upon root colonisation by AMF affecting the interactions with a wide range of organisms below- and above-ground. In fact, during mycorrhiza establishment, modulation of plant defence responses occurs, thus achieving a functional symbiosis after AM fungal colonisation, and an effective activation of the plant immune responses seems to occur, not only locally but also systemically.

Priming sets the plant in an "alert" state in which defences are not actively expressed but in which the response to an attack occurs faster and/or stronger compared to plants not previously exposed to the priming stimulus, efficiently increasing plant resistance. Priming seems to be the mechanism underlying the induced systemic resistance (ISR) observed in plants interacting with beneficial microorganisms (Conrath et al. 2006; Goellner and Conrath 2008; van Wees et al. 2008). Interestingly, priming of the plant immune responses by beneficial microbes is often dependent on a functional JA signalling pathway, as has been described for rhizobacteria and AMF (Pozo et al. 2004, 2010; Van der Ent et al. 2009; Verhagen et al. 2004).

The priming of jasmonate-regulated plant defence mechanisms plays a central role in the induction of resistance by arbuscular mycorrhizae, and the higher resistance of mycorrhizal plants to a wide range of below-ground attackers such as soil-borne fungal and bacterial pathogens, nematodes or root-chewing insects (Azcón-Aguilar and Barea 1997; Whipps 2004) or induced resistance against shoot pathogens has also been reported (Campos-Soriano et al. 2012; Koricheva et al. 2009; Pozo and Azcón-Aguilar 2007).

Induction of ISR in the host plants may be critical for bacteria's ability to mitigate both biotic and abiotic stress effects. Thus, studies on ISR against plant pathogens will be very useful in decoding signalling cascades induced by rhizosphere bacteria, resulting in enhanced abiotic stress tolerance. In fact, mechanisms triggering ISR and those leading to enhanced tolerance against abiotic stresses overlap.

Plant defence responses are coordinated by small molecules that act as signal transducers and tailor the coordinated expression of genes that code for defence-related proteins and compounds (Ausubel 2005; Jones and Dangl 2006). Among these molecules, the phytohormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) play key roles (Pieterse et al. 2009).

The identification of defence regulatory elements that may operate in priming of plant defences in plants with a mycorrhizosphere optimised may have important practical implications regarding the effectiveness of AMF and PGPR in the biological control and integrated management of pests and diseases.

The role of microbial associations in growth, stress tolerance and plant establishment under nutritional and other stress conditions is based on a range of physiological and cellular mechanisms. There is evidence that AM fungi and PGPR help plants grow under stress conditions by reducing stress and by increasing physiological and nutritional plant status.

6.6 Conclusions

The establishment of the arbuscular mycorrhizal (AM) fungus in the root cortex is known to change many key aspects of plant physiology and the mineral nutrient composition in plant tissues, the hormonal balance and the patterns of C allocation. Consequently, AM colonisation induced many plant changes which affect the microbial populations, both quantitatively and qualitatively, in either the rhizosphere or the rhizoplane. However, there are specific modifications in the environment surrounding of the AM extraradical mycelium itself. Thus, the mycorrhizosphere formed around AM plants has features that differ from the rhizosphere of a nonmycorrhizal plant. Bacteria living in the soil root surface or inside cortical cells can interact with AM fungi. The cooperative plant–microbial interactions have focused their attention on plant growth-promoting bacteria (PGPB) and AM fungi and processes involved in the establishment and functioning of the mycorrhizosphere. AM and PGPB inoculation improved plant performance at low levels of nutrients, water potential, at high amount of heavy metals and under diseases caused by photogenic associations. All the detrimental effects in plants of these stresses can be compensated by specific microbial associations in the mycorrhizosphere based on a range of physiological and cellular mechanisms here reported.

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Chapter 7
How Root Structure Defines the Arbuscular Mycorrhizal Symbiosis and What We Can Learn from It?

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7.1 Introduction

7.1.1 Mycorrhizal Anatomy

Mycorrhiza is a mutualistic association between fungi and plant roots, in which the fungal partner facilitates mineral nutrient acquisition by the plant and in turn host provides carbohydrates to the fungus (Smith and Read 2008). Up to seven different mycorrhizal types have been distinguished, depending on the fungal symbiont and the type of anatomy that it forms in the root (Peterson et al. 2004; Smith and Read 2008). The arbuscular mycorrhiza (AM) formed by fungi of the division *Glomeromycota* is one of the most widespread in terrestrial ecosystems, since it is formed by almost 80 % of plants (Brundrett 2009). This is characterised by the intracellular growth of the fungal partner, forming arbuscules in the cells and, under some circumstances, vesicles. Among AM, a high variability of mycorrhizal anatomies can be distinguished, sometimes described as a continuum between the classical *Arum* and *Paris* anatomical types (Dickson 2004). However, most authors who have studied mycorrhizal anatomy classify the different plant species, genera and families as either *Arum* or *Paris* type (Smith and Smith 1997), and a reassessment considering this continuum of AM anatomical types is still pending in most cases.

The different AM types are usually highly consistent within a certain host plant and even within a certain plant family (Smith and Smith 1997; Dickson et al. 2007).

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However, the factors determining their formation are not well understood, and the AM anatomical type formed may be under host control (van Aarle et al. 2005; Ahulu et al. 2006), under AM fungal control (Cavagnaro et al. 2001a; Smith et al. 2004) or under the control of both plant species and AM fungus (Dickson 2004).

7.1.2 *Root Features as "Barriers" to the Entrance or Modifiers of the Development and Anatomy of Arbuscular Mycorrhizae*

The entrance of an AM fungus into a root, its development therein and formation of the mycorrhizal anatomical type and the mycorrhizal susceptibility of the different root orders present in a root system depend on many factors.

Some of the anatomical features present in roots act as barriers to the entrance of AM fungi, so that no entry points are formed. For example, the rhizodermis of some species presents extremely thickened cell walls that AM fungi cannot penetrate. Also, the presence of an exodermis or hypodermis, or thickened cells in the outer cortex, may hinder the AM fungi reaching the cortex (Brundrett and Kendrick 1990b; Brundrett et al. 1990). Modifications of the cell walls of these root tissues could determine the way in which AM fungi enter the roots. For example, in species with a dimorphic exodermis, AM fungi enter through the short cells after long cell suberinisation is complete (Brundrett and Kendrick 1988). But, in the cortex, AM development may also be hampered by root anatomical features, e.g. idioblast cells that contain crystals (Brundrett and Kendrick 1990b) or aerenchyma lacunae, which clearly reduce the tissue available for colonisation (Dreyer et al. 2010).

Other root anatomical features, like the Phi-thickenings mentioned by some authors as potential barriers to fungal growth in ectomycorrhizae (see Fernández-García et al. 2014), should not act as barriers in AM as the presence of Phi-thickenings even in the innermost cortical layer in *Ginkgo biloba* did not impede the AM fungus *Glomus epigaeum* from forming arbusculate coils in these cells (Fontana 1985).

Another root anatomical feature that may influence the mycorrhizal formation is the presence of intercellular spaces. Brundrett and Kendrick (1990a) suggested that the AM anatomical type formed is determined by the presence of continuous intercellular airspaces along the root cortex. When they are present, the *Arum* type is formed; otherwise, when they are absent, the *Paris* type develops. For plant species forming discontinuous intercellular airspaces, intermediate types would be expected (Smith and Smith 1997). However, although the correlation between the size of the airspaces within the root cortex and the AM anatomy was suggested as early as 1904 with the study of Gallaud (Dickson et al. 2007), there have been no concerted attempts to quantify this, although the few studies conducted do not support this hypothesis. For example, it has been shown that a

tomato cultivar that can form either *Arum*, *Paris* or intermediate AM anatomical types, depending on the AM fungus used as inoculum, may also present discontinuous airspaces (Bago et al. 2006; Dickson et al. 2007). In addition, Li (2008) showed that the proportion of airspaces in the inner and outer cortex of Dandelion and Chive did not have any influence on the AM anatomical types (*Arum*, *Paris* and intermediate) developed. The higher abundance of intercellular hyphae in dandelion did not correlate with bigger intercellular airspaces in the cortex. However, care has to be taken with these results, as only a small number of root sections were examined and the plants were not inoculated with a known isolate of AM fungus, so that different AM fungi may have been present (Li 2008).

It has been also found that other properties of the cortex may have a considerable influence on mycorrhizal anatomy and development of the AM fungi, since in many plant species with an *Arum*-type anatomy, the intercellular hyphae with arbuscules are localised within the roots preferentially in the inner cortex (Abbott 1982; Fisher and Jayachandran 1999; Allen et al. 2006), while in plant species with a *Paris*-type anatomy, extensive intracellular hyphal coils are formed but predominantly in the outer cortex (Cavagnaro et al. 2001b). Further, AM fungi are never present in the endodermis, although these cells do not offer physical restrictions to the radial passage of fungi when they are in State I of suberinisation (Brundrett and Kendrick 1990b). Other factors, like the nutrient content or gas concentration, might further determine the way in which the AM fungi spread in the root.

It has been suggested that in roots presenting physical barriers to their passage, AM fungi would penetrate the root subapical regions, from where they would colonise the entire root. However, in heterogeneous root systems, some root orders are colonised and others are not, although they all have non-differentiated root zones, in which the cell walls have no secondary modifications. Thus, there must be other reasons for the non-colonisation of some root orders, other than merely physical ones. This hypothesis is further emphasised by studies conducted with mutants that can block AM colonisation at different AM fungal developmental stages. Such studies should help unravel the events in recognition and early colonisation (Harrison 2005).

Further, root anatomical features may change in response to the presence of AM fungi or with abiotic factors like water. For example, some AM fungi are able to digest the cell wall material in order to facilitate their passage through the intercellular spaces (Bonfante and Perotto 1995), while others have no such ability.

The debate as to whether particular regions of a root are preferentially colonised by AM fungi, cells in these regions showing unique structural or physiological properties, will go further until more is known on the molecular dialogue between plants and AM fungi. It has been affirmed that the root segments are only colonisable for a limited time, after which they become permanently non-mycorrhizable (Schwab et al. 1991), although detailed analysis of AM entry points to clover and leek roots has shown that this is not the case, since a region immune to colonisation behind the root apex does not exist (Smith et al. 1992). However, it has been shown that an established arbuscular mycorrhizal symbiosis

suppresses further mycorrhization, demonstrating the presence of an autoregulatory mechanism linked with the extent of root colonisation (Vierheilig 2004).

What makes this type of study even more complicated is the highly dynamic nature of the processes involved in the establishment of a functioning AM. The growth of the root system is constant and there are always developing roots representing new colonisation sites for AM fungi. Curiously, this could be under the control of the AM fungal partner, as, among the biotic and abiotic factors that influence the root development, it has been shown that the presence of AM fungi can induce important changes in root systems (see Sect. 7.1.4).

7.1.3 Root Order Variation in Root Structure and Function and Mycorrhizal Susceptibility of Root Systems

Most plant species show a highly heterogeneous root system with a high diversity of root functions. Even the apparently homogeneous root systems of herbaceous plants show variability among root orders. For example, in maize root systems, seminal and nodal roots can be distinguished. The former play a role in the water supply and acquire less P, while the latter are for P acquisition (Hodge et al. 2009). In other plants, the real measured uptake rates suggest that only 10 % and 30 % of the total root system lengths are involved in nitrate and water uptake, respectively (Hodge et al. 2009). In trees, shrubs and other perennial plants, this variation in root function among root orders is expected to be even more marked. Unfortunately, no accompanying data have been presented on the mycorrhizal susceptibility and presence of the different AM fungal structures in these root types, although it has been repeatedly suggested that it would be highly revealing to examine root traits by root order in mycorrhizal studies (Valenzuela-Estrada et al. 2008). Many studies have shown that the root orders most susceptible to being colonised are the higher-order or ultimate roots (Janos 1977; Nadarajah 1980; Fisher and Jayachandran 1999; Dreyer et al. 2010).

The main reason for the lack of information on the mycorrhizal susceptibility of the root orders with different root functions may be the difficulty of distinguishing them. Roots, conversely to aboveground tissues, are very difficult to classify (Valenzuela-Estrada et al. 2008). Traditionally, the roots have been classified arbitrarily by their diameter, designating the ones narrower than 1 or 2 mm as ephemeral fine roots and assuming an absorptive function for them and those wider than 1 or 2 mm as perennial coarse roots with anchorage and transport functions. Recently, the characterisation of roots by their branching order has been reported as a useful approach to identify anatomical, morphological and functional differences within a root system (Pregitzer 2002; Pregitzer et al. 2002; Guo et al. 2008; Valenzuela-Estrada et al. 2008). However, care has to be taken here, as various root types of the same age and order exist in the same root system (Bagniewska-

Zadworna et al. 2012) and no study so far has determined whether branch order effectively distinguishes roots with an absorptive capacity in the entire root system.

As root functions are difficult to measure directly (Lucash et al. 2007), indirect methods, such as anatomical methods, have been used to determine root function, the assumption being that anatomy and physiology are tightly linked. The presence or absence of secondary xylem could be used as an indicator of the transition from absorptive to transport functions in temperate trees (Guo et al. 2008). Based on anatomical traits, it was estimated that 75 % and 68 % of the fine-root lengths in temperate forests were absorptive and mycorrhizal, respectively. In monocotyledonous plants such as *Arecaceae*, other anatomical and morphological features could be proposed, e.g. the absence of aerenchyma and sclerenchymatic ring, as indicators of absorptive functions (Dreyer et al. 2010).

Such studies are further complicated by the fact that not all parts of the root systems are active at the same time since tissue ageing and differentiation occur. Further, this may vary with nutrient availability. These differences have a clear impact on the pattern of AM colonisation. However, very few studies have been devoted to studying AM colonisation, from a root order point of view.

More studies are needed in which the heterogeneity of roots as regards their physiological capacity is coupled with the spatial pattern of resource availability and their interactions with soil microorganisms like AM fungi (Hodge et al. 2009), e.g. the study of Comas and Eissenstat (2009). Otherwise, the possibility exists that the data gained will remain highly confusing, because roots with different patterns and functions are being compared.

7.1.4 Arbuscular Mycorrhizal Fungi Induce Changes in the Root System Morphology and Physiology

The anatomical features of a plant root are genetically determined and no absolute change in response to biotic factors has been described. Of course, there may be minor changes, e.g. the colour change of the roots due to AM colonisation in onions (Becker and Gerdemann 1977), maize (Klingner et al. 1995) and other plants (Fester et al. 2002); enlargement of the root cortex with extra cell layers to accommodate the AM fungal structures; or minor modifications of the plant cell wall to allow the passage of AM fungi.

What may indeed be under AM fungal control is the quantity of the different root orders that constitute the highly heterogeneous root system, whose structure is determined by an interplay between the intrinsic developmental programme and external biotic and abiotic stimuli (Lynch 1995). The ability of plant root structures to adapt to the encountered environmental conditions varies greatly, depending on the plant species, soil composition and, particularly, on water and mineral nutrient availability (Malamy 2005). Much less is known about the effect of biotic factors on

the temporal-spatial distribution and structure of root systems, but one of the most important biotic interactions is that which involves AM fungi.

It has been recognised for a long time that AM colonisation could enhance root growth. Further, it has been described that nutrients could affect root morphology in non-mycorrhizal plants (Drew 1975), and it was found that the morphological changes in the root systems of mycorrhizal plants could be correlated with the improvement in nutrient acquisition that they experience. One of the first studies that showed that mycorrhizal dependency was related to root morphology was that of Baylis (1975). Since then, numerous studies have pointed out that AM fungi have an important impact on the root morphology and architecture of plants and consequently on plant physiology. This aspect has been reviewed by Atkinson et al. (1994), Berta et al. (1993), Hetrick (1991) and, more recently, Berta et al. (2002).

Morphological modifications have been divided into structural, spatial, quantitative and temporal modifications (Atkinson 1992). Analysis of the spatial morphology of *Allium porrum* using the topological method showed that the branching pattern was not affected by AM fungi despite the strong impact they had on lateral root numbers per unit of root length (Berta et al. 1993). However, in other studies, changes in the root branching pattern have been observed, with control plants presenting a herringbone-like root morphology, while mycorrhizal plants were characterised by a dichotomous root system (Atkinson et al. 1994). Conversely to these two examples, most studies have determined only the quantitative morphology of root systems. It is well known that AM fungi decrease the root biomass in relation to the aerial biomass of the host plant (Berta et al. 1990; Smith and Read 2008; Torrisi et al. 1999). However, higher root to shoot ratios have also been found in mycorrhizal plants compared to non-mycorrhizal ones like *Prunus cerasifera* (Atkinson et al. 1994), *Andropogon gerardii* (Hetrick et al. 1988), *Populus* sp. (Hooker et al. 1992), *Arachis hypogaea* and *Cajanus cajan* (Yano et al. 1996). Although the mechanisms underlying this effect are not clear, it has been observed that the addition of P to *A. porrum* plants inoculated with *G. mosseae* diminished the root to shoot ratio (Amijee et al. 1989). In non-mycorrhizal plants, the nutrient deficiency increases the root to shoot ratio (Lynch 1995). Further, it has also been suggested that plants that are less mycorrhizal dependent have a higher root to shoot ratio, although this is not supported by the study of Manjunath and Habte (1991).

Although root biomass is the root growth parameter most measured in mycorrhizal studies, studying this parameter alone, without taking into account the root morphology and architecture, can lead to important changes and differences in biomass allocation being overlooked (Hetrick 1991). Root mass can seldom be correlated with the nutrient absorption capacity, as the main roots that contribute most to total mass are those that contribute least to nutrient acquisition. For this reason, it is also important to study other morphological features of the root systems.

AM fungi have been seen to cause multiple changes in root morphology and the alteration patterns may vary highly among plant species. In most associations, AM colonisation increases the number of adventitious and lateral roots (Aguín

et al. 2004; Berta et al. 1990, 1995; Hetrick et al. 1988; Hooker et al. 1992; Torrisi et al. 1999; Yano et al. 1996) and usually results in greater branching, although this may be not a general trait of mycorrhizal roots, as a reduction in branching in *A. gerardii* has been observed (Hetrick et al. 1988). AM fungi also lead to an increase in root diameter in *Lycopersicon esculentum* (Fusconi et al. 1999), *A. gerardii* (Hetrick et al. 1988) and *P. cerasifera*, the latter only in association with *G. intraradices* but not with *G. mosseae* (Berta et al. 1995). Conversely, in *Gossypium hirsutum*, the diameter was not affected by AM colonisation (Torrisi et al. 1999).

Hooker et al. (1992) observed an increase in the branching of plants inoculated with *Glomus* sp. E3 and *G. caledonium*, but no effect was registered when they were inoculated with *Scutellospora calospora*. The results of Berta et al. (1993) showed that the root systems of *A. porrum* plants inoculated with *Glomus* E3 were more branched and contained shorter and more branched adventitious roots, with a higher proportion of roots of higher orders, with greater diameter and less specific root length. In other studies, it has been determined that the differences between mycorrhizal and control plants increased with the root order. Hooker et al. (1992) observed that inoculation with *S. calospora*, *Glomus* sp. E3 or *Glomus caledonium* of *Populus* did not affect the total root length, while the length of second- and third-order roots increased in mycorrhizal plants. The branching of second- and third-order roots was greater in mycorrhizal than in non-mycorrhizal plants. The branching of second-order roots increased with *Glomus* E3 and *G. caledonium* by 81 % and 60 %, respectively. The increase in branching of third-order roots with *Glomus* E3 and *G. caledonium* was 616 % and 500 %, respectively.

Other studies suggest that the response may not be universal. Trotta et al. (1996) found a reduction in the branching of the root system of mycorrhizal *Lycopersicon esculentum* plants in comparison with non-mycorrhizal ones. However, Vigo et al. (2000) observed no effect on root system morphology in tomato due to AM fungi. Gamalero et al. (2002) observed that AM colonisation increased total root length only in soils low in nutrients and that the branching was reduced in mycorrhizal plants, but not the number of apices.

As the AM colonisation of roots normally affects plant nutrition, the effect of AM fungi on root system morphology has been ascribed to growth effects related to nutrition (i.e. the direct effect of nutrition on plant development).

This is difficult to determine, due to the difference in growth between mycorrhizal and non-mycorrhizal plants. Using plants of similar growth, Hooker et al. (1992) showed that AM colonisation modifies the root system in a different way to the modification induced by high P levels in non-mycorrhizal plants. Conversely, Tisserant et al. (1996) indicated that the increase in branching of the root system of *Platanus acerifolia* coincided, from the fifth week after inoculation with *G. fasciculatum*, with the development of the active fungal biomass in all the lateral root orders and with a significant increase in P acquisition. Also, Amijee et al. (1989) found that high P absorption in mycorrhizal plants influenced the root geometry. Price et al. (1989) indicated that the specific root length increased with the increase in soil P concentration, but was reduced by the AM association. In

non-mycorrhizal plants of *Hordeum vulgare* cv. Proctor, Drew (1975) observed that in localised zones with a high availability of P, NO^{3-} and NH^{4+}, lateral root number and branching increased. Therefore, the greater P content in mycorrhizal plants compared to non-mycorrhizal plants can at least partially explain the induced modifications in root systems of mycorrhizal plants. However, other additional or alternative effects cannot be excluded, such as modifications in the phytohormone balance or differences in mitotic index of the root apices in mycorrhizal plants due to blocked meristematic activity (Berta et al. 1990, 1991; Fusconi et al. 2000).

7.2 Case Study: The Palm *Phoenix canariensis* Chabaud

7.2.1 Some Considerations and Definitions

In case of palms, few studies about root morphology and anatomy have been carried out. Tomlinson (1990) made a brief review of these studies, while the book of von Guttenberg (1968) provides numerous examples on the root anatomy of different palm species. The most complete studies on palm root anatomy are those of Seubert (1996, 1997) devoted to the subfamilies *Calamoideae* and *Coryphoideae*. Other studies provide specific details of certain palm species; for example, the anatomy of adventitious roots of *P. canariensis* (Cabrera et al. 1990), some aspects on root morphology and anatomy of *P. dactylifera* (Oihabi 1991) and *Metroxylon sagu* (Nitta et al. 2002) or the aerial roots of different palms (de Granville 1974). Further, the root architecture of *Elaeis guineensis* has also been studied (Jourdan et al. 1995, 2000; Jourdan and Rey 1997). However, none of these studies had proposed comparing the morphology and anatomy of mycorrhizal and non-mycorrhizal root systems.

One of the prerequisites to be able to study the modifications induced by AM fungi in a given root system is to have sufficient knowledge about the morphology and anatomy of such a root system before modification has taken place. As the mycorrhizal condition is the rule in nature for most plant species, this can only be achieved under controlled conditions that allow the plant to grow without the presence of AM fungi. The modifications induced by AM fungi in the root architecture can be very extensive but difficult to identify and quantify. A detailed comparative analysis of mycorrhizal and non-mycorrhizal root systems is required, ensuring that all roots with all their intact connections to lower-order roots are sampled (Hooker et al. 1998). This was not possible to achieve for *P. canariensis* as almost all the third-order roots were loosened from the root system during the harvesting process, despite the fact that the plants were grown in silica sand to facilitate sampling and the extreme care taken in the process. Thus, of the different types of root parameters required according to Atkinson (1992) to describe root morphology or architecture (structural, spatial, quantitative and temporal morphology), we have collated data on the quantitative morphology.

Here, the term "order" is used to define the branching degree, with first-order roots being the adventitious roots, i.e. the roots originating from the shoot. Second-order roots are those originating from first-order roots, and third-order roots are the roots originating from second-order roots. Any root formed as a result of injury to the parent root (replacement roots) is designated as the same root order as the injured root from which they originated.

7.2.2 Root Morphology and Anatomy

The root system features of *P. canariensis* are those expected for plants with high mycorrhizal dependency (Brundrett 1991), i.e. a low specific root length (SRL), few root orders, sparse branching, absence of root hairs and protective features like the lignification of primary root structures (see below and Sect. 15.2.4 for quantitative data). Third-order roots show the thinnest diameter and the highest SRL and represent the largest proportion of total root length (TRL).

P. canariensis is characterised by a homorrhizal root system that can develop up to three root orders (Dreyer et al. 2010). An abrupt change in diameter takes place with the development of each root order (Table 7.1). The third-order roots are morphologically distinct and can be further divided into five different groups: short thick roots, mycorrhizal thickened roots, fine short roots, fine long roots and pneumatorhizas. The short thick roots are lateral modified roots, strongly swollen, and bottle-shaped. The mycorrhizal thickened roots are also a type of swollen third-order roots but of a deep yellow colour that can be observed only in mycorrhizal root systems (Dreyer et al. 2010). It has been suggested that the precursor roots of the mycorrhizal thickened roots could be the short thick roots which, once colonised, undergo an elongation and colour change (Dreyer et al. 2010).

Along the roots of all orders, numerous pneumathodes, pneumatozones or pneumatorings are found. These zones or rings, of mealy aspect, loose tissue and bright white colour, are clearly distinguishable from the normal root segments and persist for a long time after the root abscission. In addition, the root system also presents numerous pneumatorhizas and pneumatophores. The pneumatorhizas are extremely short modified lateral roots, in which the loosening of the rhizodermis and the outer cortex forms a "hat" on the apex, while pneumatorings are present at their base. The pneumatophores are second-order aerial roots that develop with negative geotropic growth, with generally more than one pneumatoring at their surface.

As is to be expected, most of the anatomical aspects also vary considerably with and within each root order (Table 7.1). The first- and second-order roots consist, in general, of the same tissues, with the difference that the second-order roots are composed of fewer cell layers. The outermost root tissue is a one-layered rhizodermis consisting of large persistent cells with a thickened lignified outer cell wall. Beneath the rhizodermis lies the exodermis composed of two layers of equally thickened lignified cells. The outer cortex appears to be homogeneous, in

Table 7.1 Morphological and anatomical features of the different root orders of *Phoenix canariensis*

	Root order			
	First	Second	Third	
Diameter (mm)[a]	3.27 (2.08–4.44)	1.22 (1.01–1.57)	0.54 (0.45–0.63)	
Number of roots[a]	4.45 (3–7)	277.88 (22–713)	1598.86 (185–4461)	
			Mycorrhizal thickened roots	Fine roots[b]
Rhizodermis (μm)	240	20	24	24
Exodermis (μm)		25	40–52	32
Outer cortex (μm)		24–28		28
Inner cortex (μm)	2,080	88–110	200–360	80
Endodermis	− PC	− PC	+ PC	− PC
Vascular cylinder (μm)	2,040	80–130	68–120	84
NO. of phloem poles	34	4–16	3–4	4–8
Parenchymatic pith	Yes	No	No	No
Raphia-type fibres	Yes	Yes/no	No	No
Aerenchyma	Yes	Yes	No	Yes/no
AM colonisation	No	No	Yes	Yes[c]/no

[a]Mean of 40 plants; the diameter and number of second- and third-order roots were calculated based on 100 segments for each plant. In brackets, maximum and minimum values
[b]Both short and long fine roots together
[c]Intraradical hyphae and spores were observed, but no arbuscules. +/− PC, with or without passage cells

the form of a continuous lignified sclerenchymatic ring, but is mostly composed of two zones: an outer zone consisting of thin-walled cells and an inner zone of strongly thickened cells.

The inner cortex is divided into three zones. The outer zone is built up of small cells, with few intercellular spaces; the middle zone of larger cells, with wider intercellular spaces mostly forming aerenchyma lacunae; and the inner zone of small cells, radially oriented in concentric rows, again with few intercellular spaces. The walls of all the inner cortex cells are thin, with the exception of the *Raphia*-type fibre bundles. The outermost layer of the vascular cylinder is a one-layered pericycle with equally moderately thickened walls. The rest of the vascular cylinder is formed of sclerotic tissue, in which the xylem and phloem elements are embedded. The vascular cylinder is polyarch, with a mean number of phloem and xylem poles of 34 and 6–16 in first-order roots and second-order roots, respectively (Table 7.1). In the centre, a parenchymatic pith can be observed.

The mycorrhizal thickened roots are also characterised by a one-layered rhizodermis consisting of unequally thickened lignified cells. No exodermis is present. The outer cortex is formed of two to three, more or less lignified, cell layers. The inner cortex is homogenous and no aerenchyma is present. The tertiary endodermis always displays passage cells (Table 7.1). The vascular cylinder is

triarch or tetrarch, with sclerotic pith (Table 7.1). The short thick roots show the same anatomy as the mycorrhizal thickened roots. The long fine roots are characterised by a more or less developed aerenchyma lacuna system in the inner cortex and a two-zoned outer cortex. In contrast, the short fine roots show a much reduced cortex of only four cell layers, with no division into outer and inner cortex.

7.2.3 Root Function Diversity

Based on the root anatomical features, it can be indirectly stated that the function of the first- and second-order roots, as well as of the long fine third-order roots, is anchorage and conductance. In these roots, no AM colonisation has been observed due to the presence of the sclerenchymatic ring in the outer cortex, which represents a physical barrier against AM fungal penetration, and of the aerenchyma lacunae in the inner cortex, which considerably reduce the tissue available for AM colonisation (Table 7.1; Dreyer et al. 2010). The third-order root-denominated pneumatorhizas have an aeration function, as do the pneumatorings, pneumatozones and pneumatophores. It is unclear whether an absorption function can be assigned to all other third-order roots, mycorrhizal thickened roots, short thick roots and short fine roots or just to the mycorrhizal thickened roots. However, what is clear is that only the mycorrhizal thickened roots formed mycorrhizae with arbuscules in *P. canariensis* (Dreyer et al. 2010), from which it can be deduced that only these roots harbour "functional" mycorrhizae. The AM fungal structures were found in the entire inner cortex, with the exception of the two inner layers adjacent to the stele (Dreyer et al. 2010). 89 % of the arbuscules present in transverse root sections of mycorrhizal thickened roots were succinate dehydrogenase active (Dreyer et al. 2006; Dreyer and Morte 2009). The rhizodermis and outer cortex lacked AM colonisation, except when the transverse section revealed entry points and the formation of coils. The AM anatomical type observed was characterised by the presence of intercellular hyphae and vesicles in the mature developmental stages of AM colonisation. The arbuscules were generally intercalated, although terminal arbuscules were observed as well (Dreyer et al. 2010). The arbuscules generally formed on the surface of hyphal coils and looked like arbusculate coils. Intracellular hyphae connecting two intercalated arbuscules were observed. The hyphae did not always choose the most direct way of intracellular passage, but crossed the cell wall at the corner of the cortex cells and, in transverse sections, could be distinguished as intercellular hyphae. Thus, two types of intercellular hyphae could be distinguished: those extending linearly along the roots and parallel to the root axis, called long-distance hyphae, and those that form intercalated arbuscules as a result of passing from one cell to another, called short-distance hyphae. Because of these features, the AM anatomical type of *P. canariensis* has been classified as intermediate (Dreyer et al. 2010; Fig. 7.1). Palms have been described to form *Arum*, *Paris* and intermediate AM anatomical types (Smith and Smith 1997; Fisher and Jayachandran 1999, 2005; Sengupta and Chaudhuri 2002;

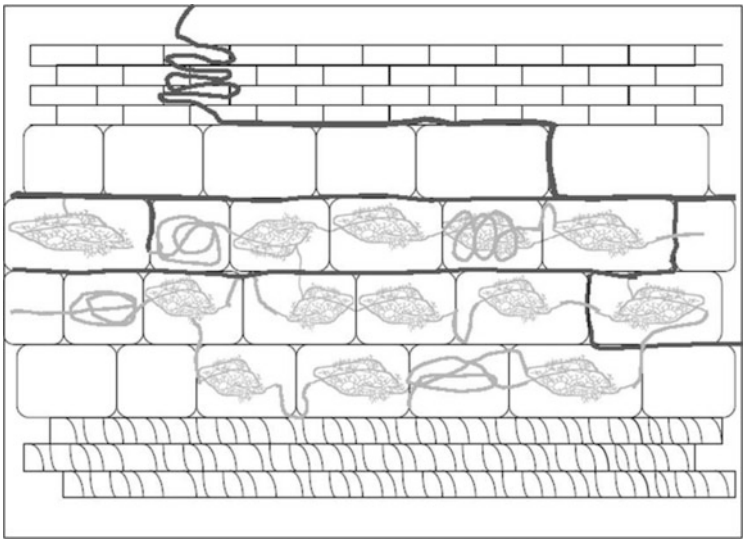

Fig. 7.1 Diagram of the intermediate AM colonisation found in *Phoenix canariensis*. The mycelium spreads intracellularly and sometimes intercellularly by long- and short-distance hyphae and forms intercalated arbusculate coils, rarely terminal

da Silva and Cardoso 2006; Ramos-Zapata et al. 2006; Dickson et al. 2007; Dreyer et al. 2010). However, in most studies, little emphasis has been placed on the aspect and formation of arbuscules, i.e. whether they were intercalated or terminal and simple or compound.

The other type of third-order roots, the short fine roots, although also colonised by AM fungi, presented only intraradical hyphae and spores or vesicles but no arbuscules (Dreyer et al. 2010). Further, it has been observed that the better aeration characteristics of the pneumatodermis in the pneumatorings of the second-order roots seem to trigger the massive sporulation of the AM fungi, leading to the formation of a spore pseudomantle (Dreyer et al. 2010). The exact meaning of the physical separation of different AM development patterns along the *Phoenix* root system, functional colonisation in mycorrhizal thickened roots, endophytic activities in the fine roots and spore proliferation at pneumatorings remains unclear. Muthukumar et al. (1997) suggested that mycotrophic nonfunctional plants, i.e. those with endophytic activities, may help increase the number of propagules in soils, since they observed that the association of a mycotrophic with a non-mycotrophic plant enhances arbuscular colonisation in the former plant and vesicular colonisation in the latter. *Phoenix* palms have been suggested as a good model for studying these different "endophytic" and "functional" activities of AM fungi because they bring together processes in the same plant and at the same time that normally occur separately in different plants or in the same plant at different times (Dreyer et al. 2010). The fine short roots and the pseudomantles could act as

inoculum reservoirs for newly developing mycorrhizal thickened roots (Dreyer et al. 2010).

Although other authors have shown that the higher-order roots in other palms are also the most susceptible to AM colonisation (Janos 1977; Nadarajah 1980; Fisher and Jayachandran 1999; Carrillo et al. 2002; Dreyer et al. 2010), they did not present the differentiation in AM function described for *Phoenix* spp. (Dreyer et al. 2010). Further, these other palms do not have the high degree of heterogeneity among the ultimate- or third-order roots that lead to different functions, such as ventilation through pneumatorhizas, which is a characteristic of *Phoenix* spp.

7.2.4 *Effect of AM Colonisation on Root Morphology*

In an experiment carried out with *Glomus mosseae* and the combination of *G. mosseae*, with either the isoflavonoid formononetin or the phytohormone indole-3-butyric acid (IBA), it was shown that the root diameter of the first-order roots increased in all mycorrhizal treatments in comparison with the non-mycorrhizal plants, while the diameter of second- and third-order roots did not differ (Fig. 7.2a). Conversely, the specific root length (SRL) of third-order roots, calculated by dividing the total root length by the fresh root mass, was clearly reduced by AM colonisation in all mycorrhizal treatments (Fig. 7.2b), although the total SRL for all root orders together showed a significant decrease only in the treatment with *G. mosseae* alone (results not shown).

The total root length (TRL) to shoot dry weight (SDW) ratio was much reduced by AM colonisation, showing that less biomass is invested in root development in mycorrhizal plants (Fig. 7.2c).

Specific phosphorus uptake (SPU), calculated by dividing the phosphorus acquisition efficiency (μg P plant^{-1}) by the total root length of third-order roots, was clearly increased in all mycorrhizal treatments (Fig. 7.2d).

It was supposed that formononetin enhanced the AM colonisation (Siqueira et al. 1991; Nair et al. 1991) and that this led to an increase in the number of mycorrhizal-susceptible roots (Torrisi et al. 1999). An enhanced mass and total length of third-order roots were observed in mycorrhizal *P. canariensis* palms treated with formononetin compared with mycorrhizal palms without formononetin (results not shown). However, this only led to a small increase in SRL in formononetin-treated palms (Fig. 7.2b). Similarly, the application of IBA should lead to the formation of more lateral roots (Blakely et al. 1988; Muday and Haworth 1994; Reed et al. 1998; Torrey 1986). The application of IBA led to modifications in the maize root system similar to that induced by AM fungi (Kaldorf and Ludwig-Müller 2000). Conversely, mycorrhizal *P. canariensis* palms showed different morphological features, e.g. a higher SRL, when treated with IBA compared to non-treated mycorrhizal palms (Fig. 7.2b), suggesting that the mechanism inducing the morphological changes may differ between IBA and AM fungi.

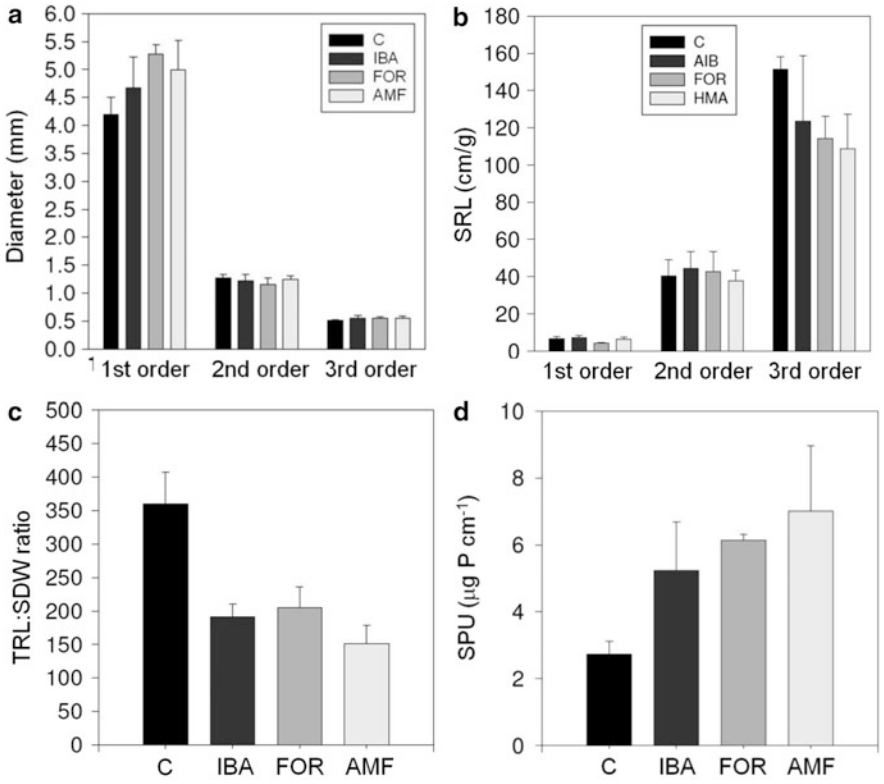

Fig. 7.2 Effect of IBA and formononetin on the root morphology of *Phoenix canariensis*, 9 months after inoculation with *Glomus mosseae*. (**a**) Diameter of different root orders, (**b**) specific root length (SRL), (**c**) total root length (TRL) to shoot dry weight (SDW) ratio, (**d**) specific P uptake (SPU). *C* control; AMF, *G. mosseae*; IBA, *G. mosseae* and IBA application; FOR, *G. mosseae* and formononetin application. The columns represent means of four repetitions with standard error

To study more specifically the effect of P and N on the root morphology, a further experiment was conducted, in which mycorrhizal and non-mycorrhizal *P. canariensis* palms were treated with five different fertilisation regimes. Independent of the fertilisation treatment, the AM colonisation led to a reduction in the SRL (Fig. 7.3a). The overall effect of the different fertilisation levels was similar for both mycorrhizal and non-mycorrhizal palms, with the palms treated either without P or without N showing the lowest SRLs.

The TRL:SDW ratio was also reduced by AM colonisation, and again, the results showed the same trend among fertilisation treatments (Fig. 7.3b).

A lower TRL:SDW ratio is a general feature of mycorrhizal plants (Marschner and Dell 1994) and is due to the lower investment in root development. This effect was not so evident in the root to shoot ratio (results not shown) and stresses the

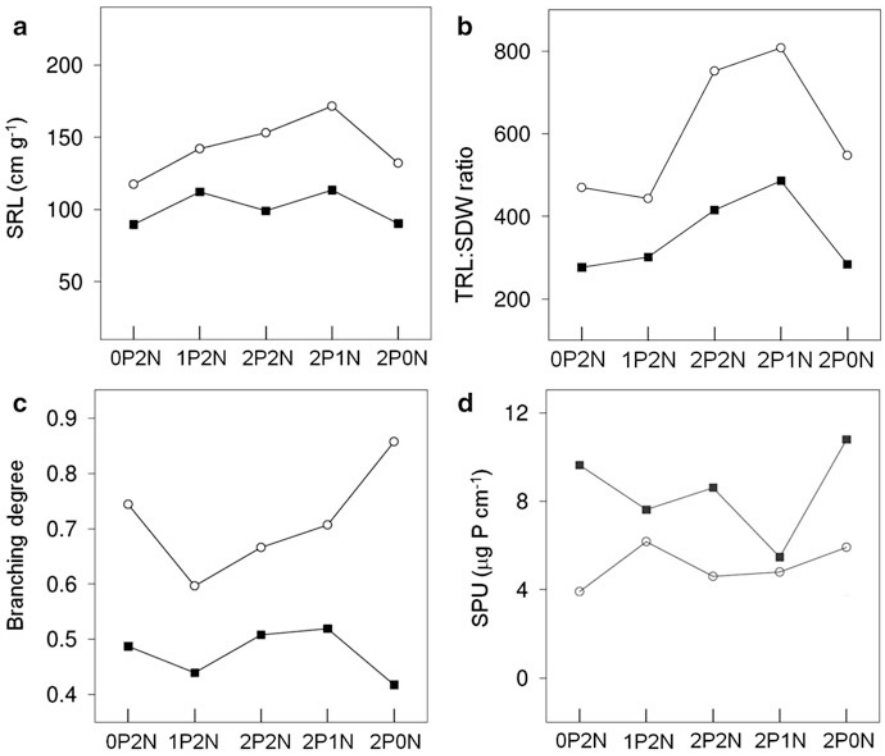

Fig. 7.3 Effect of different levels of P and N on the root morphology of *Phoenix canariensis* inoculated with *Glomus mosseae*. (**a**) Specific root length (SRL), (**b**) total root length (TRL) to shoot dry weight (SDW) ratio, (**c**) branching degree, (**d**) specific P uptake (SPU). *Hollow circles*, non-mycorrhizal; *filled squares*, mycorrhizal. Fertilisation with a Hewitt solution modified as follows: 0P2N, 0 mM P and 3.5 mM N; 1P2N, 0.67 mM P and 3.5 mM N; 2P2N, 1.33 mM P and 3.5 mM N; 2P1N, 1.33 mM and 1.75 mM; 2P0N, 1.33 mM P and 0 mM N

importance of complementing mycorrhizal studies with root morphological measures.

The branching degree of the root system, calculated by dividing the total root number by TRL, was reduced in mycorrhizal palms (Fig. 7.3c). The overall effect of the different fertilisation levels was the same in mycorrhizal and non-mycorrhizal palms, except in the case of the fertilisation without N, when the mycorrhizal palms showed the lowest branching degree, while the non-mycorrhizal palms resulted in the highest branching degree (Fig. 7.3c). The branching of root systems has been shown to decrease or be unaffected when plants are inoculated with AM fungi (Berta et al. 1995; Gamalero et al. 2002; Hetrick et al. 1988; Hooker et al. 1992; Tisserant et al. 1996; Trotta et al. 1996), although the results of the different studies are difficult to compare as branching is expressed as intensity (root numbers of order *n*/root numbers of order $n - 1$), degree (total root number/TRL) or frequency (root numbers of order *n*/root length of order $n - 1$). In palms,

branching is less in mycorrhizal root systems than in non-mycorrhizal ones, whether expressed as branching degree (Fig. 7.3c) or branching frequency (results not shown), perhaps because these plants invest more in building the external mycelium.

The lower SRL found in mycorrhizal palms compared with non-mycorrhizal ones means that more biomass is invested in the root system in mycorrhizal palms, especially in third-order roots, maybe as a strategy to harbour more AM colonisation in this moderately mycorrhizal-dependent palm. Species with high SRL have a lower cortical area available for mycorrhizal symbiosis and are normally less dependent on mycorrhizae, while plants that are more mycorrhizal dependent have a lower SRL, as they reduce the high metabolic cost of their roots by developing coarser root systems (Hetrick et al. 1988; Brundrett 1991; Hetrick 1991). Further, mycorrhizal root systems with low SRL are associated with a greater length of external AM fungal hyphae (Miller et al. 1995). The cost of producing fine roots may be superior to that involved in producing external hyphae. Extensive root systems required 20–47 % of all photosynthetic products for their production and maintenance (Smucker 1993). The maintenance of mycorrhizal root systems costs 4–20 % of additional photosynthetic products (Douds et al. 2000; Graham 2000).

It has been suggested that high SRLs are characteristic of plants grown under conditions of low P availability or, generally, in soils of low fertility (Hetrick et al. 1988). Further, Fusconi et al. (2000) found an increase in root diameter and TRL in mycorrhizal *A. porrum* plants, but only at low P concentrations. However, our results show that the *P. canariensis* palms grown without P or N presented the lowest SRLs (Fig. 7.3a). It has also been observed here that the application of P and N induced changes in the root morphology of non-mycorrhizal palms in a different way to that seen in mycorrhizal plants, as the changes induced by AM fungi were not reproducible by any of the fertilisation treatments. This has been observed in mycorrhizal *Populus* plants as well (Hooker et al. 1992). It would be important to test a higher range of nutrient concentrations in future studies with both mycorrhizal and non-mycorrhizal palms, to find the threshold, above which no additional morphological changes occur, in order to be absolutely certain about the changes induced by the AM fungi or by fertilisation (Hetrick 1991). However, this may be not so easy to do as the increase in nutrient availability may lead to an increase in lateral root numbers susceptible of being mycorrhized, which could lead also to an increase in AM colonisation and, consequently, to further morphological and physiological changes. However, this does not seem to occur as it has been shown that a high nutrient concentration, especially of P, diminished the percentage of AM colonisation. Our results show that fertilisation with a high concentration of P and N and fertilisation without N or without P have a negative impact on AM colonisation (Fig. 7.4a, b). The highest AM colonisation was achieved when the P concentration was halved, correlating with a higher number of mycorrhizal thickened roots (Fig. 7.4a). However, the palms in this treatment were not those with the highest SPU (Fig. 7.3d). Although the SPU was increased in all mycorrhizal treatments in comparison with non-mycorrhizal ones, the highest SPU was

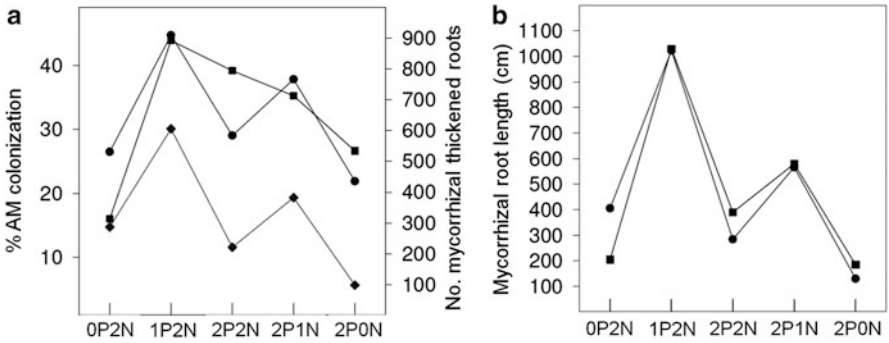

Fig. 7.4 Effect of different doses of P and N on the arbuscular mycorrhizal (AM) colonisation of *Phoenix canariensis*. (**a**) Percentage of AM colonisation. (**b**) Arbuscular mycorrhizal root length. *Squares*, estimation with trypan blue-stained roots; *circles*, direct count of mycorrhizal thickened roots; *diamonds*, total number of mycorrhizal thickened roots

observed in mycorrhizal palms not fertilised with N, followed by those not fertilised with P (Fig. 7.3d).

What seems to be the case is that the total number of mycorrhizal thickened roots was in agreement with their percentage in relation to all third-order roots, showing that the effect of the different fertilisation levels was similar for both third-order roots and mycorrhizal thickened roots (Fig. 7.4a). Thus, it may be that the interplay between AM fungi and the nutrient content in root cells alters the root morphology in order to adapt it to the prevailing soil conditions.

It may not be possible to achieve similar responses with nutrients and with AM fungi, because although both activate the same morphogenetic mechanisms, the intentions are different. In the case of nutrients, the production of more fine roots is a plant response to enhance nutrient absorption, while in the case of AM fungi, they increase root volume in order to accommodate more AM colonisation and, indirectly, increase nutrient absorption and interchange.

Our results show clearly that the AM fungi induce changes in the root morphology of *P. canariensis* palms and that the mechanisms underlying the morphological changes are not entirely due to improved host plant nutrition. The increase in ACP activities in roots of *P. canariensis* when inoculated with *G. mosseae* (Dreyer et al. 2008) may partially explain the increases in SPU seen here in both experiments.

7.2.5 *Inclusion of a Knowledge of Root Structure in the Methods for Evaluating AM Colonisation*

As stated above, the mycorrhizal thickened roots of *P. canariensis* presented a highly distinctive morphology and colour that could be estimated visually without the need for staining and subsequent assessment under the microscope.

It has been shown that it is possible to determine the AM colonisation level of *P. canariensis* by counting the mycorrhizal thickened roots directly (Fig. 7.4a, b). The reliability of the method can be demonstrated by comparing both methods, the visual assessment with assessment of stained roots (Fig. 7.4a, b).

The percentage of AM colonisation estimated from stained roots was in good agreement with the mycorrhizal thickened roots expressed as a percentage of the total third-order roots. Only in the treatment involving normal P and N fertilisation levels was the percentage estimated visually lower than that estimated by staining (Fig. 7.4a). The results expressed as total mycorrhizal root length estimated with both methods were even more similar (Fig. 7.4b).

It is possible that the lower AM colonisation level determined by directly counting mycorrhizal thickened roots in some treatments compared with the percentage estimated by staining is due to the fact that the whole root system was quantified in the first case, while only a subsample was subjected to staining, i.e. it was an effect caused by the sample size. Another explanation could be the time it takes since the first AM colonisation units are formed in the pre-mycorrhizal thickened roots until the synthesis of the yellow pigments is not known. For example, in maize, it has been observed that the yellow root segments are formed 1 week after the initial colonisation (Fester et al. 2002). Thus, it is possible that mycorrhizal palm roots were evaluated as non-mycorrhizal because of the lack of pigmentation. To know the limits of the visual method, the development of AM colonisation with respect to the production of the yellow pigment should be studied, e.g. by using a colorimetric method like the one used by Becker and Gerdemann (1977).

The method proposed here for *Phoenix* species is important from a practical point of view, as it could save a great deal of time in the mycorrhizal assessment of these palms and could facilitate the monitoring of the AM colonisation in vivo.

Regardless of the method chosen, what we have also learnt from this study is that a profound knowledge of the roots susceptible to being colonised by AM fungi is needed. Otherwise, the wrong sample, e.g. roots that will never be colonised by AM fungi, could be taken, leading to an underestimation of the AM colonisation level. This was probably the case in the first studies conducted with palms, which resulted in an extremely low AM colonisation percentage (Dreyer et al. 2001; Morte and Honrubia 2002). The contrary may also occur, as the subjective observer could be tempted to sample only mycorrhizal thickened roots as, we think, might be the case in the study of Oihabi et al. (1993), in which an AM colonisation level of 90 % was registered.

One could ask what purpose the data of the studies mentioned above serve (Dreyer et al. 2001; Morte and Honrubia 2002; Oihabi et al. 1993), besides verifying that the palms were colonised by AM fungi, if the percentages of AM colonisation levels are not related to the ratio of roots susceptible to being mycorrhized to the total roots of the root system. Further, it is surprising that when expressing AM colonisation as mycorrhizal root length, the total root length is used for the calculations since this factor could vary among treatments, as shown by our morphological studies, and no direct relation has been demonstrated between percentage of AM colonisation and total root length. Instead, we believe that there is a relation between mycorrhizal roots and a certain type of root order. The good agreement between our results using the visual assessment method and assessment by staining was because in neither of them were the second-order and first-order roots considered; the number of mycorrhizal roots was related to the total number of third-order roots.

Although many attempts have been made to quantify the degree of mycorrhizal colonisation of root systems (Giovannetti and Mosse 1980; Trouvelot et al. 1986; McGonigle et al. 1990), none of the methods developed until now allow an accurate estimation of the high plasticity and dynamics as well as functional diversity of a given root system, which may be the main reason why in most of the studies no correlation between AM colonisation and physiological parameters was found. Thus, the tedious quantification work carried out normally ends with just the observation that the mycorrhizal system is colonised by AM fungi or not, something that could be achieved more quickly with a subjective estimation. The methods used were developed for the study of herbaceous plants, which were assumed to have homogenous root systems, and may only be accurate for them. For perennial plants, however, they are of little value, without a careful consideration of root morphological features.

7.3 What Can We Learn from These Studies?

In most mycorrhizal studies, root anatomy, root morphology and mycorrhizal anatomy are treated separately. Our results show that these three fields should be brought together to provide more information on mycorrhizal symbiosis. While *P. canariensis* may represent a very curious case, much of the information presented here would have been overlooked if an integrative approach had not been chosen.

Further, it should be remembered that the way in which root system architecture is studied has its origin in hydrology, assuming that all roots act as streams (Horton 1945). This is clearly a simplification and may be not the best option in root systems showing a high heterogeneity of roots with different functions. Studying the root system of *P. canariensis* by means of a topological model would have led the different third-order roots present being overlooked.

Different root types among root orders may also be present in other palms, but may have been overlooked due to the approach chosen. For example, Zona (1996) mentioned that the root system of *Roystonea* sp. presents tuberised roots that are mycorrhizal colonisation sites. However, no information on the anatomy or morphology of this type of roots in *Roystonea* is available because this observation was made by chance (S. Zona, personal communication).

Regarding the intermediate-type AM anatomy found in *P. canariensis*, we have suggested that a degree of adaptation exists to the slow growth of palms. As most palm studies have contributed little information on AM anatomy, a reassessment of this aspect may be necessary. The impression is that the intermediate and *Paris* AM anatomical types may be more widespread in palms than was formerly believed. We encourage further mycorrhizal studies in the plant family *Palmae* (*Arecaceae*) as less than 1.2 % of the existing palm species have been studied in regard to their mycorrhizal condition.

Further, the possible "autoregulation" of the soil AM inocula levels shown here for *P. canariensis* and by other authors between mycotrophic and non-mycotrophic plants should receive more attention.

And last, but not least, mycorrhizologists should rethink the way in which they evaluate mycorrhizal colonisation. Sampling of the bulk root system may be suitable for assessing the percentage of mycorrhizal colonisation in fast-growing annual plants, but in the case of tree and shrub species, the evaluation of the percentage of mycorrhizal colonisation should be preceded by a morphoanatomical study of the different root orders present.

7.4 Conclusions

The colonisation of a root system by arbuscular mycorrhizal (AM) fungi depends on different root anatomical characteristics, e.g. thickening of the cell walls of the rhizodermis, exodermis and outer cortex or the presence of aerenchyma in the inner cortex. As a result, only some root orders are susceptible of being colonised. The type of mycorrhizal anatomy formed ranges between the two extremes of a continuum, the *Paris* and *Arum* type, and it has also been suggested that this depends on features of the root anatomy.

For over two decades, it has been known that AM fungi alter the root morphology of their host plants, in most cases reducing root branching and decreasing specific root length and the total root length to shoot dry weight ratio.

Despite this knowledge in all mycorrhizal studies to date, mycorrhizal colonisation has been expressed as a percentage colonisation of the total root length and there has been no attempt to modify the methods of AM colonisation assessment. Here, the results obtained with the palm species *P. canariensis* are presented as a case study. As stated by other authors, a root order-oriented approach may expand the information gained from mycorrhizal studies. An alternative way of assessing AM colonisation for this palm species is suggested, the main objective being to

provoke a rethinking of the methods used in mycorrhizal research and to move towards a more integrative approach.

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Chapter 8
How Ectomycorrhizae Structures Boost the Root System?

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Please note the Erratum to this chapter at the end of the book.

8.1 Introduction

The word mycorrhiza has its origin in the Greek words fungus (*mukés*) and root (*rhiza*), and it defines its symbiotic association. The plant obtains water and minerals from the fungus, and the fungus obtains photosynthetic products and vitamins from the plant (Allen 1991). There is the possibility that one fungus forms mycorrhizae with more than one species of plant and it is usual that different fungi form mycorrhizae with the same plant species (Brundrett 2002; Simard et al. 2012).

Mycorrhizae are divided into several groups according to their morphology. The two main groups are endomycorrhizae and ectomycorrhizae, according to the penetration or not (respectively) of the fungi in the root cortex cells. In more detail, there are seven important mycorrhizal types (Smith and Read 2008), arbuscular mycorrhizae, ectomycorrhizae, ectendomycorrhizae, arbutoid mycorrhizae, monotropoid mycorrhizae, ericoid mycorrhizae, and orchid mycorrhizae,

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depending on whether they have intracellular colonization. Ectomycorrhiza is the only type that does not have intracellular colonization and is always formed by septate fungi, Ascomycota and Basidiomycota, and occasionally by Glomeromycota. They cover the root, forming a fungal mantle, and an intracellular space, forming the Hartig net in gymnosperms and angiosperms.

About 95 % of the trees in temperate areas (mainly Pinaceae, Fagaceae, Dipterocarpaceae, and Caesalpiniaceae families) form ectomycorrhizae with a number of Ascomycota and Basidiomycota species, including edible fungi with a high commercial value such as *Tuber* sp., *Boletus* sp., and *Lactarius* sp. (Smith and Read 2008).

Fungal structures formed in those associations (mantle, cystidia, extraradical mycelium, and rhizomorphs) increase the root exploration area in the soil and allow both the tree and the fungi to thrive (Agerer 1998).

The study of ectomycorrhizal anatomy and morphology has always been limited by it requiring access to the microscopic structures in the soil. Although mycorrhizae were discovered by Albert B. Frank in 1885, their detailed description did not begin until the 1980s. Since the beginning of the twenty-first century, molecular tools that can detect and measure the presence of a fungus in the soil have significantly increased available knowledge on fungal identification and dynamics. Nowadays, there is abundant data about ectomycorrhizal fungal communities in a forest, but there is little knowledge about how these organisms interact with each other, simply because there is scarce knowledge about their structures and about how they live together in the roots.

Mycorrhizae are classic examples to explain the mutualistic interaction between two different organisms in nature: a plant and a fungus. Both species establish a permanent relationship, they live together in symbiosis, and that differentiates the nature of mycorrhizae from other plant–fungus interactions. Although the line between parasitism and mutualism is fine and negative interactions between plant and fungus can occur with changing environmental conditions, the relationship is generally positive for both symbionts (Allen 1991).

8.2 The Huge Diversity of Ectomycorrhizal Fungi in the Forest Ecosystems and Its Relevance

There are more than 3,500 land plant species that have the ability to form mycorrhizae (Wang and Qiu 2006). A relatively small number are exclusively ectomycorrhizal (probably around 3 % of seed plants) (Meyer 1973), but their global importance is greatly increased by their disproportionate occupancy of terrestrial land surfaces and their economic value as the main producers of timber. Smith and Read (2008) and Wang and Qiu (2006) counted the plant genera reported to contain at least one species of ectomycorrhizae. They reflect that there are 195 terrestrial plant genera belonging to 65 families which could form

ectomycorrhizae, including the ones that form conifer forests, deciduous forests, and sclerophyll forests, three of the main forest types on Earth.

Over 5,000 fungal species form ectomycorrhizae (Agerer 2006). Within Basidiomycota exclusively Hymenomycetes and within Ascomycota exclusively Ascomycetes contribute to this type of symbiosis. Pezizales are mostly responsible for ascomycetous ectomycorrhizae, with their hypogeous derivates, whereas Boletales, Gomphales, Thelephorales, Amanitaceae, Cantharellaceae, Cortinariaceae, Russulaceae, and Tricholomataceae form ectomycorrhizal relationships within Hymenomycetes (Agerer 2006).

Wild edible fungi are an important socioeconomic resource in many regions of the world. More than 2,000 fungi are known to produce edible sporocarps (Boa 2004). Over the last decade, the market value, consumer demand, and interest in managing forests for non-timber products have increased (Díaz-Balteiro et al. 2003; Pilz et al. 1999). This resource not only is a food source but also could be an important income generator in rural forest areas if used properly (Barroetaveña et al. 2008; Boa 2004). In addition, edible mushrooms also represent the basis of multiple products made by manufacturers, including medicine (Table 8.1), and are the source of a new wave of tourism resulting from recreational programs linked to nature.

Nowadays, the commercial value of forest fungi may equal or even surpass that of timber, especially in the Mediterranean area (Alexander et al. 2002; Arnolds 1995; De Román and Boa 2006; Reyna 2012); therefore, fungi have become strategic in the conservation and management of forest systems. Where mushroom picking is a significant forest resource, it should be included in forest management and planning (Palahí et al. 2009). Paradoxically, only in the last 10 years has this resource begun to be integrated within forest planning, most of the time sporadically (Martínez de Aragón et al. 2007). The lack of information and the low predictability of harvesting sporocarps may be partly the cause of this absence.

Sporocarp formation of these fungi is linked to habitat characteristics and climate conditions, but this data alone does not explain all the trends of fungal fruiting and dynamics. Factors influencing sporocarp formation are not yet apparent. Sporocarp formation is probably the most complicated stage in the life cycle of fungi. This situation is even more complex for ectomycorrhizal fungi, which require symbiotic association with a host plant. Fungal and host genes, environmental and physiological conditions, and nutritional state of mycelium and the host trigger sporocarp formation in ectomycorrhizal fungi, but the process is not fully understood to date (Murat et al. 2008).

Different factors are known to influence the sporocarp formation of fungi. Several studies have shown that fructification is linked to habitat characteristics (Alonso Ponce et al. 2011) and climate conditions, mainly soil moisture and temperature (Barroetaveña et al. 2008; Bonet et al. 2010; Martínez de Aragón et al. 2007; Pinna et al. 2010; Salerni et al. 2002). However, the same studies stated that this data alone does not explain all the trends in the dynamics of fungal fruiting. Host conditions also obviously affect this process (Kües and Martin 2011; Ortega-Martínez et al. 2011). In early successional or disturbed habitats, nutrients are

Table 8.1 Ectomycorrhizal species used as food and medicine all over the world, extracted from Boa (2004)

Ectomycorrhizal genus	Number of edible species	Number of medicinal species
Amanita Dill. ex Boehm.	83	7
Boletus L.	72	7
Cantharellus Adans. ex Fr.	42	3
Cortinarius (Pers.) Gray	50	10
Laccaria Berk. & Broome	14	4
Lactarius Pers.	94	7
Leccinum Gray	26	–
Morchella Dill. ex Pers.	18	5
Ramaria Holmsk.	44	5
Russula Pers.	128	25
Suillus P. Micheli	27	2
Terfezia (Tul. & C. Tul.) Tul. & C. Tul.	7	–
Tricholoma (Fr.) Staude	52	17
Tuber P. Micheli ex F.H. Wigg.	18	–

primarily abiotic, ecosystems tend to have high entropy levels, and symbioses, primarily mycorrhizae, are not well developed; but later successional habitats have a high degree of symbiosis, organically bound nutrients, and low entropy, suggesting that mycorrhizae are important in the successional process and ecosystem development (Allen 1991).

The "universal" latitudinal gradient of diversity that characterizes the distribution of richness of most terrestrial and marine macroorganisms has a unimodal form in ectomycorrhizal fungi (Tedersoo et al. 2012). There is lower ectomycorrhizal fungal richness in tropical ecosystems, failing to conform to the "universal" pattern. Tedersoo and Nara (2010) propose three explanations for this unique fact. Firstly, the strictly temperate ectomycorrhizal fungal lineages probably evolved at higher latitudes with the Pinaceae hosts, but may be inferior competitors in tropical conditions. Secondly, when both soil and roots are regarded as habitats for ectomycorrhizal fungi, the lower diversity and abundance of these habitats may account for the lower ectomycorrhizal fungal diversity in the tropics. Thirdly, there is the effect of resource availability and fragmentation of the suitable host distribution.

8.3 Ectomycorrhizae Structures

The development of an ectomycorrhiza begins with contact between the fungus and the roots. Establishment of the symbiosis must be under the control of the genes of both partners (Smith and Read 2008). Until the symbiotic function starts, there are five stages of development (Malajczuk et al. 1990): *preinfection*, characterized by hyphal contact with the root; *symbiotic initiation*, characterized by fungal

attachment to the epidermis; *fungal colonization*, with hyphal penetration between epidermal cells and the formation of the initial mantle layers; *symbiotic differentiation*, in which the Hartig net proliferates and there is a rapid buildup of mantle hyphae; and *symbiotic function*, meaning the end of Hartig net growth and the development of a consistent mantle, tightly pressed against the epidermal cells.

The Hartig net formation is the first step in the beginning of ectomycorrhizal relationships and indicates the existence of a true ectomycorrhizal association. This is the contact zone between the symbionts, where the interchange of nutrients between fungi and host plant is produced. The structure is usually formed from the inner part of the root to the outer. Fungal hyphae penetrate between the epidermal cells, which become progressively more radially enlarged from the apex back (Massicotte et al. 1986).

Although the study of the anatomy of ectomycorrhizae began with the study of root longitudinal and cross sections, nowadays, they are considered as minor features (Agerer 2006). Their only differences are regarding host genera, based on the depth of the penetration. In the majority of angiosperms, penetration is confined to the epidermal layer, forming an "epidermal" Hartig net and with the hyphae wholly or partially encircling the epidermal root cells (Agerer and Rambold 2004–2013; Godbout and Fortin 1983). In the gymnosperms, by contrast, the Hartig net typically penetrates beyond the epidermis to enclose several layers of cortical cells, sometimes even extending to the endodermis (Agerer and Rambold 2004–2013).

Whereas the Hartig net forms the most extensive interface between fungus and plant, its biomass in most ectomycorrhizae is relatively small compared to that of the overlying mantle (Smith and Read 2008). The ectomycorrhizal fungal mantle protects the Hartig net in order to make the association between fungus and plant as stable as possible, improving the way of life for both partners. The mantle is a significant concentration of biomass for the fungus and it could also be used as a resistant structure.

Usually, the mantle can be divided into three layers in plane view: inner, middle, and outer mantle layers (Agerer and Rambold 2004–2013). The inner and outer mantle layers are always present in the ectomycorrhizae, while middle mantle layers may or may not present or there may be more than one, depending on its structure. The inner mantle layer is in full contact with the root surface, and it is usually thinner than the rest of the layers.

There are two basic types of mantles (Agerer 1991): plectenchymatous mantles, in which the hyphae can be recognized individually, and pseudoparenchymatous mantles, in which the individual hyphae cannot be distinguished because they have been enlarged and have lost their original form, thus resembling a true parenchyma. Pseudoparenchymatous mantles appear more advanced than plectenchymatous ones in a structural and evolutionary sense (Agerer 1995).

Mantle types are described in detail by Agerer (1987–2012, 1991, 1995, 2006) and Agerer and Rambold (2004–2013) and divided into 16 types: nine plectenchymatous and seven pseudoparenchymatous. Laticifers, the latex-containing, long, thick, scarcely branched hyphae, can occur in all of the mantle

types (Agerer 2006). Pseudoparenchymatous mantles prevent the formation of emanating elements, perhaps due to the great cell dimensions of the outer mantle layer (Agerer 2006). They are seemingly destined to have close contact with the soil particles surrounding them and usually increase in size due to the hydrophilicity of their cells.

Extraradical mycelium, cystidia, and rhizomorphs are emanating elements from the ectomycorrhizal mantle. These three elements may all be represented together in an ectomycorrhizal type, alternatively only one or two elements may be present without the second or third, or the case may be that none are represented at all. It all depends on fungal colonization strategies, physiological aspects, and competition abilities.

Emanating hyphae can have various shapes in the same ectomycorrhizal type (Agerer 1991). It can also form anastomoses and have simple septa or clamps, a rough surface, or even crystals.

Cystidia occur not only on the cap skin, gills, and stipe of sporocarps but also on the ectomycorrhizae (Agerer 2006). Although they are not very common, the 15 types compiled by Agerer (1991) and Agerer and Rambold (2004–2013) are distinctive for some fungal groups. The structures seem to be specialized in short-distance nutrition processes, increasing the mantle's field of influence.

Rhizomorphs are multi-hyphal linear aggregates (Agerer 1999), divided into seven types (Agerer 1999; Agerer and Iosifidou 2004) by their structure. Their function is directly related to nutrient uptake and transport and they are capable of exploring large distances from the mantle.

8.4 The Key Role of Rhizomorphs

Acquisition and transport of water and nutrients are performed exclusively by fungal mycelium in the soil, especially by the most distal parts of rhizomorphal hyphae, while the mantle is in fact an outwardly sealed compartment solely involved with storage and exchange between symbionts (Agerer 2001).

Rhizomorphs are hyphae connected by various mechanisms and growing more or less parallel and more or less closely together over a greater distance (Agerer 1987–2012), forming multi-hyphal linear aggregates (Agerer 1999). Based on structural, ontogenic, and functional similarities between all multi-hyphal linear aggregates, Cairney et al. (1991) recommend that the term rhizomorph be used to describe all those structures, irrespective of their internal organization and their ontogeny (Agerer and Iosifidou 2004). The term rhizomorph appears to be the first used to describe this type of structure and also highlights their rootlike morphology. A comprehensive typology of rhizomorph structures was first published in 1991 by Agerer, who extended it to include ontogenetical aspects in 1999. Eight rhizomorph types could be distinguished (Agerer 1999, 2006; Agerer and Iosifidou 2004): *uniform-loose*, composed of normal vegetative hyphae; *uniform-compact*, which possess uniformly shaped densely agglutinated hyphae; *thelephoroid*, with slightly

differentiated hyphae; *ramarioid*, internally differentiated; *russuloid*, which have irregularly distributed thickened hyphae with often incomplete septa; *phlegmacioid*, with a few randomly distributed slightly thicker hyphae often embedded in a matrix; *agaricoid*, highly differentiated with vessel-like hyphae; and *boletoid*, also highly differentiated and with vessel-like hyphae but ramified as split type.

Ramarioid rhizomorphs in the Gomphales, with oleoacanthocystidia and/or oleoacanthohyphae, thin-walled, and with irregular globular yellowish cells (Agerer 2006; Agerer and Iosifidou 2004), seem to be strongly influenced by the functional demands of the symbiosis because it enables the fungi to extract water from soil particles to an extremely high degree (Agerer 2006).

Boletales are characterized by the long-distance exploration-type (Agerer 2001) and boletoid rhizomorphs (Agerer 2006), which are defined by three essential characters: (1) runner hyphae that grow very fast and with few ramifications; (2) the formation of split-type hyphal ramifications to facilitate forward and backward transport in rhizomorphs; and (3) the increase of hyphal diameters with a contemporary dissolution of their septa to reduce the transport resistance of solutions (Agerer 1999; Raidl 1997). These rhizomorphs are usually hydrophobic, thus preventing leakage when water is transported from distal regions to the proximal sink (Agerer 2006; Unestam and Sun 1995).

8.5 The Increase in Volume of Exploited/Explored Soil by Ectomycorrhizae

There are three structures that ectomycorrhizae use to expand root systems: extraradical mycelia, cystidia, and rhizomorphs. Every structure that emanates from the ectomycorrhizae should perform a role mainly concerning the process of nutrition and protection against pathogens.

Due to the amount, range, and differentiation of the mycelia structures emanating from the hyphal mantle into the soil, i.e., the extraradical mycelia, several functional groups, so-called exploration types, can be distinguished (Agerer 2001): ectomycorrhizae with almost smooth mantles (*contact exploration type*), ectomycorrhizae with distinct emanating hyphae and rather limited growth into the surrounding soil (*short-distance exploration type*), and a variety of ectomycorrhizae with rhizomorphs grouped according to rhizomorph range and internal organization. The *medium-distance exploration type* has rather far-reaching rhizomorphs that either are internally undifferentiated or have internal hyphae of an enlarged diameter in certain fungal species. The highest differentiated exploration type, the *long-distance exploration type*, features very long rhizomorphs with internal vessel-like transport hyphae (Agerer 2001, 2006). Long-distance exploration types are more prevalent in areas of low root density, while short-distance exploration types are more common in areas of high root density (Peay et al. 2011).

Long-distance exploration-type fungi are also able to colonize root plants at long distances and continue to increase their biomass by tapping into multiple plants in an area, with this pattern congruent with the idea that mycelia networking is most advantageous to high-biomass structures, like the rhizomorphs related with this exploration type (Simard et al. 2012).

The anatomy and architecture of extraradical mycelial systems may determine the potential for nutrient capture (Smith and Read 2008). The extraradical mycelia of ectomycorrhizal fungi enormously increase the space plant roots occupy for water and nutrient gain (Cairney and Burke 1986; Leake et al. 2004; Read 1992; Smith and Read 2008), but the costs for ectomycorrhizae are substantial (Rygiewicz and Andersen 1994; Weigt et al. 2011). Therefore, the exploration types raise two different issues in the functioning of the mutualistic symbiosis: (1) the benefit for the plant as expressed by the range of soil occupation by the mycelium to explore and exploit the soil and finally transport water and nutrients to the roots and (2) the costs for the tree which are mainly expressed by the carbohydrates that have to be invested by the tree to support its fungal partner (Weigt et al. 2012).

While the study of exploration types requires morphological observation of mycorrhizae, extraradical mycelial study is much more difficult to carry out using observational techniques. The methods to study the structure and function of extraradical mycorrhizal mycelia, such as biochemical and DNA-based markers, in vitro and in soil observation and root-free hyphal compartmentalization were reviewed by Leake et al. in 2004. The distribution and dynamics of extraradical fungal mycelia in the soil were poorly understood until appropriate methods for their study were developed (Anderson and Cairney 2004). The development of techniques based on direct nucleic acid extraction coupled with polymerase chain reaction (PCR) amplification has provided new insights into the ecology of these soil fungi (Guidot et al. 2002, 2003). Real-time PCR allowed for the relative or absolute quantification of fungal biomass, and compared to other quantification techniques such as total hyphal length or biochemical markers, it provides a species-specific measure for mycelial biomass estimations (Landeweert et al. 2003). This technique has been adapted for monitoring plant pathogenic fungi (Gachon and Saindrenan 2004; Hietala et al. 2003) and mycorrhizal fungi (Kennedy et al. 2007; Parladé et al. 2007; Schubert et al. 2003; van der Linde et al. 2009).

Nowadays, specific markers for real-time PCR have been developed for some edible ectomycorrhizal species: *Lactarius deliciosus* (L.) Gray (Hortal et al. 2008, 2009; Parladé et al. 2007), *Boletus edulis* Bull. (De la Varga et al. 2012, 2013), *Tuber aestivum* Vittad. (Gryndler et al. 2013), *Tuber melanosporum* Vittad. (Parladé et al. 2013; Zampieri et al. 2012), and *Tuber magnatum* Picco (Iotti et al. 2012).

Several studies have tried to relate the amount of soil mycelia to the number of ectomycorrhizae and the sporocarp production for *Boletus edulis* (De la Varga et al. 2012, 2013); *Boletus* sp., *Cortinarius* sp., and *Tomentella* sp. (Kjøller 2006); *Lactarius deliciosus* (De la Varga et al. 2013; Parladé et al. 2007); *Cortinarius* sp. and *Russula* sp. (Peter et al. 2001); *Clavulina cristata* (Holmsk.)

J. Schröt., *Lactarius subdulcis* (Pers.) Gray, and *Xerocomus pruinatus* (Fr. & Hök) Quél. (Rineau et al. 2010); and *Tuber melanosporum* (Suz et al. 2008). However, no conclusive results have been obtained to date. The sporocarp production of edible species depends on both the host tree preference and the ecological environment: fungal communities, climate, soil, and tree development, among other factors (Barroetaveña et al. 2008; Bonet et al. 2010; Buée et al. 2011; Laganà et al. 2003; Martínez de Aragón et al. 2007; Martínez-Peña et al. 2012; Ortega-Martínez et al. 2011; Pinna et al. 2010; Salerni et al. 2002; Savoie and Largeteau 2011). Egli et al. (2010) showed an increase in fruit body production after thinning, relating the production of sporocarps with the previous annual tree growth. Similarly, Bonet et al. (2012) found an increase in the sporocarp production of *Lactarius deliciosus* after thinning and related it to changes in soil fertility.

The apparent uncoupling between above- and belowground fungal components has been observed in ectomycorrhizal species (De la Varga et al. 2012, 2013; Gardes and Bruns 1996; Hynes et al. 2010). It has been demonstrated that the belowground mycelial system of *Suillus grevillei* (Klotzsch) Singer (Zhou et al. 2001), *Tricholoma matsutake* (S. Ito and S. Imai) Singer (Lian et al. 2006), *Hydnellum peckii* Banker, and *Phellodon tomentosus* (L.) Banker (van der Linde et al. 2009) is not always centered on the sporocarps, and there was no quantitative relationship between the belowground abundance of mycelia and the number or distribution of sporocarps. Suz et al. (2008) compared nonproductive and productive trees in a *Tuber melanosporum* orchard, finding apparently higher quantities of mycelia in soil samples taken around nonproductive trees. Peintner et al. (2007), studying the soil fungal communities in a *Castanea sativa* Mill. forest, demonstrated that the overlap between above- and belowground fungal communities was very low. In their study, *Boletus* mycelia, compared with other soil fungi, were rare and scattered, whereas their sporocarps were the dominant in the mushroom production of that forest. *Tuber magnatum* mycorrhizae are scarce or absent even where their sporocarps are found (Bertini et al. 2005; Murat et al. 2005), but Zampieri et al. (2010) have shown that *Tuber magnatum* mycelium is widely distributed in the soil of truffle grounds.

8.6 Ectomycorrhizae Patch Dynamics in the Roots

The distribution and interaction of ectomycorrhizae in the root system are conditioned by many factors. Some of them are related with the root morphology of the host tree, and also with ectomycorrhizal morphology, but abiotic factors, such as soil properties, also have an influence.

Ectomycorrhizal communities are impressively diverse, even in stands dominated by a single plant species (Horton and Bruns 2001). Buée et al. (2009) found around 1,000 fungal molecular operational taxonomic units in 4 g of forest soil, in which ectomycorrhizal fungi represented more than 50 % of the 30 most abundant genera. Fungi initially colonize isolated points along a root system and proliferate

locally through vegetative production, so their distribution is clustered, and there are extreme changes in species presence and abundance by a few centimeters (Kennedy et al. 2009; Lilleskov et al. 2004; Taylor 2002).

There is a strong spatial variation, but there is also variation due to seasonal factors. *Boletus edulis* and *Lactarius deliciosus* present changes in its extraradical mycelium characterized by seasonal variability, with a clear increase in the amounts of biomass during the coldest months of the year and with variability strongly dependent on the weather (De la Varga et al. 2013). For both species, the minimum mycelium quantity was detected before or at the same time as the fructification period, which could indicate an allocation of resources to produce sporocarps.

Fungi are organisms that compete among themselves when resources are limited. Mycorrhizal fungi compete for two general classes of resources: host-derived carbon and soil- or detritus-derived mineral nutrients; both types of resources are arrayed in space (Bruns 1995). The creation of additional habitats for ectomycorrhizal fungi is related to small-scale natural disturbances in the roots and in the soil. There are three key abiotic factors that determine the presence of ectomycorrhizal fungi in the soil: temperature, pH, and nitrogen (Erland and Taylor 2002). There is also another factor to take into account: competitive interactions between fungi may significantly influence temporal patterns of the ectomycorrhizal community structure (Kennedy et al. 2011b).

As was stated by Bruns (1995), there are four ways in which ectomycorrhizal competitors could coexist in a small homogeneous host environment: niche partitioning, disturbance-related patch dynamics, density-dependent mortality, and competitive networks. Kennedy (2010), Kennedy et al. (2009), and Peay et al. (2011) tried to explain the coexistence among ectomycorrhizal competitors assuming that space is a limiting resource and that vacant space is recolonized by the first-available recruit and there are strong priority effects in space occupancy. These authors affirm that it could be a "lottery" for root space driven by widespread spore dispersal and rapid colonization. Spore dispersal would be related to sexual species reproduction, as the species invest in forming sporocarps, while the rapid colonization would be related to extraradical mycelium growth. Differences in colonization and competitive abilities may facilitate species coexistence in ectomycorrhizal fungal communities (Peay et al. 2007).

8.7 Developing General Predictive Models for Mycorrhizal Fungal Communities

Our understanding of distribution and community organization of ectomycorrhizal fungal communities on the roots is in its infancy. Although some regional models of ectomycorrhizae, sporocarp production, and environmental relationships have been developed over recent years (Alonso Ponce et al. 2010, 2011; Barroetaveña

et al. 2008; Bergemann and Largent 2000; Wolfe et al. 2010), sporocarps represent a biased subsample of the belowground community (Gardes and Bruns 1996). However, the transcendence of sporocarps as non-timber forest products justifies the development of sporocarp-based distribution models, particularly for edible ectomycorrhizal fungi.

Nevertheless, relatively little is known about how mycorrhizal fungi interact with large-scale environmental processes (Bingham and Simard 2012; Peay et al. 2010; Simard et al. 2012). Through the years, autoecological features of hosts have usually been accepted to be the same for the fungal associate. Nonetheless, this statement does not appear to be entirely true, at least from the sporocarp formation perspective, even in fungi with ecological host specificity (Hirose et al. 2010). For example, *Boletus edulis* sporocarps are usually collected in *Pinus sylvestris* L. stands in Spain but never in France (Alonso Ponce et al. 2011), and *Boletus aereus* Bull. sporocarps are collected in drier, warmer locations than *Boletus edulis* for the same host plant (Oria-de-Rueda et al. 2008).

Moreover, patterns of fungal distribution are governed by several factors. Structural differences between sporocarps determine dispersal ability, though most spores only travel very short distances from their point of origin (Peay et al. 2010). Ectomycorrhizal fungi possess some saprotrophic capacity, particularly if there is some selection pressure maintaining it, such as the occasional loss of connection with a living host plant (Koide et al. 2008).

The traditional view of the nature of species assemblages, derived from plant ecology, has led us to focus on predicting individual species distribution models rather than whole communities (Lilleskov and Parrent 2007). They also predict current distribution in relation to static environmental variables, assuming equilibrium conditions. This assumption may not be valid when modeling fungal communities in a changing environment. One of the next challenges for mycorrhizal ecologists will be to develop dynamic community models to define baseline species distribution data in the context of rapid global change (Lilleskov and Parrent 2007).

8.8 How Ectomycorrhizal Types Share Root Systems

While there are genetic and physiological barriers to certain plant–fungus associations (Molina and Trappe 1982), host specificity of ectomycorrhizal fungi does not appear to be absolute (Águeda et al. 2006; Dickie 2007). Thus, the host preference of mycorrhizal fungi reflects a realized, rather than a fundamental, niche.

Host receptivity and host range of mycorrhizal fungi will clearly limit fungal colonization from the limited propagule banks in primary succession systems. The plant community structure will also influence the identity of fungal taxa residing in the propagule bank and those fungi that establish and sustain colonization in a host root system (Jumpponen and Egerton-Warburton 2005).

As was stated by Horton and Bruns in 2001, the most abundant and frequent taxa on ectomycorrhizal roots in conifer communities in both Europe and North

America, and other angiosperm forests, are members of Russulaceae, Thelephoraceae, and non-thelephoroid resupinates. Later studies by Águeda et al. (2010), Buée et al. (2009), De Román and De Miguel (2005), García-Barreda and Reyna (2012), Genney et al. (2006), Kennedy et al. (2011a), Pickles et al. (2010), Põlme et al. (2013), Pritsch et al. (2010), and Roy et al. (2013) have confirmed this fact.

Regardless of the host and condition, ectomycorrhizal fungal communities usually include the presence of *Cenococcum geophilum* Fr. (the one notable exception to the rule of niche differentiation) across soil profiles at all stages of stand development, at every distance from forest edges, and in every season of the year (Dickie 2007; Ishida et al. 2007). This Ascomycetous, belonging to short-distance exploration type, could be one of the species specialized in primary nutrition processes due to its sclerotia being able to resist the worst of conditions until it establishes symbiosis with a host plant.

There are few differences found in comparing the ectomycorrhizae associated with coniferous forests to those with deciduous forests. Besides *Cenococcum geophilum*, ectomycorrhizal fungal communities in coniferous forests are dominated by Russuloid and Thelephoroid groups and also by Amanitaceae, Boletaceae, Clavulinaceae, *Cortinarius* sp., *Inocybe* sp., and *Piloderma* sp. (Auèina et al. 2011; Genney et al. 2006; Izzo et al. 2005; Koide et al. 2005; Pickles et al. 2010). In those fungi, the dominance of medium-distance exploration types (Thelephoroid, Russuloid, Clavulinaceae, and *Cortinarius* sp.) could be remarked upon, combined with short-distance types (*Inocybe* sp. and *Piloderma* sp.) and long-distance types (Boletaceae and Amanitaceae).

Deciduous mixed forest in France is dominated by Sebacinales, *Lactarius* spp., *Scleroderma* sp., *Russula* spp., *Inocybe* sp., *Cortinarius* sp., *Amanita* sp., *Pseudotomentella* sp., and *Boletus* sp. (Buée et al. 2009), with this community very similar to that belonging to coniferous forests.

In angiosperm Mediterranean forests, ectomycorrhizal fungal communities seem to be dominated by Thelephoroid, Pezizales, Boletales, and *Pisolithus* sp. (Águeda et al. 2010; Benucci et al. 2011; De Román and De Miguel 2005; García-Barreda and Reyna 2012; Hynes et al. 2010). The presence of long-distance and short-distance exploration types is more relevant, with medium-distance exploration types in a secondary role. Differences could be more related to abiotic factors than to host preference. Long-distance exploration types could be more resistant to the harsh conditions of a Mediterranean climate, and short-distance types need to invest less energy in developing their structures.

Recent studies about the ectomycorrhizal fungal communities in alder forests on a local scale (Pritsch et al. 2010), regional scale (Kennedy et al. 2011a; Roy et al. 2013), and global scale (Põlme et al. 2013) show that they are not primarily rich, regardless of sampling intensity. The special characteristics of the *Alnus* spp. forest habitats seem to be the ones that make its ectomycorrhizal diversity low, contrasting to the rest of the stands (Horton et al. 2013). Russuloid and other short-distance exploration types dominate this community, with a very low rate of long-distance exploration types.

8.9 The Need to Continue Studying Anatomy and Morphology of Ectomycorrhizae in Molecular Evolution

Over the last 20 years, knowledge regarding the ectomycorrhizal fungal community has evolved greatly due to the use of molecular tools, incomparable with the slow development of ectomycorrhizal science which started with the discovery of the structures in 1885 (Frank 2005).

The first work using PCR to study ectomycorrhizal fungi was carried out by Gardes and Bruns (1993). Nowadays, the use of PCR is commonplace for the study of ectomycorrhizal fungi, with practical applications like the quality control of black truffle mycorrhizal seedlings (Fischer et al. 2013) and population studies (Murat et al. 2004). Progressively, other biotechnological molecular tools have been added to the study of ectomycorrhizal fungal individuals and communities such as sequencing (Martin et al. 2008, 2010), quantitative PCR (de la Varga et al. 2012; Parladé et al. 2007, 2013; Schubert et al. 2003), and 454 pyrosequencing (Buée et al. 2009; Janos 2007; Jumpponen and Jones 2009; Tedersoo et al. 2010; Wallander et al. 2010).

Interpretation of results regarding how ectomycorrhizal fungi live in host roots requires knowledge about the structures and function of the mycorrhizal symbiosis, in order to better understand their ecological function.

8.10 Conclusion

Mycorrhizae are classic examples to explain the mutualistic interaction between two different organisms in nature: the roots of a vascular plant and a fungus. Both species establish a permanent relationship, they live together in symbiosis, and that differentiates the nature of mycorrhizae from other plant–fungus interactions. Ectomycorrhizal associations increase the root exploration area in soil, boosting the potential for mineral nutrition, water availability, and mutual survival of plant and fungus. The diversity of ectomycorrhizal fungal communities in the roots of spermatophyte plants is impressively high and means a complex diversity of structures in the root system, including emanating hyphae and rhizomorphs, which enlarge its area of influence. The distribution of the ectomycorrhizae living in the root system in an ever-changing balance is conditioned by many factors. Some of them are related with the root morphology of the host tree and also with ectomycorrhizal morphology, but abiotic factors (such as soil properties) also play a role.

Knowledge of the distribution and organization of ectomycorrhizal fungal communities in the rhizosphere is still in its infancy. Development of regional models of ectomycorrhizal sporocarp–environment relationships and molecular tools and the

study of anato-morphological structures are helping to increase levels of understanding.

Ectomycorrhizal associations increase the root exploration area in the soil, improving the potential for mineral nutrition, water availability, and mutual survival of plant and fungus. Every structure that emanates from the ectomycorrhizae (extraradical mycelia, cystidia, and/or rhizomorphs) performs a role in symbiosis process (nutrition, water transport, protection against pathogens). Ectomycorrhizal fungi form complex communities in the roots and in the surrounding soil living in an ever-changing balance which changes with the host age and abiotic ecological factors, mainly with those related with soil properties.

The diversity of ectomycorrhizal fungal communities in the roots of higher plants is impressively high and involves a complex diversity of structures in the root system, which enlarge its area of influence. These structures are organized in the soil to optimize the area for nutrient and water uptake. Ectomycorrhizae anato-morphological structures are very diverse, with the differences having evolved to create competitive advantages.

Distribution and dynamics of the ectomycorrhizae in the root system are conditioned by many factors. Some of them are related with the root morphology of the host tree and also with ectomycorrhizal morphology. Abiotic factors, such as soil properties, also have an influence and usually prevail over root-specific structure.

Our understanding of the distribution and community organization of ectomycorrhizal fungal communities on the roots is still in its infancy. Although some regional models of ectomycorrhizae, sporocarp production, and environment relationships have been developed over the last few years, sporocarps represent a biased subsample of the belowground community. The development of regional models of ectomycorrhizal sporocarp–environment relationships and the study of fungal structures by both morphological and molecular tools are helping to increase this knowledge.

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Chapter 9
Functions of Root Fungal Endophytes

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9.1 Introduction

Plant roots in their natural habitat are symbiotically associated with diverse fungal endophytes (Petrini 1986). These fungal symbionts can play a major role in shaping plant communities (Clay and Holah 1999) and influence ecology, health, evolution of the plant (Brundrett 2006), and diversity of other organisms with which they interact (e.g., bacteria, nematodes, and insects; Omacini et al. 2001). The endophytic fungi (Krings et al. 2007) and mycorrhizal fungi (Redecker et al. 2000) were likely to have been associated when plants colonized the land, thus implying their long and important role in the evolution of plant life on land (Rodriguez et al. 2009).

The term endophyte refers to the intracellular symbiotic microorganisms in the host organism which do not cause any visible sign of damage or adverse effects on the host (Schulz and Boyle 2005). Endophytic fungi differ from mycorrhizal fungi in the fact that they are present within plant tissues, may grow inside the roots, and emerge to sporulate at host-tissue senescence (Stone et al. 2004). These fungi can be classified into two major groups based on their evolutionary relatedness, taxonomy, plant hosts, and ecological functions. One group is the clavicipitaceous endophytes, which infect grasses, and the second being nonclavicipitaceous endophytes, which can infect tissues of nonvascular plants, ferns, conifers, and angiosperms.

Clavicipitaceous endophytes represent a small group of phylogenetically related species that are fastidious in culture and restricted to some grasses (Bischoff and

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White 2005). Typically, these endophytes are present within plant shoots, where they cause systemic intercellular infections (Clay and Schardl 2002). These include class I type of fungal endophytes.

Nonclavicipitaceous endophytes are very diverse, primarily representing ascomycetous fungi with diverse and ill-defined ecological roles. Some basidiomycetous fungi also exist as endophytes (Verma et al. 1998; Barazani et al. 2005). These endophytes have been found to be associated with every major lineage of land plants and from all terrestrial ecosystems (Arnold and Lutzoni 2007). The root-inhabiting fungi that are non-mycorrhizal and the dark septate endophytes (DSE) are included in this group. The fungi that possess intracellular hyaline structures when colonizing the hosts (Narisawa et al. 2003) but are without typical DSE structures are also included. Their diversity, ecological roles, evolutionary significance, and their potential applications (Selosse et al. 2008) are catching the attention of scientists from different fields. Concentrating on the root fungal endophytes, in this chapter, we will focus on these nonclavicipitaceous endophytes.

9.2 Nonclavicipitaceous Endophytes

Many of the nonclavicipitaceous endophytes establish symbiotic relation with plants, imparting health and fitness benefits such as increased growth and yields, biotic and abiotic stress tolerance, and nutrient acquisition (Waller et al. 2005; Rodriguez et al. 2008). These diverse fungi can be classified into three functional groups based on their differences in life history, ecological interactions, and functions (Rodriguez et al. 2009).

9.2.1 Classification of Nonclavicipitaceous Endophytes

This group of endophytes can be classified into three functional classes based on their pattern of host colonization, mechanism of transmission through host generations, *in planta* biodiversity levels, and ecological role. In spite of the broad host range of all three classes, class 2 endophytes grow in both above- and belowground plant tissues, whereas class 3 and 4 endophytes are limited to aboveground tissues and roots, respectively. In terms of host-tissue colonization, class 3 endophytes cause highly localized infections, while class 2 and 4 endophytes show extensive tissue colonization (Rodriguez et al. 2009). The diversity of class 2 (Rodriguez et al. 2008) endophytes in a single host is limited, whereas that of class 3 endophytes can be as high as more than 20 species in a single tropical leaf (Arnold et al. 2003). The diversity of class 4 endophytes in individual plants has not been evaluated sufficiently. Class 2 endophytes are the most extensively investigated fungi for their role in imparting fitness benefits to the host plant.

9.2.2 *Functions of Nonclavicipitaceous Endophytes*

9.2.2.1 Plant Promotional Activity

Most of the nonclavicipitaceous endophytes promote plant growth in terms of increase in host shoot and/or root length and biomass (Varma et al. 2012a; Unnikumar et al. 2013) possibly as a result of the induction of plant hormones by the host or biosynthesis of plant hormones by the fungi (Tudzynski and Sharon 2002). *Piriformospora indica*, an endophytic mycorrhiza-like fungus, has been shown to increase the rate of seed germination, induce early seed germination and flowering, and increase seed production in the host plant (Varma et al. 2012a).

9.2.2.2 Nutrient Uptake

Like mycorrhizal fungi, *P. indica* has been shown to play a role in acquisition of phosphorous by the plant root especially in the semiarid and arid soils. Involvement of a high-affinity phosphate transporter from this endophyte was reported (Yadav et al. 2010). Recently, the crystal structure of the PiPT transporter was elucidated in detail (Pedersen et al. 2013). Investigations have also shown that this fungus stimulates the metabolism of sulfur (Varma et al. 2012a).

9.2.2.3 Host Disease Resistance

Endophytic fungi protect hosts to certain extent against fungal pathogens (Campanile et al. 2007). This may be due to the production of secondary metabolites (Schulz et al. 1999), fungal parasitism (Samuels et al. 2000), or induced systemic resistance (Vu et al. 2006) or inability of pathogens to compete for the niche with endophytes (Rodriguez et al. 2009). A few of class 2 endophytes have been examined for their interaction with host defenses. Endophytic isolates of *Fusarium oxysporum* conferred disease resistance which was correlated to increase in the concentration of phenolic metabolites upon pathogen attack in barley and larch (Schulz et al. 1999).

9.2.2.4 Ecological Adaptations of Plants

Class 2 endophytes have the unique ability to asymptomatically colonize and impart habitat-adapted fitness benefits to host species (Rodriguez et al. 2008). Comparing the fitness benefits conferred by class 2 endophytes in plants growing in geothermal soils (*Curvularia protuberata*), coastal beaches (*Fusarium culmorum*), and agricultural fields (*Colletotrichum* sp.), it was observed that *C. protuberate* conferred heat but not salt or disease tolerance, *F. culmorum*

conferred salt but not heat or disease tolerance, and *Colletotrichum* sp. imparted disease resistance but not heat or salt tolerance (Rodriguez et al. 2008).

It was observed that the plants inoculated with *P. indica* were tolerant to various abiotic stresses, viz., drought, salinity, nutrient deficiency, and heat stress (Varma et al. 2012b). Recently, a cyclophilin A-like protein was isolated from *P. indica* (PiCypA), which expressed to a higher level under salinity stress. This protein exhibited a novel RNA binding activity as detected by NMR spectroscopy and crystal structure analysis (Trivedi et al. 2013). It is interesting to note that *P. indica* which was isolated from the hot deserts (maximum temperature, +45 °C) of Thar, Rajasthan, India, showed cold stress alleviating and growth promotional properties in the fungus-inoculated plants in the cold and high altitude deserts (minimum temperature,−20 °C) of Leh-Ladakh, India (Varma et al. 2012b).

9.2.2.5 Interaction with Other Microflora

Interaction of the endophytic fungi with the microbes in the rhizosphere plays an important role in sustainable soil environment. In a recent study (Varma et al. 2012b), it was reported that *P. indica* interacts positively with rhizobacteria. Interactive colonization of barley roots with *P. indica* and *Pseudomonas putida* as detected by confocal laser scanning microscopy and FISH analysis showed plant growth promotional property.

9.3 A Case Study

Rann of Kutch is a salt marsh located in the western tip of Gujarat, India. It is one of the hottest areas of India, with summer temperatures averaging 41 °C and peaking to 49.5 °C. Winter temperatures reduce dramatically and can go below 0 °C (Fig. 9.1). The fungal root endophytes associated with the vegetation of this extreme climate were screened.

9.3.1 Endophyte Isolation and Screening

9.3.1.1 Collection of the Host Plants

Small cuttings (about 10 cm) of the roots were collected from specific locations in the Rann of Kutch. The plant material was transported to the laboratory in sterile polythene bags and stored at 4 °C until further processing.

Fig. 9.1 A view of the salt desert of Bhuj, Gujarat

9.3.1.2 Isolation of Endophytes

Plant root cuttings were thoroughly washed with running tap water, cut into small pieces (2–3 cm long) and surface sterilized with 1 % sodium hypochlorite followed by 90 % ethanol. The internal tissues were cut into smaller pieces of 0.5–1 cm and plated on different microbiological media such as water agar and potato dextrose agar. The plates were incubated at 25 °C for 3 weeks. The hyphal tips of fungi emerging out of the plant tissues were picked and grown on potato dextrose agar as pure culture.

9.3.1.3 Identification of Endophytes

For preliminary identification, microscopic slides of each endophyte were prepared by staining with lactophenol cotton blue (Vainio et al. 1998) and were examined under light microscope. As many as 103 fungal isolates were isolated. Further, the cultures were submitted to Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, Pune, for characterization: *Fusarium moniliforme*, *Fusarium sporotrichioides*, *Fusarium solani*, *Curvularia* sp., *Drechslera* sp., and *Aspergillus niger*, to name a few.

9.3.2 Interaction of Identified Fungal Isolates with Pearl Millet Plants

Pearl millet (*Pennisetum glaucum*), an economically important cereal crop of traditional farming systems in tropical and subtropical Asia and sub-Saharan Africa, accounts as the sixth most important crop after wheat, rice, maize, barley, and sorghum. It is a staple crop with high nutritional value and is also used as feed, fodder, and construction material. Its potential as a source of biofuel is also being explored (Wu et al. 2006). Its cultivation is commonly practiced in drought-prone rainfed agriculture areas.

9.3.2.1 Seed Treatment

Pearl millet seeds were surface sterilized with 0.1 % $HgCl_2$ for 3 min and washed with sterile distilled water for five rinses. The cultures of *P. indica* and *F. moniliforme* were grown in potato dextrose broth for a week.

The mycelium was crushed for further use. Pearl millet seeds were incubated overnight with the mycelium of *P. indica* and *F. moniliforme*, individually or in combination. As many as 70 seeds were sown in each pot with the dimensions 92 cm × 35 cm × 32 cm. Control seeds were incubated in distilled water for overnight. The pots were then placed in greenhouse at a temperature of 22–30 °C, 12–14 h daylight, and 70–75 % relative humidity.

9.3.3 Growth-Promoting Effect

After the 10th day of sowing, it was observed that the seed germination was maximum in pot containing seeds inoculated with both *P. indica* and *F. moniliforme* (Table 9.1). The seed germination percentage increased by 15.71 % over control. The overall growth of the plants in this treatment was also enhanced as compared to other treatments and the control (Fig. 9.2).

As reported earlier and from our present observation, some of the *Fusarium* species interact positively with plants (Damicone and Manning 1982; Hallmann and Sikora 1994; Blok and Bollen 1995; Elsharkawy et al. 2012). These are reported to be associated with plant roots as intracellular, symptomless fungi (Stone et al. 2000) and may be useful for plant growth.

9.4 Conclusion

The endophytic fungi are a diverse group of microorganisms. They differ from each other in their symbiotic behavior and their ecological functions. As detailed in this chapter, the endophytic fungi impart health and fitness benefits to the plants using different mechanisms. They promote plant growth, increase nutrient uptake by the plant, alleviate biotic and abiotic stresses, and also interact with other members of the rhizosphere microflora. A vast majority of the endophytes are yet to be isolated and characterized which leaves the path open for addition of many more beneficial features of this biologically diverse group of fungi.

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Table 9.1 Effect of fungal endophytes on germination of *Pennisetum glaucum* seeds

Treatments	Number of seeds sown	Number of seeds germinated	Percentage of seed germination (%)	Percent change over control (%)
Control	70	40	57.14	–
Fusarium moniliforme	70	35	50.00	−7.14
Piriformospora indica	70	49	70.00	+12.86
Piriformospora indica + *Fusarium moniliforme*	70	51	72.85	+15.71

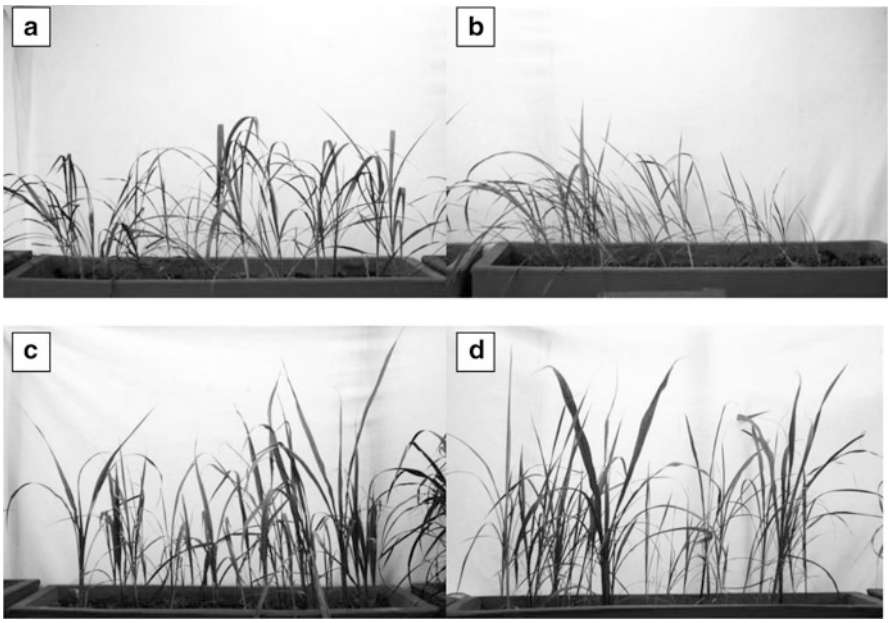

Fig. 9.2 Effect of fungal endophytes on the growth of *Pennisetum glaucum* plants. Prior to sowing the seeds were treated overnight with (**a**) distilled water (**b**) mycelium of *Fusarium moniliforme* (**c**) mycelium of *Piriformospora indica* (**d**) mycelium of both *Fusarium moniliforme* and *Piriformospora indica*

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Chapter 10
Strigolactones Involvement in Root Development and Communications

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10.1 Introduction

Strigolactones (SLs) are a family of substances produced by plants, with diverse biological roles. They are involved in communication with both parasites and symbionts in the rhizosphere. They are stimulators of seed germination of parasitic plant (*Striga* and *Orobanche;* Cook et al. 1966; reviewed by Xie et al. 2010) and hyphal branching of the symbiotic arbuscular mycorrhizal fungi (AMF; reviewed by Koltai et al. 2012). SLs were recently described to be plant hormones, since they were shown to be regulators of shoot development, acting to repress lateral buds outgrowth (Gomez-Roldan et al. 2008; Umehara et al. 2008). SLs are terpenoid lactones derived from carotenoids (Matusova et al. 2005). They all have a common structure consisting of two lactones connected by an enol–ether bridge (reviewed by Xie et al. 2010) and were found to be synthesized, mainly in roots, in a wide variety of plant species, including dicots, monocots, and primitive plants (e.g., Xie et al. 2010; Proust et al. 2011; Delaux et al. 2012; Liang et al. 2010; Koltai et al. 2010b).

SLs are synthesized in few biosynthesis steps; some of them are known. A cytochrome P450 and two carotenoid cleavage dioxygenase (CCD) enzymes (CCD7/MAX3 and CCD8/MAX4) were suggested to encode for enzymes that catalyze SL biosynthesis (reviewed by Dun et al. 2009a; Leyser 2009). Also, in rice, a mutant in Dwarf27 (D27), a β-carotene isomerase, was deficient in SL levels (Lin et al. 2009). Moreover, D27 was found to catalyze conversion of all-*trans*-β-carotene into 9-*cis*-β-carotene; the latter might be a substrate for cleavage by CCD7. CCD8 was suggested to act downstream by incorporation of oxygen, producing carlactone; carlactone was suggested to be an SL-like plant hormone (Alder et al. 2012). Putative regulators of the SL-biosynthesis pathways in rice and

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Medicago were suggested to be NSP1 and NSP2, GRAS-type transcription factors (Liu et al. 2011).

The activity of SLs on inhibition of growing buds was demonstrated to apply to a wide range of bud growth states in pea (*Pisum sativum*) and was demonstrated to directly act in the bud itself (Dun et al. 2012). A direct target of SLs was shown to be the axillary bud specific transcription factor *BRANCHED 1* (*BCR1*) (Braun et al. 2012; Dun et al. 2013). An alternative model for SL activity was suggested, in which SLs modulate polar auxin transport to control branching by reducing the basipetal transport of auxin, a second branch-regulating hormone. Inhibition of branch activity is derived from the prevention of the establishment of auxin transport out of axillary branches due to a restricted capacity for auxin transport in the main stem. SLs are thus suggested to act by dampening auxin transport, enhancing competition between branches (e.g., Crawford et al. 2010; Domagalska and Leyser 2011).

The signal transduction associated with SL response is still mostly unknown. Mutations in MAX2 confer an overshooting phenotype (Stirnberg et al. 2002), and the *max2* mutant over branching phenotype was not repressed by GR24 application (GR24 is a bioactive, synthetic SL; Johnson et al. 1981; Umehara et al. 2008). Therefore, MAX2 was suggested to be a component of SL signaling (Umehara et al. 2008). It encodes an F-box protein that might be part of the ubiquitin-mediated degradation of as-yet unknown protein targets (Stirnberg et al. 2007). Another component of SL signaling was suggested to be Dwarf14 (D14). D14 is a putative α/β hydrolase that was shown to be associated with the SL response; in mutants in both rice (*Oryza sativa*) and *Arabidopsis,* D14 had a hyper-branching phenotype and insensitivity to SLs (Arite et al. 2009; Waters et al. 2012). In addition, a petunia (*Petunia hybrida*) ortholog of the rice and *Arabidopsis* D14 genes designated DAD2 was demonstrated to have an α/β hydrolase fold and in the presence of GR24 to interact with the petunia MAX2A to confer SLs' perception (Hamiaux et al. 2012).

In addition to SL activity as regulators of shoot branching, they were also shown to be involved in the regulation of shoot secondary growth (Agusti et al. 2011). Shoot secondary growth is dependent on activity of the vascular cambium and involves the lateral growth of plant axes, leading to production of secondary vascular tissue and wood production (Miyashima et al. 2012). SL signaling in the vascular cambium was shown to provoke cambium stimulation, and SL mutants displayed a reduction in secondary growth. Also, exogenous supplementation of synthetic SL stimulated the cambium-specific stem cell niche and vascular tissue formation. Interestingly, the same positive effect of SL exogenous application was found in eucalyptus and pea, suggesting the SL-dependent cambium regulation to be conserved between species (Agusti et al. 2011). Thus, SLs may act as general modulators of shoot growth, controlling shoot branching and the secondary growth of stems.

In addition to SL activity in the shoot, SLs were shown to act also in root. SLs affect lateral root formation and positively regulate root hair elongation (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011). They also repress adventitious root

formation (Rasmussen et al. 2012). The activity of SLs in root development and communication with symbionts in the rhizosphere will be the subject of this chapter. This chapter will not cover topics related to the role of strigolactones in parasitic plant interaction. The reader is kindly referred to Koltai et al. (2012) for this topic.

10.2 Strigolactones as Plant Hormones Regulating Root Development

10.2.1 Lateral Root

SLs negatively regulate lateral root formation in Arabidopsis, under conditions of sufficient phosphate nutrition (Kapulnik et al. 2011a). Treatment of seedlings with synthetic SLs repressed lateral roots formation (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011) in wild-type and SL-biosynthesis mutants but not in the SL-response mutants. Also, mutants, deficient in either SL response (i.e., *max2*) or biosynthesis (i.e., *max3* and *max4*), displayed more lateral roots than the wild type (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011). Thus, the negative effect of SLs on lateral roots formation is MAX2 dependent (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011). The branching of roots to lateral roots involves formation of auxin gradients with maxima at the primordia tips (Benkova et al. 2003). These auxin gradients are formed due to asymmetrical localization of PINs, auxin efflux carriers (Benkova et al. 2003; Marhavy et al. 2013).

Exogenous application of SLs was shown to decrease PIN1-GFP intensity in lateral root primordia, suggesting that the SL effect on lateral roots involves PIN1. Also, in the presence of exogenously applied auxin, SL application induced, rather than reduced, lateral root development. Under these conditions no reduction in PIN1-GFP intensity was observed (Ruyter-Spira et al. 2011). Moreover, SL signaling in the root endodermis alone was shown to be sufficient for lateral root response (Koren et al. 2013). This is in accordance with Marhavy et al. (2013) findings suggesting that root endodermis plays an important role in the emergence of lateral roots. SL response was also shown to include the involvement of SHY2 (Koren et al. 2013), a prominent component of the interplay of cytokinin and auxin in the determination of root development (Dello Ioio et al. 2008). Together, the results point to a role for SLs in modulating auxin flux in root tips that is associated with lateral root initiation, thereby altering the auxin optima necessary for lateral root formation (Koren et al. 2013; Ruyter-Spira et al. 2011).

10.2.2 Root Hairs

SLs increase root hair elongation; their exogenous supplementation led to an increase in root hair length in wild-type and SL-deficient mutants (*max3* and *max4*), but not in the SL-response mutant (*max2*), suggesting that the SL effect on root hair elongation is MAX2 dependent (Kapulnik et al. 2011a). The elongation of root hair tip is a result of hormonal balance in epidermal cell layer. Ethylene, by promoting auxin biosynthesis (Swarup et al. 2007) and/or auxin transport (Ruzicka et al. 2007), promotes root hair elongation while inhibiting root epidermal cell elongation by directing auxin into the epidermal cell layer (Strader et al. 2010), in an AUX1 (auxin influx carrier)-dependent way (Pitts et al. 1998; Jones et al. 2009).

An analysis of the root hair response to SLs suggested that auxin signaling is required, at least in part, for SL positive effect on root hair elongation (Kapulnik et al. 2011b). Also involved with the root hair-SL response was ethylene. Ethylene-signaling mutants had reduced sensitivity to SLs, and aminoethoxyvinylglycine (AVG, an ethylene-synthesis inhibitor) had a negative effect on the response of root hairs to SLs. Also, treatment with SLs induced transcription of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthases, involved in ethylene biosynthesis (Kapulnik et al. 2011b). Together, the results suggested that SLs affect root hair length at least partially through the ethylene pathway. However, a functional involvement of SLs with auxin efflux in regard to root hair elongation was shown in tomato roots, in which the inhibitory effect of SLs on root hair formation was reversed by 2,4-D only; 2,4-D is a synthetic auxin that is not secreted by efflux carriers (Koltai et al. 2010a).

10.2.3 Primary Root

SLs were shown to also affect primary root growth. First, under conditions of carbohydrate limitation, which usually lead to a reduction in primary root length (Jain et al. 2007), the SL-deficient and SL-response mutants have a shorter primary root than wild type. This reduction in meristem cell number was accompanied by reduced number of cell number of the primary meristem in the SL mutants than in wild type (Ruyter-Spira et al. 2011). Accordingly, under these conditions, exogenous supplementation of SLs had a positive effect on both primary root elongation and meristem cell number, in a MAX2-dependent way (Ruyter-Spira et al. 2011).

The positive effect of SLs on meristem size was demonstrated also under sufficient carbohydrate conditions (Koren et al. 2013). Also, under these conditions, endodermal expression of MAX2 in *max2-1* mutants was sufficient to allow SLs' effect on meristem size (Koren et al. 2013). Based on this and additional data, it was suggested that this SLs' effect on meristem size is a result of interference with auxin flux in the root tip (Koren et al. 2013). Another case to demonstrate this effect of SLs on auxin efflux was SLs' effect on root directional growth. SL treatments

induced asymmetric root growth in both tomato and Arabidopsis (Koltai et al. 2010b; Ruyter-Spira et al. 2011). In this case the SL interference with auxin flux might have led to asymmetric auxin distribution, perhaps as a result of distorted expression of the PIN auxin efflux carriers (Ruyter-Spira et al. 2011). However, SL might also affect auxin levels; exogenous application of SLs decreased GUS staining in the aerial parts of the plant from the auxin-response reporter DR5-GUS (Ruyter-Spira et al. 2011). Furthermore, SLs were suggested to affect also auxin sensitivity in the primary root. This notion comes from studies of SL regulation of root development under conditions of phosphate starvation (detailed below).

10.2.4 Adventitious Roots

SLs were shown to negatively regulate adventitious root formation from stem, in both *Arabidopsis* and pea (*Pisum sativum*). SL-deficient and response mutants of both species have enhanced adventitious rooting, and SL treatments reduced adventitious rooting in the SL biosynthesis and wild type, but not in the SL-response mutant. Moreover, based on analysis of mutant sensitivity, it was suggested that SL activity to suppress adventitious rooting is independent of cytokinins yet is at least partially auxin dependent (Rasmussen et al. 2012). In accordance, transgenic tomato plants with reduced *SlCCD8* expression, and thereby reduced SL levels, were with increased number of adventitious roots (Kohlen et al. 2012), further supporting a negative role for SLs in this process.

10.3 Strigolactones as Signals for Plant Interactions in the Rhizosphere

10.3.1 Mycorrhiza

The arbuscular mycorrhizal (AM) symbiosis is an association between the roots of higher plants and the soil AM fungi (AMF). Under suitable conditions, symbiotic associations are formed with most terrestrial vascular flowering plants. AM symbiosis is suggested to be an ancient plant association, and this symbiosis played an important role in plants' ability to colonize the land (Smith and Read 2008).

In general, AMF-host association can be discerned by two functional stages: (i) the "pre-symbiotic stage," in which the fungal spore germinates in the soil and hyphae developed towards the root surface, attached to the epidermal cell layer, and penetrates through a pre-penetration apparatus to the root cortical cells, and (ii) the "symbiotic stage" where the fungus grows within the root cortex and forms morphologically distinct structures, the arbuscules (Smith and Read 2008). The

two functional stages require morphological changes in both the host and the fungus and assumed to depend on reciprocal exchange of molecular signals between the fungus and the plant.

During the symbiosis, bidirectional exchange of signals and metabolites between the two organisms is recognized; nutrient exchange between the fungus and the host is taking place in the arbuscules (reviewed by, e.g., Paszkowski 2006). While, in return, the fungus receives photoassimilates from the host. The fungal carbon demand has been shown to affect carbon partitioning in the plant. Nevertheless, under suboptimal growth conditions, the fungi affect plant growth, e.g., induction in leaf area and reduction in root-to-shoot biomass, whereas within the root, mycorrhization affects the level of root carbon and its distribution between soluble and insoluble forms (Smith and Read 2008 and references therein).

Towards completion of the fungal life cycle, most fungi produce an extraradical mycelium, in the soil, from which spores are eventually formed (Smith and Read 2008).

10.3.2 Strigolactones as Signal Molecules of AM Symbiosis

The ability of the AMF to get in touch with the host plant involves, in many cases, hyphal development and branching which are found to be accelerated in response to root exudates exposure. These responses enhance probability of the fungus to meet their host's roots (Harrison 2005).

In the absence of a host, a limited development of the fungal hyphae is usually observed. However, AM hyphae undergo extensive growth and branching in the presence of host roots. Many research groups have tried to identify the most effective factor(s) associated with this phenomenon. It was discovered that the existent "brunching factor" in the root exudates might be SLs (Akiyama et al. 2005, 2010; Akiyama and Hayashi 2006). A reduction in AMF hyphal branching was found in the presence of root exudates of SL-deficient mutants compared to the WT root exudates (e.g., Gomez-Roldan et al. 2008). Moreover, purified SLs from root exudates of *Lotus japonicus* are capable of inducing hyphal branching of *Gigaspora margarita* at low molar levels (Akiyama and Hayashi 2006; Akiyama et al. 2005). Yet, the synthetic SL, GR24, was shown to effectively induce AMF branching at 10^{-8} M (Gomez-Roldan et al. 2008).

SLs were shown to induce mitosis in the AMF *Gigaspora rosea*, as well as mitochondrial activation in *Glomus intraradices* (Besserer et al. 2006, 2008). It is also shown that following the application of the synthetic SL, GR24, rapid changes in the shape, density, and motility of the mitochondria within the fungal hyphae are evident within 60 mins from application. In *G. rosea* hyphae, it was shown that ATP content, NADH concentration, and dehydrogenase activity were all induced within minutes from application. These observations lead to the conclusion that SLs may rapidly enhance the fungus's energy metabolism (Besserer et al. 2006, 2008).

Interestingly, it has been demonstrated that AMF reduce SLs' concentrations in mycorrhized plants. This, in turn, reduces SLs' production and/or exudation to the rhizosphere and reduces susceptibility to *Striga* or *Orobanche* due to lower levels of seed germination induction (López-Ráez et al. 2011 and references therein). Consequently, SLs may be involved in the regulation of AMF colonization in the roots via a feedback loop: where initially SLs promote AMF colonization to assure colonization level up to a certain root colonization rate which above that, SLs' production or secretion is reduced by the host and by that the capacity for new colonization attempts in the rhizosphere is regulated.

10.3.3 Rhizobia

The induction of nitrogen-fixing nodules in legume roots by soil bacteria from the genera *Rhizobium* and *Bradyrhizobium* is host plant specific. This specificity is expressed at an early stage of the infection process and results from multiple interactions between bacterial and plant products. Among these are root exudates, lectins, and flavonoides that are involved in the earliest stages of the bacteria interaction with its hosts.

The putative involvement of strigolactones in the *Rhizobium*-legume interaction has been recently suggested: in pea it has been shown that exudates from non-nodulated plants exhibit a higher seed germination activity on seeds of *Orobanche* than exudates from nodulated plants. These observations lead to a suggestion that levels of seed germination stimulants—predominantly strigolactones—are reduced in nodulated plants (e.g., Mabrouk et al. 2007).

The role of strigolactones in the establishment of *Sinorhizobium meliloti* in alfalfa (*Medicago sativa*) roots, primarily the formation of nodules, was also tested. It was demonstrated that alfalfa nodulation was positively affected by the presence of the strigolactone analogue GR24. However, this increase in nodulation could not be linked with a stimulatory effect of GR24 on the growth or the expression of the nodulation (*nod*) genes of *S. meliloti*. It was further suggested that the strigolactone analogue, GR24, acts on the plant side but not on the bacteria nor on any of the symbiotic stages (e.g., Soto et al. 2010).

Foo and Davies (2011) have demonstrated that a pea mutant, *rms1*, which has a reduced level of strigolactone, exhibits a significant reduction in nodule number that could be elevated by the application of the synthetic SL, GR24. The grafting of wild-type shoots was unable to modify nodulation in *rms1* mutant roots, indicating that shoot-driven factor(s) are not involved in the nodule reduction process, but more interestingly, the shoot SLs are not contributing to the SL content of the roots. It was concluded that strigolactones influence, but not required for Rhizobium nodulation.

10.3.4 Other Interactions in the Rhizosphere

The ability to use SLs as a signal molecule to initiate specific interaction(s) in the rhizosphere is now limited to a signal only for AMF and parasitic weeds. No effect of SLs on hyphal branching has been recorded in soilborne fungi, including *Rhizoctonia solani*, *Fusarium oxysporum,* and *Verticillium dahliae* (Steinkellner et al. 2007).

Nevertheless, the response of various phytopathogenic fungi to the synthetic strigolactone GR24 was notified under in vitro conditions when tested on agar media. According to this report, GR24 inhibits growth of the root pathogens *Fusarium oxysporum* f. sp. *melonis*, *Fusarium solani* f. sp. *mango*, and *Sclerotinia sclerotiorum* and of the foliar pathogens *Alternaria alternata*, *Colletotrichum acutatum,* and *Botrytis cinerea*. These observations suggest that strigolactones can also have a more general effect on fungi (Dor et al. 2011).

10.4 Strigolactones Movement

10.4.1 Strigolactones Transport

SLs are synthesized mainly in roots, and they, their metabolites, or other unknown secondary messengers move in the root-to-shoot direction to confer inhibition of bud outgrowth (reviewed by Dun et al. 2009a). Moreover, in *Arabidopsis,* the presence of the SL orobanchol (Kohlen et al. 2011) in the xylem sap, suggesting orobanchol to be produced in the root and move towards the shoot through vasculature, was found as evidence. The findings of Hamiaux et al. (2012) in petunia, in vitro, reinforce the suggestion that SLs themselves are moving, rather than any degradation products. First, the petunia DAD2 was shown to interact with PhMAX2A in an SL concentration-dependent manner. Second, DAD2 is able to hydrolyze GR24, in association with the ability of DAD2 to interact with PhMAX2A. The GR24 hydrolysis products did not stimulate the DAD2-PhMAX2A protein interaction nor modulate branching (Hamiaux et al. 2012). Therefore, SLs, rather than their degradation products, may move in plant and be recognized as a signal for inhibition of shoot branching.

Furthermore, SLs may be actively transported to their target organs (e.g., shoot buds) for their activity. An active transporter for SLs was found in petunia. This is an ABC transporter, designated PDR1, of which overexpression in *Arabidopsis* resulted in increased tolerance to high concentrations of GR24, due probably to increased export of SLs from the roots. PDR1 was found to be present in cells in the plasma membrane, consistent with a suggested role in secretion, and to be expressed in root and shoot tissues, consistent with PDR1 functioning as an SL transporter (Kretzschmar et al. 2012).

However, SLs are produced in shoots, in addition to roots. The pea *rms1* (CCD8) is expressed in other plant tissues (epicotyl tissue and internode tissue) then roots, although to a lesser extent (Foo et al. 2005; Dun et al. 2009b). Therefore, it might be that SLs do not always need to move along the plant for their activity. Rather, they may act locally to directly repress bud outgrowth (Brewer et al. 2009) and in the root, their main site of production. In these cases no movement or a short distance movement of SLs, e.g., between cells, may be involved.

10.4.2 Strigolactones Secretion and Exudation

Root-secreted secondary metabolites are used to regulate the rhizosphere. They either are used to the detriment of neighboring plants through allelopathy or are exploited by other plants and microorganisms to initiate their development. However, despite the ecophysiological significance of plant-secreted compounds and the large number of compounds produced by plant roots, very little is known about the molecular mechanisms involved in the regulation of root exudation.

Several reports suggest a higher secretion of SLs when mycotrophic plants are exposed to low Pi levels relative to plants exposed to higher Pi levels (Yoneyama et al. 2007a, b, 2012, López-Ráez and Bouwmeester 2008, López-Ráez et al. 2008). It was shown that under limited supply of Pi (and N), SL contents in both sorghum root tissues and root exudates increase, suggesting that low Pi and N conditions increase both SL production and secretion; once produced in the roots, SLs appear to be rapidly secreted. In addition, under Pi deficiency, the exudation of SLs in red clover was significantly stimulated (Yoneyama et al. 2007a, b).

It is not yet known where exactly along the longitudinal root axis SLs are exuded. It is widely recognized that the gradual maturation of root tissues along the root axis is not the only variation in metabolic activity. Yet, from available information, it can be concluded that the pattern of exudation is not homogeneous along the root axis. For example, the release of phytosiderophores in response to Fe deficiencies appears to concentrate in the apical root zone (Marschner 1995), while the release of organic anions follows a heterogeneous pattern along the roots (Hoffland et al. 1989).

To fulfill a role in plant interactions, following root exudation, SLs have to have a reasonable level of stability to be recognized by microorganisms or other plants in the rhizosphere; a critical factor in host location for AMF and parasitic weeds is the lifetime of individual SLs in the rhizosphere. Although little is known about SL stability in soil, some indication of it was revealed by water-degradation experiments, suggesting that SL stability differs considerably between natural and synthetic SLs (Akiyama et al. 2010).

10.5 Strigolactones Feedback Regulations

10.5.1 Feedback Regulations with Other Plant Hormones

SL activity in different plant parts affecting growth and development may necessitate a creation of a state of SL homeostasis in the plant. This is shaped by the interplay of SLs with other plant hormones, including mutual influence on biosynthesis, transport (detailed above), and response. Moreover, this state of SL homeostasis should correlate to growth conditions, such that when needed, SL level would increase to confer inhibition of shoot and root branching. On other instances their level would decrease to confer increase in lateral buds outgrowth and lateral root formation.

SL feedback regulation and homeostasis are carried by at least three groups of molecules. One is auxin, which positively regulates SL levels in roots. This regulation is carried in Arabidopsis by induction of transcription of both MAX3 (CCD7) and MAX4 (CCD8) by auxin. Both of these transcripts are also upregulated in SL-response and synthesis mutants, consistent with the findings of increased polar auxin transport in these mutants (Bennett et al. 2006). Moreover, once auxin was depleted, reduced SL-biosynthesis gene expression was found in pea (for RMS5 and RMS1; Foo et al., 2005; Johnson et al. 2006) and *Arabidopsis* (Hayward et al. 2009). This feedback regulation of auxin on SL biosynthesis was suggested to involve auxin signaling (Hayward et al. 2009).

SLs themselves consist of a second group of molecules to regulate SL levels. Evidently, in several plant species, including pea and *Arabidopsis*, SL mutants show higher levels of SL-biosynthesis gene expression and/or SL content (e.g., Foo et al. 2005; Dun et al. 2009b; Hayward et al. 2009; Umehara et al. 2010), and GR24 treatments can reduce expression of SL-biosynthesis genes (Mashiguchi et al. 2009; Dun et al. 2013).

Evidence is presented to a third group of molecules that regulates SL levels; however, these were not specified up to date. Reduced levels of the major cytokinins in xylem sap in comparison to wild-type plants (Foo et al. 2005, 2007) were found in SL-biosynthesis and SL-response mutants in Arabidopsis and pea. An exception is the hyper-branching mutant *rms2* in pea. It was suggested, based on grafting experiments and double mutant analysis, that in pea, the regulation of export of root cytokinin is mediated by an inhibitory signal, which might be downregulated by local signaling and response to SLs (Foo et al. 2007). This long-distance signal might regulate expression of SL-biosynthesis genes and reduce cytokinin export from root and is probably absent in *rms2* (Foo et al. 2001, 2005, 2007).

To conclude, SL levels seem to be feedback regulated by a number of molecules, to confer a carefully controlled state of SL homeostasis. This homeostasis may be affected by growth conditions as discussed below.

10.5.2 Feedback Regulations in Response to Growth Conditions

The ability of SLs to regulate both shoot and root development positions them as potential coordinators of plant development for regulation of growth under a changing environment. For this role to be fulfilled, SL levels or response should be altered under different growth conditions, to confer appropriate plant response. The best example for a clear role for SLs in coordination with plant development in response to growth conditions is under the conditions of inorganic phosphate (Pi) starvation.

Phosphorus (P) is an essential nutrient, required by plants for growth and development. It is a major and essential part of macromolecules and participates in many life processes. Roots function as the main organ in the plant for Pi acquisition; the source for P for plant is the soil, mostly in its Pi form. However, usually Pi levels are relatively low and are a limiting factor for plant development (Bieleski 1973; Maathuis 2009).

To exceed Pi absorbance from the soil, the root structure is altered to increase root surface. One change taking place under low Pi conditions is an increase in lateral root formation and length and inhibition of primary root growth (reviewed by López-Bucio et al. 2003; Peret et al. 2011). Another change taking place under reduced Pi levels, suggested to be an efficient strategy for P acquisition, is the elongation of root hairs and an increase in their number (Bates and Lynch 2000; reviewed by Gilroy and Jones 2000).

As described above, SLs were shown to positively regulate root hair length (Kapulnik et al. 2011a). Also, under reduced Pi levels, SLs exert a positive effect on lateral root formation (Ruyter-Spira et al. 2011). Moreover, SLs have an essential role in the ability of roots to sense or respond to low Pi conditions following germination, by an increase in root hair density. SL-biosynthesis (*max4*) or SL-response (*max2*) mutants in *Arabidopsis* were unable to increase their root hair density under low Pi conditions, whereas supplementation of GR24 to the SL-biosynthesis mutant, but not SL-response mutant, led to restoration of wild-type response to low Pi in these mutants (Mayzlish Gati et al. 2012).

Following germination, SL mutants have reduced expression of several Pi transporters under low Pi conditions in comparison to wild type (Mayzlish Gati et al. 2012). However, P concentrations in the SL-insensitive mutant *max2* plants were similar to those of wild type under both low and high Pi conditions (Mayzlish Gati et al. 2012). These results suggest that SL response is not needed for P acquisition; however, despite low levels of P under low Pi growth conditions, and unlike the wild type, the SL mutant does not provoke an increase in root hair density. Taken together, the activity of MAX2 and wild-type levels of SLs is probably required for root sensing of or response to low Pi (Mayzlish Gati et al. 2012).

In addition, it was shown that the SL pathway is involved in shoot response to low Pi conditions. In both Arabidopsis and rice reduced branching phenotype of the

shoot under reduced Pi growth conditions, apparent in wild type, was absent in SL-deficient or SL-insensitive mutants (Kohlen et al. 2011; Umehara et al. 2010).

Moreover, it was shown for several plant species that SL production is induced under low Pi conditions (e.g., Yoneyama et al. 2007a, b; López-Ráez and Bouwmeester 2008; Kohlen et al. 2011) and the shoot P levels correlated to SL exudation in several plant species (Yoneyama et al. 2012). In Arabidopsis, SL (orobanchol) was detected in xylem sap and was found to be enhanced under Pi deficiency (Kohlen et al. 2011). In rice, root SL (2′-epi-5-deoxystrigol) levels were increased under Pi deficiency (Umehara et al. 2010; reviewed by Umehara 2011).

To summarize, SL is suggested to mediate the low Pi response in both root and shoot. This is carried by changing plant architecture: shoot branching is inhibited, root branching is induced, and root hair density is stimulated under low Pi growth conditions. These changes may correspond to the observed reduction in the ratio of shoot to root under Pi deficiency (Ericsson 1995). The low Pi responses of root and shoot are carefully regulated in the plant. They are suggested to involve local and systemic signaling in root and shoot and a precise communication between them (reviewed by Chiou and Lin 2011). It might be that SLs have a role in this communication in response to Pi growth conditions. For that purpose SL levels and response should be carefully regulated.

As indicated above, auxin is a regulator of SL biosynthesis. On the other hand, it was shown by Mayzlish Gati et al. (2012) that SLs are at least partially involved with the increased auxin sensitivity detected under low Pi conditions (Lopez-Bucio et al. 2002; Perez-Torres et al. 2008). This includes the induction of *TIR1* transcription (Perez-Torres et al. 2008), suggesting that SL signaling pathway is upstream to the TIR1-mediated low Pi response (Mayzlish Gati et al. 2012). Auxin response is a positive regulator of SL biosynthesis, and SLs seem to positively regulate auxin sensitivity under low Pi conditions. Thus, it might be that the induction in SL levels evident under low Pi conditions is a result of increased auxin sensitivity, suggesting a positive feedback loop for SL level and auxin sensitivity under these conditions. However, as indicated above, SLs themselves regulate their level. Thus, the increased SL levels under low Pi conditions might suppress SL biosynthesis, via a negative feedback loop.

10.6 Conclusion

SLs are recognized now as plant hormones that regulate both root and shoot development. They may coordinate development of both and act to regulate several aspects of plant growth under different environmental conditions. However, SLs fulfill an additional role in communication between plant as a host and parasitic plants or symbiotic mycorrhiza. SLs were found to be produced in green algae (Delaux et al. 2012), aquatic plants that do not form symbiosis with mycorrhiza. Therefore, SLs probably served initially as plant substance for the regulation of development. Only later in evolution, upon the emergence of land plants, exuded

SLs became a "flag" identified by parasitic plant or other soil microorganisms for the presence of a host plant. As for AM symbiosis, its ability to promote the plant's ability to acquire Pi (e.g., Bucher 2007) may give another aspect for SL activity. In addition to being regulators of development, SLs may benefit plants under Pi-deprived conditions also by promoting the mycorrhizal association, for increased Pi acquisition. As a result, SLs may be positioned as a key component of a whole plant response to nutrient status, involving regulation of both nutrient uptake and developmental responses in both root and shoot.

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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.

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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

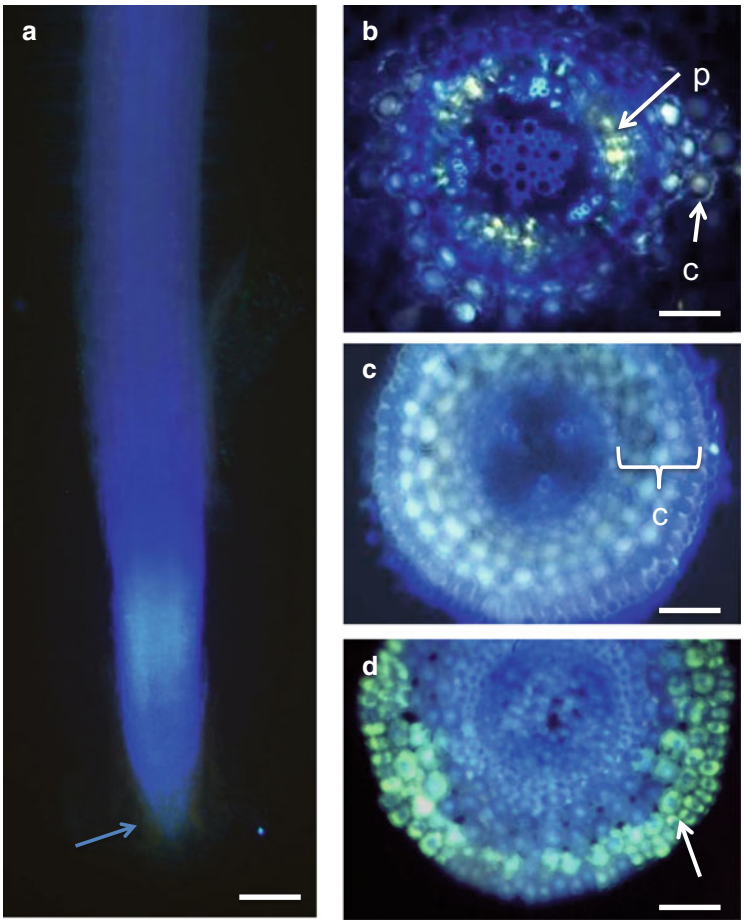

Fig. 11.1 Flavonoid accumulation in roots is cell type specific. (**a**) Flavonoid accumulation in root tips of *Medicago truncatula*. *Blue* and *orange* autofluorescence is due to the presence of flavonoids. Note the high accumulation of flavonoids in the root tip and in root cap cells (*orange*, *arrow*). (**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

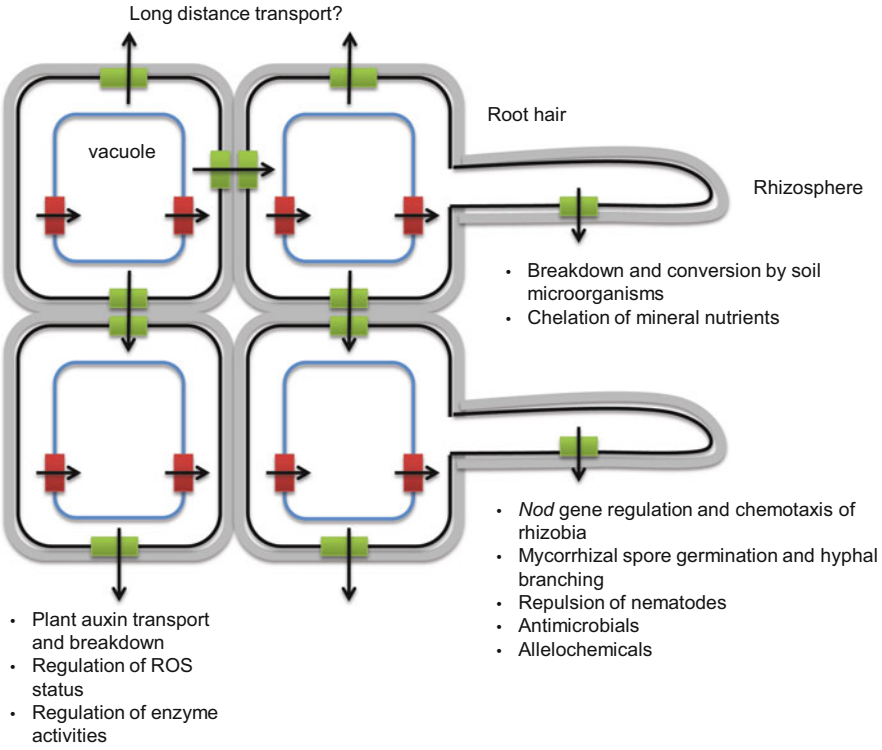

Fig. 11.2 Model for the transport of flavonoids within, between and out of root cells. Flavonoids are likely to be transported across the plasma membrane (*black line*) and tonoplast (*blue line*) by ABC-type or MATE transporters (*red* and *green boxes*). It is not known whether different transporters 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

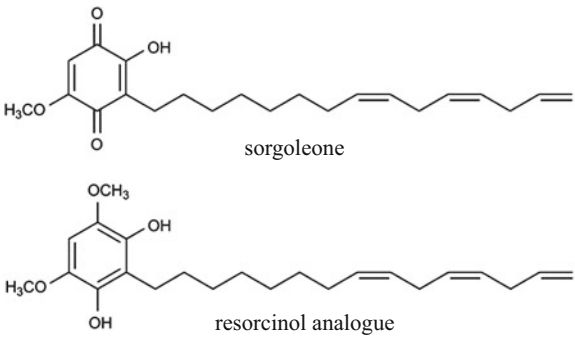

Fig. 11.4 Structure of sorgoleone and its resorcinol analogue

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

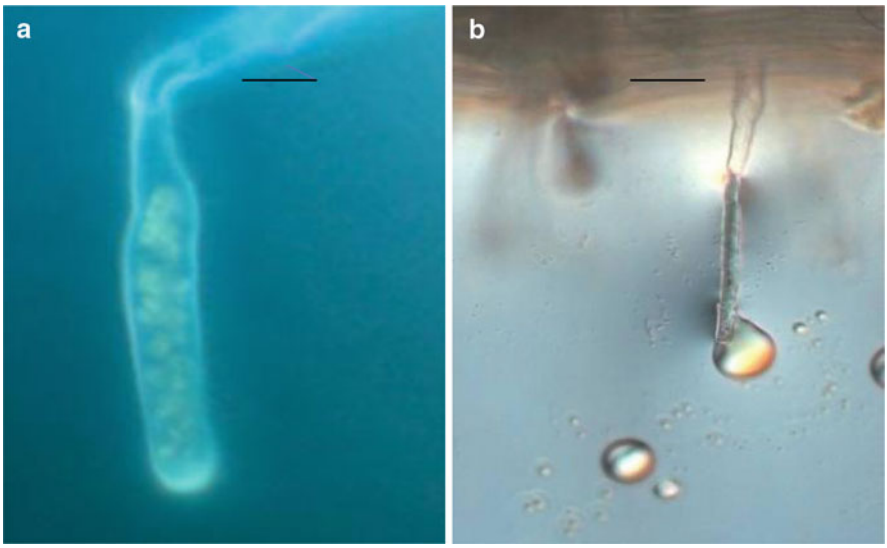

Fig. 11.5 (**a**) *Sorghum bicolor* root hair containing large numbers of vesicles containing sorgoleone, light microscopy. (**b**) Sorghum root hair exuding a droplet of sorgoleone, insoluble in water, light microscopy. Taken by Michelle Watt, CSIRO Plant Industries, Black Mountain, ACT, Australia and 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

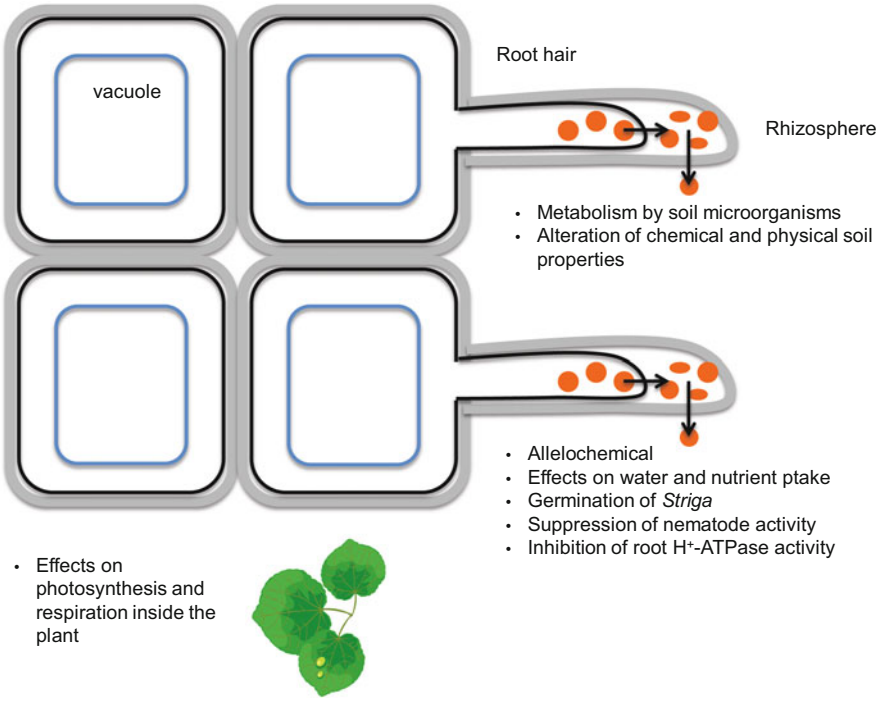

Fig. 11.6 Model for the exudation of sorgoleone from sorghum root hairs. Sorgoleone containing vesicles (*orange*) are formed in young root hairs and fuse with the plasma membrane. They accumulate in the apoplast and are exuded passively into the surrounding rhizosphere where they affect abiotic and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

Chapter 12
Root Strategies for Nitrate Assimilation

José S. Rubio-Asensio, Carmen López-Berenguer, Jesús García-de la Garma, Martin Burger, and Arnold J. Bloom

12.1 Introduction

Nitrogen is an essential element in biological molecules, such as nucleotides, amino acids, and proteins, and therefore fundamental for plant growth and development (Marschner 1995). In fact, plant performance, fitness, yield, nutrient efficiency, or susceptibility to biological and environmental stresses is highly dependent on nitrogen mineral nutrition (Epstein and Bloom 2005). Some plants can fix atmospheric nitrogen into organic forms through symbiotic relationships with soil microbes (not covered here), but most plants obtain their nitrogen directly from the soil via absorption of inorganic and organic forms of nitrogen. The concentrations of various forms of N (e.g., organic N, ammonium, nitrate, nitrite, nitrous oxide) in the soil depend on soil type, temperature, and the activities of microorganisms (Nasholm et al. 1998; Jackson et al. 2008).

Nitrate (NO_3^-) is the major N source for higher plants in most agricultural and temperate zone soils (Epstein and Bloom 2005). Distribution and concentration of NO_3^- in soils show substantial spatial and temporal heterogeneity (Jackson and Caldwell 1993), and NO_3^- is the object of intense competition among neighboring plants (Cahill et al. 2010) and between plants and microorganisms (Jackson et al. 1989; Hodge et al. 2000; Miller et al. 2007a). Under conditions of limited NO_3^- availability, the ability of a plant to acquire NO_3^- from its surroundings largely depends on the amount of root area in contact with

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NO_3^- in the soil solution and on the efficiency with which a root can transport NO_3^- from its surroundings into the plant. Under conditions of unlimited soil NO_3^- availability, roots absorb superfluous amounts, and root and shoot NO_3^- concentrations can reach up to 100 mM, most of which is stored within vacuoles (Miller and Smith 1996). Despite considerable variation in the concentration of NO_3^- in the soil solution, root cells keep cytosolic NO_3^- at a controlled level, with values of approximately 3 mM in maize and 4–5 mM in barley (Miller and Smith 1996, 2008; Huang et al. 2012), possibly to minimize oxidative stress (Huang et al. 2012). Nitrate assimilation plays a central role in this homeostasis, along with NO_3^- uptake, efflux, and xylem and vacuolar loading (Crawford and Glass 1998). Nitrate, in addition to being an important nutrient, may serve as an osmoticum for supporting root elongation and may act as signal molecule regulating nitrogen and carbon metabolism and coordinating whole-plant development (Redinbaugh and Campbell 1991; Crawford 1995; Miller et al. 2007b; Krouk et al. 2010a; Dechorgnat et al. 2011; Bloom et al. 2012a). Root strategies for NO_3^- acquisition must cope with soil NO_3^- heterogeneity and cytosolic NO_3^- homeostasis as well as coordinate root growth with NO_3^- sensing, uptake, translocation, and finally, assimilation to organic N.

The relationship between nitrogen acquisition and roots has long been of interest in plant biology and agriculture because of its influence in plant growth and food production (Oaks and Hirel 1985; Oaks 1992). The topic has been reviewed by Miller and Cramer (2005), with an emphasis in the molecular mechanisms that plants use in accessing N in the soil pools; by Jackson et al. (2008), with an emphasis on physiological and ecological functions that contribute to plant–microbe–soil N cycling; by Kraiser et al. (2011), focusing on the integration of nitrogen acquisition strategies from the ecosystem to molecular level; by Forde and Walch-Liu (2009) and Krouk et al. (2010a), with an emphasis in the role of NO_3^- as a signal and its influence on root behavior and NO_3^- regulation, respectively. Here, we review the different strategies that plant roots follow to acquire NO_3^- from the soil.

12.2 Nitrate in the Soil

12.2.1 Origins and Fates

In natural ecosystems, NO_3^- is the dominant form of nitrogen available to plants in all but very acidic and anaerobic soils, because of both the ability of particular soil microorganisms (e.g., *Nitrosomonas*, *Nitrosospiras*, and *Nitrobacter* species) to convert ammonium ion (NH_4^+) to NO_3^- and the ubiquitous distribution of these organisms (Oaks 1992; Hiorns et al. 1995). In agricultural systems, the baseline level of NO_3^- is supplemented by the addition of N fertilizers to the soil. Soil NO_3^- concentration averages 1 mM in natural ecosystems (Andrews 1986), whereas it

averages 10 mM, ranging from 0 to 70 mM, in most agricultural systems (Reisenauer 1966). In both natural and agricultural ecosystems, NO_3^- originates (i) through microbial decomposition of soil organic matter via intermediates (nitrification), (ii) through biological fixation, (iii) from atmospheric deposition, and (iv) only in agricultural ecosystems, from the incorporation of fertilizers. The major pathways of NO_3^- losses from soil include (i) leaching to surface and ground water, (ii) microbial conversion to N_2O and N_2 (denitrification), (iii) microbes immobilization, and (iv) soil erosion.

The appearance and disappearance of soil NO_3^- rapidly changes with rainfall and other factors influencing microbial activity such as pH, temperature, and oxygen concentrations, which in turn affect mineralization, nitrification, and denitrification (Haynes 1986). Nitrate with its negative charge is not adsorbed on negatively charged soil particle surfaces, allowing it to move relatively freely through the soil. For these reasons, NO_3^- concentrations in natural and agricultural soils fluctuate greatly both temporally, even diurnally, and spatially even over short distances (Burger and Jackson 2004).

12.2.2 Nitrate in the Rhizosphere

In natural ecosystems, root acquisition of exogenous NO_3^- is a function of its availability, whereas in croplands the addition of fertilizers containing NO_3^- decreases the element of chance (Oaks 1992). NO_3^- enters the rhizosphere because of NO_3^- mobility in the soil (the NO_3^- reaches the root) or activities of the root system itself (the root reaches the NO_3^-). In the first case, NO_3^- mobility in soils results in rapid diffusion to roots and thus easier plant access to the available NO_3^- (Boudsocq et al. 2012). NO_3^- can also reach the rhizosphere by mass flow, linked to transpiration and the depletion of solution near the root surface (Marschner 1995). In the second case, (i) the roots can directly intercept the NO_3^- through their growth or (ii) the roots of some plants can change the rhizosphere's conditions such as releasing oxygen (Kirk and Kronzucker 2005; Li et al. 2008) and exudates (Bais et al. 2006) that greatly influence the density and activity of microbial populations, which in turn can accelerate nitrification of NH_4^+ in the rhizosphere.

12.3 Root Strategies for Nitrate Acquisition

12.3.1 Ecosystem Level

12.3.1.1 Associations with Microorganisms

Roots form associations with microorganisms as a strategy to enhance resource capture (Hodge 2009; Kraiser et al. 2011). The majority of plants are capable of associating with arbuscular mycorrhizal fungi, which induce modifications in root system architecture (RSA) (Gutjahr et al. 2009). These modifications can increase plant NO_3^- uptake and improve the nitrogen nutrition of plants, mainly those growing at low levels of nutrients (Cruz et al. 2004). Plant roots also associate with bacteria, which can increase nutrient accessibility, uptake, or both (Bertrand et al. 2000), improving plant growth. These bacteria are referred to as plant growth-promoting bacteria and can produce phytohormones affecting RSA (Persello-Cartieaux et al. 2001) or increase the activity of NO_3^- uptake systems (Bertrand et al. 2000).

12.3.1.2 Competing for Nitrate

Nitrate in the soil is very dynamic and an "object of desire" to individuals of the same species (intraspecific competition) and among different species, regardless of taxonomic affiliation (interspecific competition) (Hodge et al. 2000; Schenk 2006; Cahill and McNickle 2011). The rapid diffusion of NO_3^- through the soil allows different individual plants to be highly efficient at acquiring NO_3^- even when they have restricted or simple RSA (Fitter et al. 2002). Some plants display pronounced proliferation in response to locally applied NO_3^- (Drew 1975; Guo et al. 2002). Because of the mobility of NO_3^- ions in soil, such proliferation increases NO_3^- acquisition by plants when they are in competition with neighbors for a finite, spatially restricted, mixed N source (Robinson et al. 1999).

Not all plant species respond to the nutrient-rich zones in the same way (Campbell et al. 1991). Competitively dominant plants exploit nutrient-rich patches to a greater extent simply because they are larger and have higher growth rates rather than because they have greater flexibility within their root system. In contrast, competitively inferior plants, although smaller, allocate more of their new root growth to nutrient-rich areas; that is, they place their new roots with greater precision. Among plants and microorganism, Jackson et al. (1989) showed that in annual grasslands, the NO_3^- pool is consumed as rapidly as it is produced, and microbial uptake is the major factor controlling NO_3^- availability to plants. In the short term (hours), soil microorganisms do compete better than plants for

NO_3^- (Jackson et al. 1989), but after longer periods (days to weeks), plants absorb more NO_3^- than microbes do because of microbial turnover (Inselsbacher et al. 2010). Recent work in which substantial microbes turnover was prevented, however, suggested that plants compete directly and more efficiently for NO_3^- than microbes (Inselsbacher et al. 2010). Spatial differences in nitrogen availability, distribution of root and microorganisms, relative turnover times of roots and microorganisms, and changes in the soil C:N ratio are the key determinants of "success" in the competition for NO_3^- (Hodge et al. 2000).

12.3.2 Organism Level

12.3.2.1 Resource Allocation to Roots

All plants face a basic economic decision: where best to invest their resources (Bloom et al. 1985). The costs associated with getting this wrong may lead to diminished nutrient capture; less resources for reproduction and hence reduced fitness; and at the extreme, competitive exclusion from the particular environment (Hodge 2009). When the roots of some plants encounter a NO_3^--rich patch, their growth becomes enhanced at the expense of the growth in poorer resource areas (Drew 1975). This mean that root development, and especially lateral root initiation, depends on the integrated effects of the local environment and the internal correlative relations between the roots (Gersani and Sachs 1992).

Allocation of resources to above vs. belowground structures or to different parts of the root systems can make budgetary sense. In general, resource allocation to belowground structures relative to aboveground structures increases when N is limiting (Miller and Cramer 2005) (Fig. 12.1). This compensates for N deficiency by increasing the plant's opportunity to obtain N for sustaining growth (Reynolds and Dantonio 1996; Sims et al. 2012). Such an acclimation response derives from metabolic changes in the shoot and an adjustment of carbohydrate transport to the root (Hermans et al. 2006). NO_3^- in the shoot acts as a long-range signal molecule that regulates root growth (Adgo and Schulze 2002). Among the several changes that result in NO_3^- accumulation in the shoot and could contribute to altered allocation patterns include an inhibition of starch synthesis and turnover in the leaves and a decrease of the transport of sucrose to the roots, resulting in an increase in the root-to-shoot ratio (Rufty et al. 1988). In *Arabidopsis* plants where tissue N is plentiful, NO_3^- can specifically inhibit root system growth while having no effect on shoot system growth (Roycewicz and Malamy 2012). This suggests that plants regulate root-to-shoot ratio not specifically in response to nitrogen starvation but as a general mechanism to tailor their growth to environmental nitrogen supply.

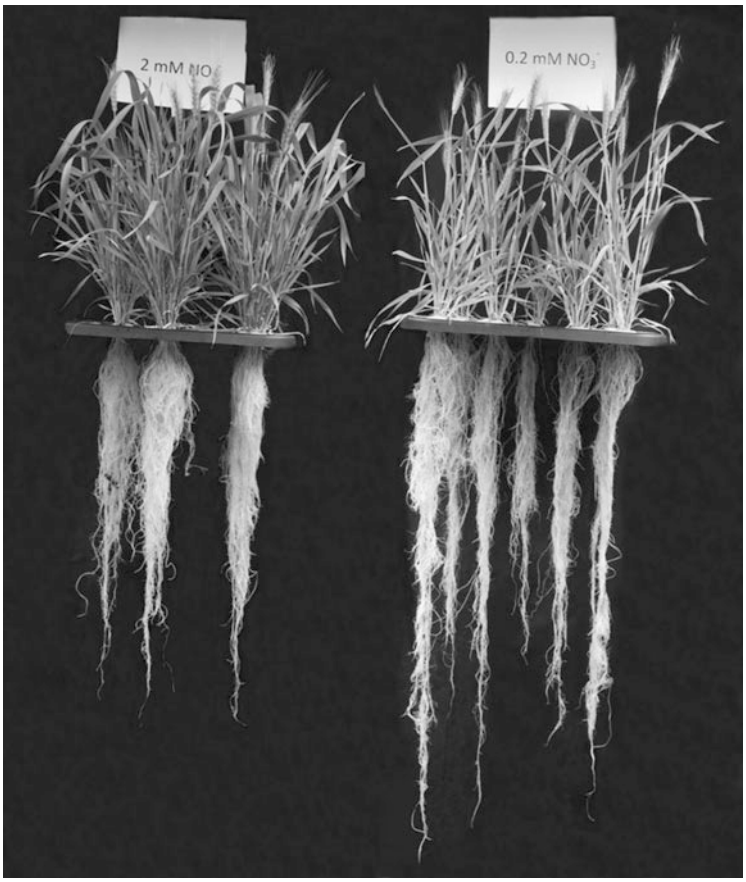

Fig. 12.1 Two months old wheat plants growth in nutrient solution containing 2 mM NO_3^- (*left*) and 0.2 mM NO_3^- (*right*) as solely nitrogen source

12.3.2.2 Root Architecture

The root system is fundamentally important for plant growth and survival because of its role in water and nutrient uptake. The architecture of the root system is determined by the pattern of root branching, which in many species displays a high degree of plasticity to enable plant survival under variable environmental conditions (Sultan 2000; Hodge 2009). Soil NO_3^- concentration and overall nutrient status of the plant in concert with the genetic makeup of the plant will determine the pattern of root branching and define the root strategies for NO_3^- acquisition (Hodge 2004; Desnos 2008; Vidal et al. 2010).

First of all, when encountering a NO_3^--rich patch, a plant maximizes N capture through upregulating inflow and making a more competitive root system (Robinson

et al. 1999). The strategies affecting RSA involve lateral root elongation, lateral root initiation, and primary root growth (Kraiser et al. 2011). A localized area of high NO_3^- stimulates the elongation of lateral roots through a dual-affinity nitrate transporter, CHL1, that not only transports NO_3^- but also senses external NO_3^- concentrations (Ho et al. 2009) and activates the *NITRATE REGULATED* 1 (*ANR1*) (an MADS-box gene) that control changes in root architecture (Guo et al. 2002). ANR1 initiates a local-range signaling pathway and regulates NO_3^--stimulated lateral root elongation (Remans et al. 2006a). When plants are grown on high NO_3^- or have high levels of N metabolites, the expression of *ANR1* decreases and lateral root elongation is suppressed (Zhang et al. 1999; Gansel et al. 2001). The net result is that, if roots grown on low NO_3^- are exposed to a localized region of high NO_3^-, then lateral roots proliferate specifically in that region of the roots. The putative high-affinity NO_3^- transporter NRT2.1, like NRT1.1, serves as a NO_3^- sensor to coordinate the development of the root system. It acts directly on lateral root initiation under NO_3^--limiting conditions (Lejay et al. 1999; Little et al. 2005; Remans et al. 2006b).

It is interesting to note that root proliferation occurs in localized patches of NO_3^-, which is a relatively mobile nutrient, but not in localized patches of NH_4^+, which is a relatively immobile nutrient (Leyser and Fitter 1998). A possible explanation for this paradox might be the very mobility of the NO_3^- ion, which makes it an ideal subterranean signal molecule (Forde and Zhang 1998). In unfertilized soils, a major source of nitrogen is from decaying organic matter, which under aerobic conditions releases both NH_4^+ and NO_3^-. The relative immobility of NH_4^+ means that it is the NO_3^- ion that will be the first to reach nearby roots through the soil solution. In this scenario, NO_3^- provides the signal that allows roots to proliferate towards areas where less mobile forms of N are localized within the soil.

Finally, NO_3^- itself stimulates primary root growth, both directly and by antagonizing the inhibitory effect of L-glutamate (Walch-Liu and Forde 2008). This highlights that relative abundance of inorganic nitrogen (e.g., NO_3^-) and organic N (e.g., glutamate) influences RSA and therefore NO_3^- acquisition. In several studies RSA adaptation to external NO_3^- availability was observed whereby NO_3^- was shown to interact with auxin, abscisic acid, and cytokinin signaling pathways (Signora et al. 2001; Garnett et al. 2009; Krouk et al. 2010b; Vidal et al. 2010; Ruffel et al. 2011; Wang et al. 2012).

There are other attributes of a root system in addition to its architecture that dictate its capacity and efficiency for NO_3^- acquisition (Gastal and Lemaire 2002; Miller and Cramer 2005; Volder et al. 2005; Garnett et al. 2009; Hodge 2009). These include the proportion of active roots, root longevity, rooting depth, proportion of fine roots versus thick roots, and number, size, and location of the root hairs.

12.3.3 Molecular Level

12.3.3.1 Root Uptake Systems

To cope with the heterogeneity and dynamic variations of NO_3^- concentrations, plants have evolved at least three NO_3^- transport systems that function according to enzyme kinetics (Crawford and Glass 1998): (i) constitutive (active even when plants have not been previously supplied with NO_3^-) high-affinity transport systems (CHATS) with low values of both K_m (concentration of substrate that gives "half-maximum" absorption) and V_{max} (maximum rate of absorption), typically 6–20 μM and 0.3–0.82 μmol g^{-1} h^{-1}, respectively; (ii) NO_3^--inducible (hours to days of exposure to NO_3^-) high-affinity transport systems (IHATS) with higher K_m and V_{max} values, typically 20–100 μM and 3–8 μmol g^{-1} h^{-1}, respectively; and (iii) constitutive low-affinity transport systems (LATS), which become evident when NO_3^- is plentiful (above 250 μM). Uptake by HATS and LATS is mediated by two families of NO_3^- transporters, NRT1 and NRT2, respectively (Wang et al. 2012). The relative contribution of these transporters to NO_3^- uptake is regulated by negative feedback, linking the expression and activity of NO_3^- uptake to the C:N status of the plant (Lejay et al. 1999; Miller et al. 2007b): under low external NO_3^- concentration, plants upregulate HATS, while under high external NO_3^- concentration or when fertilizers are applied, plants change their dependence from the HATS pathway and root–microbe associations to the LATS pathway. Like RSA, local and long-range signaling pathways regulate the activity of NO_3^- transport system in response to both external NO_3^- and sugars transported from the shoot (Forde 2002).

12.3.3.2 Vacuolar Loading and Long-Distance Transport

Once inside the root cell cytoplast, NO_3^- can be translocated across the tonoplast and stored in the vacuoles, be loaded into the xylem vessels and subsequently unloaded in plant aerial tissues, or enter the amino acid biosynthesis pathway (Wang et al. 2012). Nitrate may be an important osmoticum solute in the vacuole of root cells (Zhen et al. 1991). Also in the base of the root growth zone, NO_3^- can be considered a significant component of the osmotic pool supporting its expansion (Bloom et al. 2012a). Long-distance NO_3^- transport (e.g., root-to-shoot NO_3^- transport) is finely tuned in response to various environmental conditions (Smirnoff and Stewart 1985). The influence of the environment on long-distance NO_3^- transport (e.g., transport from root to shoot) is evident in the different shoot and root NO_3^- concentrations found during different times of the day and between plant species (Fig. 12.2). *AtNRT1.5* mediates the first step in loading of NO_3^- into xylem vessels and facilitates root-to-shoot xylem NO_3^- transport (Lin et al. 2008). *AtNRT1.8* and *AtNRT1.9* regulate root-to-shoot NO_3^- translocation; in specific, *AtNRT1.8* mediates NO_3^- removal from xylem and may also diminish root-to-shoot

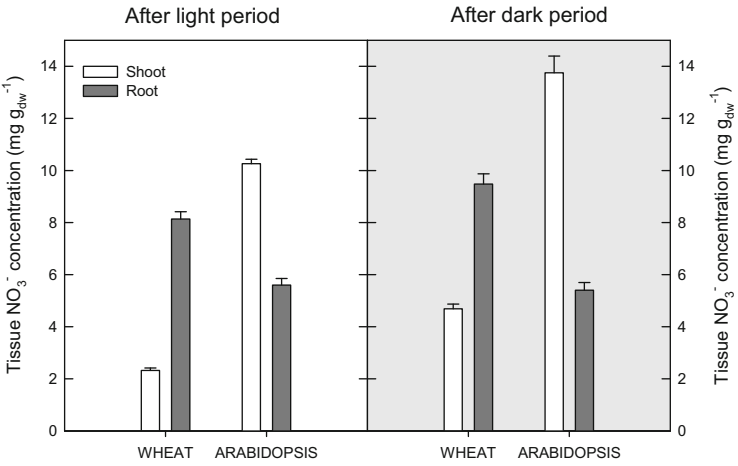

Fig. 12.2 Shoot and root nitrate concentration of wheat (14 days old) and *Arabidopsis* (40 days old) plants after the light and dark period. Wheat and *Arabidopsis* plant growth in nutrient solution containing NO_3^- (0.2 mM) as solely N source and in a light/dark cycle of 16/8 and 9/15 h, respectively (Bloom et al. unpublished data)

NO_3^- transport (Li et al. 2010), whereas *AtNRT1.9* facilitates the loading of NO_3^- into the root phloem and enhances downward NO_3^- transport in roots (Wang and Tsay 2011). *AtNRT1.9* prevents excess amounts of NO_3^- being transported to the shoot. For example, in different ecotypes of *Arabidopsis*, the capacity to maintain NO_3^- reserves under low NO_3^- supply confers higher tolerance to low NO_3^- environments (North et al. 2009). All together these studies show that roots play a key step in regulating NO_3^- distribution in the plant and again highlight that NO_3^- is a key component in the regulation of plant development and growth.

12.3.3.3 Assimilation: Where and When?

NO_3^- that is not transported to the shoot or vacuoles is reduced to nitrite (NO_2^-) in the cytosol *via* NO_3^- reductase and then further to NH_4^+ by NO_2^- reductase in the plastids (Crawford et al. 2000). NH_4^+ is then added to C skeletons to produced glutamine and glutamate through the sequential actions of glutamine synthetase and glutamate synthase, which are located in the root plastids (Lam et al. 1996). In the root, carbohydrate oxidation provides the approximate 10 mol ATP and reductants needed for NO_3^- assimilation (Neuhaus and Emes 2000). The biochemical reactions responsible for root NO_3^- uptake and assimilation are so energy intensive that this process largely determines the carbon balance of a plant as well as its nitrogen budget (Bloom et al. 1992). Despite considerable research effort, the relative proportion of NO_3^- that is reduced in the shoot and root is still a matter of

considerable debate (Nunes-Nesi et al. 2010). It is known that NO_3^- assimilation may vary between the root and the shoot tissue depending on the species and the growth conditions (Miller and Cramer 2005). Generally, species native to temperate regions rely more heavily on root NO_3^- assimilation than do species of tropical or subtropical origins (Andrews 1986). Roots also appear to be the predominant site of NO_3^- assimilation when plants are grown under low external NO_3^- availability (Gojon et al. 1991; Scheurwater et al. 2002).

Timing, as well as location, of NO_3^- assimilation will have significant implications for the plant energy budget and therefore for plant performance and adaptation (Nunes-Nesi et al. 2010). During the day, shoots can use surplus light to assimilate NO_3^- and divert relatively little energy away from photosynthetic carbon assimilation and thus detract little from plant growth (McDermitt and Loomis 1981; Bloom et al. 1989). Under these conditions, NO_3^- assimilation will have lower energy cost in the leaf than in the root, because the reducing equivalents and ATP for NO_3^- assimilation are obtained without any decrease in the rate of CO_2 fixation. When light limits photosynthesis or during the night, however, no advantage will be gained in assimilating NO_3^- in the shoot, because NO_3^- assimilation and CO_2 fixation will directly compete for ATP and reductant generated by photosynthetic electron transport (Canvin and Atkins 1974), leading to a decrease in CO_2 fixation. Another disadvantage of leaf NO_3^- assimilation, independent of the light level, is that hydroxyl ions generated in the leaf during this process must be neutralized by the synthesis of organic acids (in the root, the pH balance may be maintained via decreased proton excretion or increased bicarbonate excretion) (Smirnoff and Stewart 1985). In vitro studies showed that day and night cycles do not influence NO_3^- reductase activities in roots, suggesting that rates of NO_3^- assimilation in the root are similar day and night (Stohr and Mack 2001). Still, the in vivo rates of root NO_3^- assimilation in comparison with shoot NO_3^- assimilation and the influence of light and dark cycle away its clarification. In addition, root NO_3^- assimilation will gain in both physiological and ecological importance because the elevated atmospheric CO_2 concentrations anticipated during the next few decades strongly inhibit shoot NO_3^- assimilation in C_3 plants (Bloom et al. 2010, 2012b).

Because leaf NR is a highly regulated enzyme (Lillo et al. 2004), NO_3^- assimilation is central for achieving cytosolic NO_3^- homeostasis (Cookson et al. 2005; Huang et al. 2012). Roots also maintain cytosolic NO_3^- homeostasis under deprivation and resupply of NO_3^- (Zhen et al. 1991; van der Leij et al. 1998), but the role of NO_3^- assimilation in keeping cytosolic NO_3^- homeostasis in the root has received less attention probably because the physiological and/or environmental events that lead to changes in root NR activity are less obvious.

12.4 Nitrate Use Efficiency

To achieve the doubling in global food production anticipated during the next 50 years will require a threefold increase in nitrogen fertilization rate (Frink et al. 1999). Nitrogen fertilizer will play a key role in this expansion and intensification of agriculture. Unfortunately, excess N compounds released from agricultural systems are detrimental to the environment, threatening the quality of air, water, and soil (Canfield et al. 2010). In addition, such releases are a waste of valuable resources and may cause human health problems. Today, intensification of agriculture must be done through nitrogen management strategies that do not compromise the environment (Matson et al. 1997; Godfray et al. 2010). To this end, improving the efficiency with which plants obtain nitrogen from the environment is of critical importance (Xu et al. 2012), and nitrogen use efficiency (NUE) of individual plants plays a key role. NUE has two components, nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE) (Epstein and Bloom 2005). Roots are the key plant organ for improvement of both NUpE and NUtE (Xu et al. 2012) because they determined NO_3^- uptake and have a critical role in NO_3^- assimilation and transport to other parts of the plant. Root traits can be selected using traditional breeding and marker-assisted selection (Good et al. 2004). Natural variation of NUE in genetic resources can help to select root traits (Chardon et al. 2010). Some important root traits seem to be controlled by a single dominant gene (Werner et al. 2010); for example, overexpressing cytokinin-degrading cytokinin oxidase/dehydrogenase (*CKX*) genes resulted in an elongation of primary root and an increase in root branching and root biomass. NUpE can be improved by targeting root morphology, root-to-shoot ratios, and root NO_3^- transporters (Garnett et al. 2009; Werner et al. 2010). NUtE can be improved by targeting NO_3^- assimilation enzymes (Andrews et al. 2004) and mitochondria metabolism (Foyer et al. 2011). Nevertheless, a combination of traditional breeding and transgenic approaches will be needed to make significant improvements in NUE because of the multiple interacting genetic and environmental factors that govern NUE.

12.5 Conclusions

Nitrogen (N) is a major constituent of plant macromolecules, and nitrate (NO_3^-) is the predominant form of inorganic N available to higher plants in aerobic soils. The concentration of NO_3^- in soil solutions ranges from lower than 100 μM in natural ecosystems to higher than 10 mM in agricultural ecosystems. Nitrate, both from indigenous soil resources and from N inputs contributing to the plant-available soil N pool, varies greatly temporally and spatially. This N pool becomes the object of intense competition among plants and, in some environments, between plants and microorganisms. To cope with the heterogeneity of NO_3^- concentration in the soil,

to meet the energy requirements for its assimilation, and to secure a favorable carbon/nitrogen balance within the plant, plant roots have evolved diverse strategies for NO_3^- acquisition. These strategies extend from (i) the ecosystem level, where there are associations with some microorganisms to acquire NO_3^- and competition with others for NO_3^-; (ii) the organism level, where plants optimize the allocation of resources between roots and shoots and the pattern of root system branching; and (iii) the molecular level, where the location (root vs. shoot) and time (diurnal cycles) of NO_3^- uptake, transport, and assimilation must adapt to the prevailing environmental conditions. Understanding the diverse strategies that plant roots employ to convert external NO_3^- to organic N will provide valuable information that can be used to improve plant N utilization efficiency and thereby enhance the sustainability of agricultural production under changing climates.

As sessile organisms, plants have evolved developmental and metabolic patterns that acclimate to the prevailing environmental conditions. In particular, plant roots adopt different strategies to acquire NO_3^- from the soil:

- Some plant roots establish associations with diverse microorganisms to ensure NO_3^- accessibility and uptake.
- Flexibility, precision, and relative turnover times of roots are important characteristics in the competition with other plants and microbes for NO_3^-.
- Plant roots sense NO_3^- as a signal. Local (external NO_3^-) and long-range (internal N status) signaling pathways adjust root system architecture and resource allocation, respectively, to the physiological state of the plant and the distribution of NO_3^- in the environment.
- Plant roots can modify their capacity to acquire NO_3^- in a range of concentrations by modulating the expression and function of genes in different NO_3^- uptake systems.
- The transport of NO_3^- between the root and shoot will determine the partition of NO_3^- assimilation between root and shoot.
- Location and timing of NO_3^- assimilation will be adjusted according to the energy budget and C metabolism.
- Recent progress in the understanding of the molecular basis of the root responses to external supply of NO_3^- suggests that root responses are largely regulated by hormone homeostasis and signaling pathways.

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Chapter 13
Root Aquaporins

Thorsten Knipfer and Wieland Fricke

13.1 Introduction

As the title of chapter 'Root aquaporins' suggests, this chapter is very much about roots and aquaporins (AQPs) and how the two relate to each other. To comprehend the functioning of a root, we need to know the properties of AQPs. Vice versa, to comprehend the role which AQPs play in the physiology of roots, we must have an understanding of the organisation (anatomy) and development of root tissues and overall morphology of root system. Also, any function which AQPs play in roots must be compatible with other functions which roots fulfil, for example, mechanical anchorage, mineral nutrient acquisition and being the selectivity barrier between root environment (e.g. soil) and plant.

There exist numerous studies on root AQPs. It is beyond the scope of this review to refer to all these studies. Citing or not citing a particular study does not imply any judgement on the scientific quality or importance of that study. Rather, examples of studies are given to demonstrate an underlying principle, reflect trends in experimental results or highlight controversies about particular issues. Reviews about roots, their uptake of water and mineral nutrients, and about AQPs, their basic structure, properties and role in selected root functions, can be found in Steudle (2000), Martinez-Ballesta et al. (2006), Maurel (2007), Maurel et al. (2008, 2010) and Aroca et al. (2012).

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13.2 Roots

13.2.1 The Root System

Plants exhibit complex root systems with various root types fulfilling different functions. A typical root system as we consider it here for studying AQP function would be that of an annual crop such as barley (*Hordeum vulgare)*, wheat *(Triticum aestivum)* or maize *(Zea mays*). In these root systems, the bulk of root tissues is below-ground and consists of two major types of roots, seminal roots and adventitious roots (Fig. 13.1). Seminal (primary) roots appear early and adventitious (secondary) roots later during plant development (Esau 1965). Seminal roots are present as primordia in the embryo and start to develop right after germination (Esau 1965; Heimisch 1951). Adventitious roots arise from crown nodes of main shoot tillers, where each tiller may produce two or more adventitious roots (Esau 1965). Stem-born adventitious roots make up the secondary root system. When fully mature, they can establish the main vascular system. The number of adventitious roots in the root system of a plant depends mainly on the number of growing tillers. The contribution of the different types of root (seminal, adventitious) to the total root absorbing surface area of an annual plant changes as the plant develops. The anatomy, morphology and uptake properties of the types of roots may not be the same and can differ between species.

13.2.2 Root Development and Anatomy

13.2.2.1 Root Development

A mature root can be divided into three developmental regions, a (i) root tip, (ii) root hair and (iii) mature, lateral root region (Fig. 13.1; see also Esau 1965).

(i) The root tip region (immature zone) includes the root cap and the root elongation zone. The root cap protects the root apical meristem, containing cell-producing stem cells, and reduces the friction between the root and soil particles. The elongation zone is the region of maximum cell expansion and longitudinal root growth. Only protoxylem and protophloem elements are fully functional, with a relatively undeveloped endodermis (see below).

(ii) The root hair region (transition zone) is the region where root hairs start to develop from epidermis cells. Metaxylem and metaphloem elements are generally functional, and the endodermis is further developed (e.g. Casparian bands). This region is considered to be the region of maximum water and solute uptake of the main root axis, yet this need not be the case as shown in some studies (e.g. for barley, see Sanderson 1983).

(iii) The lateral root region (mature zone) is the region where lateral roots emerge from the pericycle of the main root axis. The endodermis is fully mature

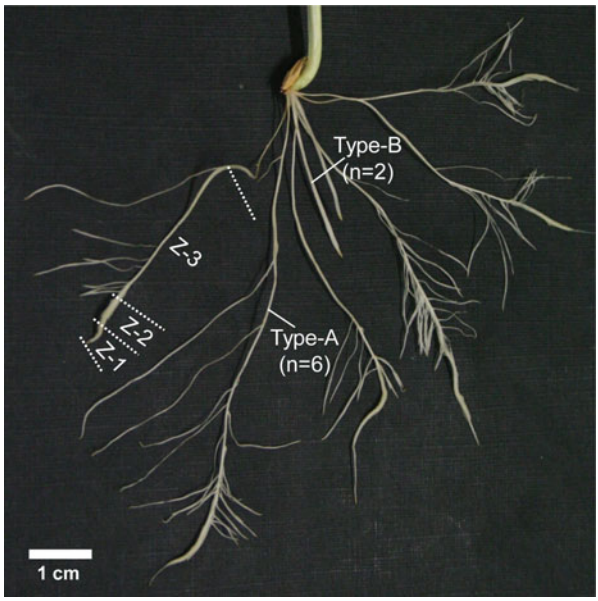

Fig. 13.1 Seminal (type A) and adventitious (type B) roots in 14-day-old barley plants. The plant shown had six seminal and two adventitious roots. Root developmental regions (Z1, Z2, Z3) are shown along a seminal root. Z1, meristematic elongation zone in the tip region; Z2, root hair zone, where root hairs appear as 'brush-like' thickening along the main axis of root; Z3, lateral root zone, which starts distal (above) the root hair zone and encompasses the area where lateral roots emerge along the main axis of root

(suberin depositions, secondary wall thickening). Branching patterns of lateral roots and the length of the main root axis are strongly influenced by water and nutrient availability in the rooting medium (e.g. Hacket and Bartlett 1971).

13.2.2.2 Root Anatomy

The root cylinder consists of different tissues. These tissues play different roles in water and nutrient transport (Esau 1965) (Fig. 13.2; shown representatively for barley).

(i) The root epidermis, or 'rhizodermis', is the outermost cell layer, which is in direct contact with the root medium. Root hairs can be formed. These increase root surface area and accessibility to water and nutrients. When roots of some species are exposed to relatively dry rooting environments, e.g. *Z. mays* grown in aeroponics or vermiculite (Hachez et al. 2006; Hose et al. 2000), or to anoxia during flooding, e.g. *Oryza sativa* or *Iris germanica* (Meyer et al. 2009; Ranathunge et al. 2003; Kotula et al. 2009), a so-called exodermis can be formed from the epidermis and underlying layer of cells (Enstone et al. 2003). The exodermis is thought to have a chemical composition similar to that of the endodermis (see below) and play a significant role in ion uptake as osmotic transport barrier (Steudle and Peterson 1998; Hose et al. 2001).

(ii) The cortex occupies the largest cross-sectional area of roots. It consists of several layers of parenchyma cells arranged in series. Intercellular air spaces are usually found between adjacent cells to prevent oxygen deficiency (Esau

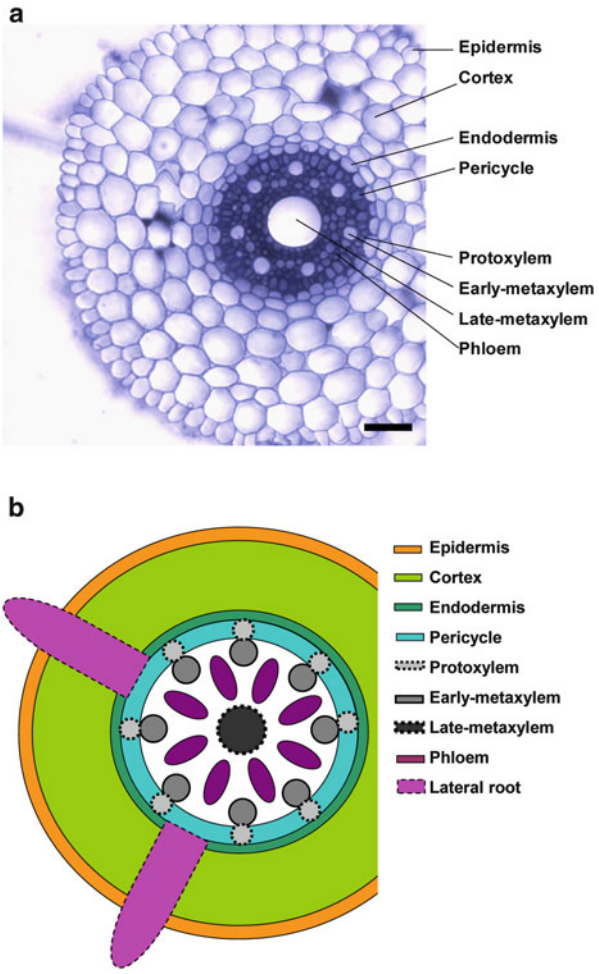

Fig. 13.2 (**a**) Anatomy of a barley (*Hordeum vulgare*) seminal root, with (**b**) main tissues highlighted in a scheme. (**a**) The Freehand cross section of a seminal root of barley (age 14 days) was taken from the mature region close to the root base where lateral roots emerge. The section was stained for 1 min with 0.5 % toluidine blue and rinsed with water before being viewed under bright light with a microscope (LEICA DM IL, Wetzlar, Germany). Scale bar 50 µm

1965). Severe anoxia of the root medium can cause formations of additional air spaces, aerenchyma, for example, in rice roots (Suralta and Yamauchi 2008).

(iii) The endodermis is, based on its ontogeny, the innermost layer of the root cortex (Bonnett 1968). The endodermis separates the outlying root cortex tissue from the central stelar compartment containing the root vascular system. Depending on the root developmental state, the endodermis goes through multiple developmental states (I–III). These are associated with significant changes in the chemical composition and, presumably, barrier properties of its cell walls (Enstone et al. 2003; Enstone and Peterson 2005). State I (primary) endodermis has Casparian strips within endodermal walls, with passage cells opposite of protoxylem pools. State II (secondary) endodermis has suberin depositions (lamella) particularly in the inner tangential walls. State III

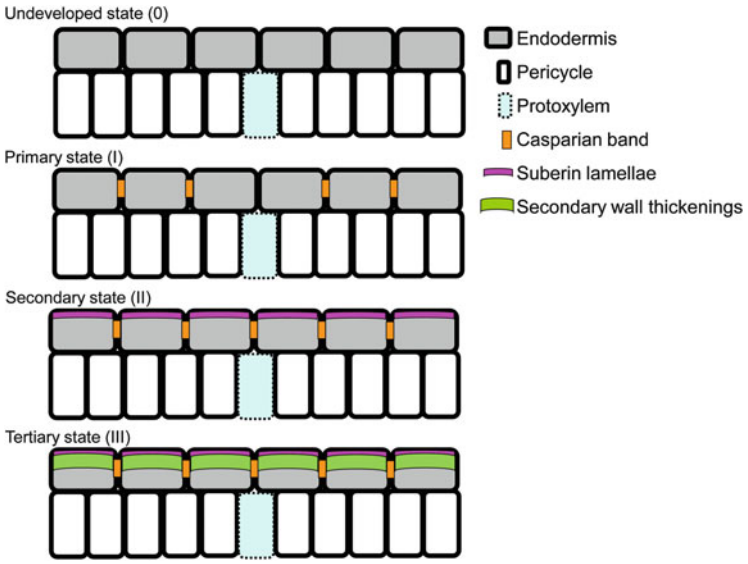

Fig. 13.3 Scheme summarising the various developmental stages of the root endodermis. In the undeveloped state (0), the endodermis shows no distinct characteristics to distinguish it from other adjacent cells in the tissue. In the primary developmental state (I), Casparian bands are initially not forming opposite of protoxylem elements within the endodermal cell walls (apoplast) containing some lignin and suberin depositions. In the secondary state (II), a suberin lamella starts to form at the inner side of the primary tangential cell wall; suberin depositions are comparatively hydrophobic and can reduce the radial transport of water also from cell to cell across the endodermis. In the tertiary state (III), secondary wall thickenings are forming by additional wall depositions on the already existing suberin lamella

(tertiary) endodermis has additional depositions of lignin and suberin depositions on the already present inner tangential suberin depositions (secondary wall thickenings, see Fig. 13.3). Passage cells are thought to provide a low-resistance pathway for water across the endodermis (Peterson and Enstone 2006).

The endodermis is thought to be a protective cell layer for the central stelar compartment and to play a major role in controlling the transfer of water and nutrients into the stelar compartment (Peterson 1998; Steudle and Peterson 1998) and, ultimately, upper plant body. Not surprisingly, the endodermis is of particular interest when studying root AQP function.

Chemical analyses of isolated endodermal walls of monocot (e.g. *Z. mays*) and dicot species have shown that Casparian bands and suberin lamella can differ in the relative contents of suberin and lignin along roots (Zeier et al. 1999). Lignin and wax-free suberin polymers in their basic form are ineffective transport barriers (Schönherr 1982), unless additional waxes are deposited into the suberin polymer. In *Z. mays*, Casparian bands in the primary endodermis are strongly lignified and exhibit a low content of aliphatic suberin

(tenfold smaller as found in the more developed endodermis), together with high amounts of carbohydrates and proteins. In contrast, in the secondary endodermis, a drastic increase in aliphatic suberin can be detected parallel to a low content of lignin (Zeier et al. 1999). Endodermis-equivalent structures can also be found in leaves (Lersten 1997).

(iv) The pericycle is in direct contact with the endodermis. It is a meristematic cell layer that forms the outermost part of the stele. The development of lateral roots is initiated in this meristematic cell layer (Esau 1965).

(v) The xylem consists of water-conducting xylem elements surrounded by xylem parenchyma cells for transfer of water and nutrients, as shown for ion transfer in barley roots by Wegner and Raschke (1994). Xylem vessels are often arranged circular in the root stele. They can be divided into protoxylem, and early and late metaxylem, according to their maturity and function (Steudle and Peterson 1998). Only dead, highly lignified xylem elements which are not anymore vacuolated are fully mature having maximum axial conductance for water and nutrients (Frensch and Steudle 1989; Bramley et al. 2009). Species and types of roots can differ in the number, diameter and maturity of vessels, which can have some impact on their maximum axial transport capacity for water according to Hagen-Poiseuille's law (Sperry 2003). For example, in barley, seminal roots have one central late and seven to nine peripheral, early metaxylem vessels (compare Fig. 13.2a); adventitious roots have typically four central and 15 peripheral metaxylem vessels (Heimisch 1951).

(vi) The phloem consists of highly vacuolated sieve tube elements surrounded by phloem parenchyma and companion cells (Esau 1965). The phloem provides root tissue with shoot-derived compounds (photosynthate, amino acids, signalling molecules) and some retranslocated ions. The phloem is generally neglected when studying water transport in roots. Although this appears justified in terms of the direction and magnitude of volume flow in phloem compared with xylem, the signalling function of phloem should not be underestimated. This applies in particular to studies where excised roots (disconnected phloem shoot connections) are used to study water transport and AQP function.

13.2.2.3 Transport Paths in Roots

Plants appear in all shapes and sizes, yet in physical terms, plants are variable hydraulic conductors which use a naturally occurring gradient in energy content of water (water potential) between root environment (soil, hydroponics) and shoot environment (atmosphere) to drive the uptake of water and dissolved mineral nutrients (Kramer 1932; Pittermann 2010). Hydraulic resistances as they occur at the root and shoot level can limit the flow of water through the plant, analogue to Ohm's law (van den Honert 1948; Landsberg and Fowkes 1978; Frensch 1997; compare also Knipfer and Fricke 2010, 2011).The main hydraulic barrier to water uptake by roots is the radial transport path, between root epidermis and stele,

compared with the axial path along xylem conduits (Frensch and Steudle 1989; Steudle and Peterson 1998, Fig. 13.4). This applies at least to fully mature root regions, where metaxylem vessels have reached their final form and function. At the very tip of the main axis of root and lateral roots, such a situation may not hold any more and axial transport becomes limiting or co-limiting (Frensch 1997). According to the composite model of water transport (Steudle et al. 1993; Steudle and Peterson 1998; Steudle 2000), the radial resistance to water flow can be divided into an apoplastic (cell wall, middle lamella and intercellular air space) and a cell-to-cell (through plasmodesmata and across membranes) component, the latter involving AQPs. Hydraulic resistances and driving forces are thought to differ between apoplast (low-resistance, hydrostatic pressure gradients) and cell-to-cell path (high-resistance, osmotic gradients). However, the (conducting) cross-sectional area perpendicular to the direction of flow is much larger in the cell to cell compared with apoplastic path. Also, the driving force for radial movement of water is ultimately a difference in water potential between root medium and xylem. Both, hydrostatic and osmotic gradients drive water movement through their effect on the water potential gradient.

The contribution to root water uptake of water flow through root AQPs depends on factors which are not necessarily directly related to AQP function. For example, the mechanisms of water uptake may differ between types of roots, and these can make a variable contribution to the entire root system during plant development (e.g. see Figs. 13.1 and 13.4). In addition, the force driving water uptake differs between day (mainly hydrostatic gradient—xylem tension) and night (mainly osmotic gradient—active solute loading into xylem) and, therefore, also the main path of water uptake (Steudle and Jeschke 1983; Steudle and Frensch 1996; Steudle and Peterson 1998). A hydrostatic gradient can move water and dissolved solutes along the apoplast by the mode of 'bulk flow' (as opposed to diffusion) only if the flow path permits this type of movement. However, the interfibrillar and matrix spaces in the wall are in such a low (nm) range that the existence of bulk flow-driven water movement across the apoplast as postulated by the composite model has to be questioned. This applies in particular to any purely apoplastic path bypassing the endodermis [for a recent discussion, see Fritz et al. (2010), Fritz and Ehwald (2011), Knipfer and Fricke (2010), Knipfer et al. (2011)].

13.2.2.4 Conclusions

Roots and root systems are highly heterogeneous structures in time and space. This heterogeneity has to be taken into account in studies on root AQP function. All too often is the existence of different types of roots, different developmental regions along roots and differences in the maturation of key diffusion barriers (exodermis, endodermis) not considered (compare Figs. 13.1 and 13.4). Before we embark on any detailed studies into the role of candidate AQPs in water and mineral nutrient uptake by roots, we should first (i) quantify the contribution which different root developmental regions make to the overall uptake of water/mineral nutrient,

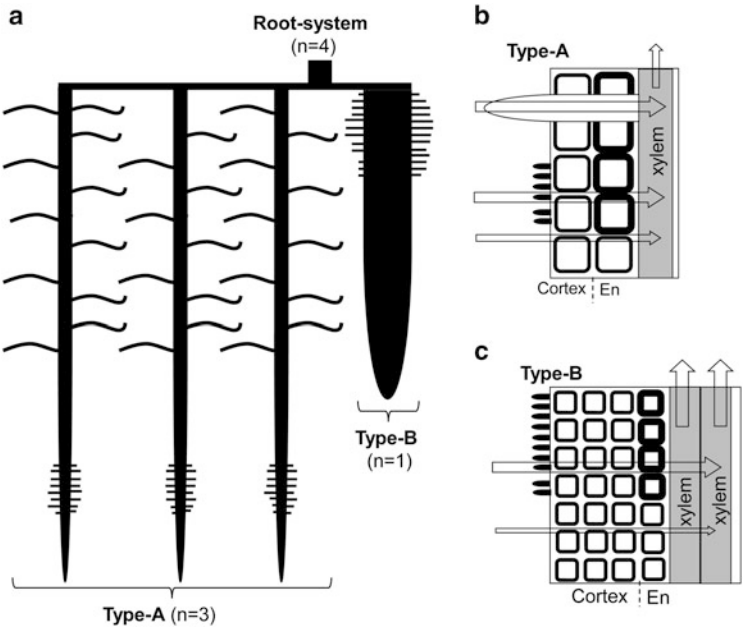

Fig. 13.4 Two types of roots, which are at different developmental stages, within the root system of a plant. (**a**) Root type A (e.g. seminal roots in 2- to 3-week-old barley plants) is represented by fully mature roots, which can be divided into tip, root hair and lateral root region. Root type B (e.g. adventitious roots in 2- to 3-week-old barley plants) is immature compared with type A, lacking a lateral root region (compare Fig. 13.1). (**b**, **c**) Besides differences in developmental states of roots, number of roots and surface area, root types can differ in their basic anatomy. This can affect the contribution of roots to plant water uptake. *En* endodermis

(ii) extent this approach to different types of roots present (e.g. Bramley et al. 2009; for barley, see Knipfer and Fricke 2010, 2011; Knipfer et al. 2011) and, finally, (iii) carry out analyses over a longer developmental period.

13.3 Aquaporins

13.3.1 Phylogeny and AQP Subfamilies

Aquaporins belong to the family of major intrinsic proteins (MIPs). They occur in all eukaryotic organismal kingdoms. In prokaryotes, the closely related aquaglyceroporins are found (Schäffner 1998; Maurel et al. 2008; Kjellbom et al. 1999). The highest diversity of AQP isoforms and substrates transported by AQPs is found in plants [for review, see Adrianus and Borstlap (2002), Maurel et al. (2008) and Bienert et al. (2011)]. This relates most likely to the sessile

evolutionary life strategy of plants. For example, compared to the 13 AQPs in humans, there exist between 33 and 37 different AQPs isoforms in *Arabidopsis thaliana*, rice (*O. sativa*), maize (corn, *Z. mays*), wheat (*T. aestivum*) and poplar (Johanson et al. 2001; Sakurai et al. 2005; Chaumont et al. 2001; Kerrie et al. 2007). In barley, 25 different AQPs have been identified so far (Besse et al. 2011). The highest number of AQP isoforms (71) has been reported for upland cotton (*Gossypium hirsutum*; Park et al. 2010).

Plant AQPs group into five major groups: plasma membrane intrinsic proteins (PIPs); tonoplast intrinsic proteins (TIPs); Nod26-like proteins (NIPs); small, basic intrinsic proteins (SIPs); and the comparatively recently classified X-intrinsic proteins (XIPs) (Johanson et al. 2001; Danielson and Johanson 2008). The latter group has only been found in dicotyledonous plants and appears to be absent from monocotyledonous plants. Based on sequence homologies, AQPs can be divided further into subgroups, in particular PIPs into a PIP1 and PIP2 subfamily.

Aquaporins are multifunctional membrane channels, but they are best known for their ability to facilitate the diffusion of water across membranes (Tyerman et al. 1999; Maurel 2007; Maurel et al. 2008). Water channel activity is displayed in particular by members of the PIP2 subgroup of PIPs and TIPs. Although members of the PIP1 subfamily show poor water channel activity when tested on their own in heterologous expression systems such as yeast (spheroplasts) and oocytes of the toad *Xenopus laevis*, they do increase water channel activity when co-expressed with PIP2s (Fetter et al. 2004; Otto et al. 2010). PIPs and TIPs are the most abundant AQPs in the plasma membrane and vacuolar membrane (tonoplast), respectively (Chaumont et al. 2001; Johanson et al. 2001; Maurel et al. 1997, 2008)—those membranes that are of particular interest when studying the uptake and transport of water and mineral nutrients across plant roots. In addition, AQPs have been reported to be present in mitochondria (e.g. Lee and Thévenod 2006). While SIPs have been localised to the endoplasmic reticulum (Maeshima and Ishikawa 2007), XIPs are also present in the plasma membrane where they facilitate the diffusion of uncharged substrates (Bienert et al. 2011). The subcellular localisation of NIPs seems to be the most diverse among plant AQPs. There are reports pointing to localisation in endoplasmic reticulum (*Arabidopsis* AtNIP2;1; Mizutani et al. 2006) but also tonoplast and plasma membrane [for review, see Maurel (2007)].

13.3.2 AQP Molecular Structure and Selectivity

Aquaporins have a highly conserved structure (Yoshinori Fujiyoshi et al. 2002; Törnroth-Horsefield et al. 2006). The molecular weight ranges from 23 to 31 kDa (243–302 amino acid residues long), and the AQP molecule, being an integral membrane protein, contains six membrane-spanning α-helices linked by two intracytoplasmic and three extracytoplasmic loops. The N- and C-terminus of AQP are located on the cytoplasmic side. The two hydrophobic loops, B and E,

are forming the central pore by dipping into the membrane from opposite sides, both having a conserved Asn-Pro-Ala (NPA) motif. This motif is a hallmark of AQPs, in particular PIPs. Each AQP monomer forms a conducting channel, yet several studies suggest that these channels only become active as AQPs assemble as homo- or heterotetramers in the membrane (Daniels et al. 1999; Törnroth-Horsefield et al. 2006; Maurel et al. 2008). Water passes as a single file of water molecules through the centre of the pore of each AQP monomer.

Substrate specificity is determined by the NPA motif at the pore constriction site and the aromatic/arginine (ar/R) selectivity filter (Murata et al. 2000). All AQP families have members with proven water channel activity as tested through functional expression and swelling assays in yeast or *Xenopus* oocytes (Maurel 2007; Maurel et al. 2008). These assays have also shown that AQPs can transport molecules other than water, in particular small neutral solutes such as glycerol, urea, formamide, acetamide, methylammonium, boric acid, silicic acid and lactic acid (Biela et al. 1999; Gerbeau et al. 2002; Holm et al. 2005; Choi and Roberts 2007; Takano et al. 2006). Furthermore, depending on their concentration, NH_3, NH_4^+, CO_2 and H_2O_2 can be transported (Bienert et al. 2007; Holm et al. 2005; Liu et al. 2003; Uehlein et al. 2008). The ability to transport these 'non-water' compounds is observed most among the NIPs, TIPs, XIPs and PIP1 subfamily of PIPs.

13.3.3 AQP Regulation and Gating

Aquaporins can be gated, that means they can be in an open (conducting) and closed (non-conducting) state. The activity of AQPs and their gating can be regulated through a range of mechanisms and in response to external factors. Water channel activity (open state) can be increased through phosphorylation and decreased through dephosphorylation (e.g. Johansson et al. 1996, 1998; Guenther et al. 2003; Wei et al. 2007). Aquaporin activity can also be regulated through intracellular pH and acidification (Gerbeau et al. 2002). Protonation of conserved His residues of loop D can result in a conformational change and closure of the pore in PIPs (Tournaire-Roux et al. 2003). As intracellular pH decreases in response to anoxia, pH-dependent regulation provides an important means for roots to adjust water uptake to changes in root medium oxygen partial pressure (Tournaire-Roux et al. 2003).

Some AQPs can facilitate the diffusion of H_2O_2, a signalling molecule in plants (Hung et al. 2005), across membranes (Bienert et al. 2007). Hydrogen peroxide may also affect AQP activity through an oxidative gating mechanism, which results in conformational changes of the pore (Henzler et al. 2004; Ye and Steudle 2006; Fenton reaction: $Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + {}^*OH$). Boursiac et al. (2008) demonstrated that H_2O_2 can act as an intermediate in regulating root water transport in *A. thaliana* roots, causing an internalisation of AQPs from root cell membranes. At low concentrations (0.25–5 mM) and incubation times of 5 h, H_2O_2 acts also as signalling molecule for AQP expression in roots of *Phaseolus vulgaris* resulting in

an increase in root hydraulic conductivity (Benabdellah et al. 2009). At high concentrations (>20 mM) of H_2O_2, cell hydraulic conductivity of *Chara corallina* decreased by 90 % (Henzler et al. 2004), which has also been observed for cells of corn roots besides a reduction in overall root hydraulic conductivity (Ye and Steudle 2006). In general, during short periods of H_2O_2 treatments of around 0.5–2 h, root hydraulic conductivity decreased (Martinez-Ballesta et al. 2006; Boursiac et al. 2008; Ye and Steudle 2006).

The most commonly used experimental inhibitor of AQP function is mercuric chloride ($HgCl_2$) (Preston et al. 1993; Henzler and Steudle 1995; Tazawa et al. 1997; Katsuhara et al. 2002; Hukin et al. 2002). It is typically applied at a concentration range of 50–100 μM over a period of 10–30 min prior to or during water channel activity measurements. For example, in the giant algal cells of *Chara corallina*, hydraulic conductivity was reduced by 75 % in response to treatment with 50 μM $HgCl_2$ (Henzler and Steudle 1995). In barley roots *trans*-osmosis was reduced by 80 % after treatment with 100 μM $HgCl_2$ (Tazawa et al. 1997). Mercuric chloride is far from being an ideal inhibitor due to the general cytotoxicity of Hg. However, $HgCl_2$ is the best inhibitor for AQPs that is currently available, and there are only few reports on AQP isoforms which are not inhibited by Hg (e.g. Daniels et al. 1994). Although AQP inhibitors such as H_2O_2 (see above), phloretin, silver (Ag^+ as $AgNO_3$) and gold (as $HAuCl_4$) have been used in the study of plant AQPs, their applicability appears to be limited (Javot and Maurel 2003; Niemietz and Tyerman 2002; Maurel et al. 2008; Dordas et al. 2000; Moshelion et al. 2002; Volkov et al. 2007). Externally applied hydrostatic and osmotic pressure pulses (0.1–0.2 MPa) have also been shown to decrease hydraulic conductivity in root cells, and a reversible inhibition of AQP activity based on conformational changes has been proposed (Wan et al. 2004). An osmotically induced gating of AQPs by a cohesion/tension mechanism of non-permeating solutes has also been suggested. Accordingly, these could create a tension in the pore region (NPA motif) and result in the subsequent closure of pore (Ye et al. 2004).

Taken together, the general lack of AQP-specific, nontoxic or easy-to-apply inhibitor (in plants and animals) has precluded any longer-term studies into the role of AQPs, for example, in root water uptake, which are based on the application of inhibitors. Such studies require other means through which AQP function can be modulated. Anoxia could be one such means, yet anoxia leads to a general decrease in cytosolic pH and this feeds back on the entire metabolism of cell and function of organ, including effects on cell expansion through changes in wall pH. An alternative approach is to study plants which have altered expression and protein levels of individual AQP isoforms. Expression levels can be altered through targeted experimental manipulation (e.g. over-expression, knockout (KO) mutants or antisense lines of particular AQP isoforms) or by selecting cultivars which have intrinsically altered expression levels of a particular AQP isoform. Great care, though, should be taken in the interpretation of results in these studies since plants show a high degree of plasticity and ability to compensate. For example, reduced expression of a key AQP with water channel activity may lead to compensatory changes in root surface

area, number of cortex cell layers or contribution of fine roots to total surface area. An absence of change in root water uptake does not necessarily rule out a water transport function of the AQP isoform of interest. Also, only those transformants can be studied which are viable. Viability can merely reflect a successful compensation of otherwise lethal consequences of transformation event.

13.4 Root AQPs

13.4.1 Root-Specific AQP Expression

There exist several reports in which the gene expression of the majority of AQP isoforms of a particular plant species has been compared between root and shoot. These studies show two tendencies. First, if there are AQPs which are almost exclusively expressed in either root or shoot tissue, then these are root AQPs. Second, compared with shoots, roots show a higher total expression of AQPs. For example, relatively (compared with shoot tissue) high or exclusive expression of AQPs in roots was observed for *O. sativa* (OsPIP2;3, OsPIP2;5, OsTIP2;1, Sakurai et al. 2005), *Z. mays* (ZmPIP2;1, ZmPIP2;5, ZmPIP1;5, Hachez et al. 2006), *A. thaliana* (AtPIP2;2, AtPIP1;1, AtPIP1;2, AtPIP2;1, AtTIP1;1, AtTIP1;2, Javot et al. 2003; Alexandersson et al. 2005), *Vitis vinifera* (VvPIP1;1, VvPIP2;2; Vandeleur et al. 2009), *H. vulgare* (HvPIP2;1, HvPIP2;2, HvPIP2;5; Katsuhara et al. 2002; Besse et al. 2011; Knipfer et al. 2011) and *Pisum sativum* (PsPIP2;1; Beaudette et al. 2007). Eighteen AQP genes were tested for differences in expression between leaf and root in barley plants (Knipfer et al. 2011). Four AQPs (*HvTIP2;1*, *HvTIP3;1*, *HvNIP1;1*, *HvNIP2;1*) showed very low or no detectable expression. One AQP (*HvPIP1;2*) was expressed almost exclusively in root tissue. The remaining 13 AQPs were expressed in leaf and root. Seven AQPs showed a statistically significant difference in expression between root and leaf. In all cases expression was higher in roots (Knipfer et al. 2011). Similarly, Alexandersson et al. (2005) observed for *Arabidopsis* that no MIP family member was expressed exclusively in leaf tissue and that expression of MIPs, in particular PIPs, was generally higher in root tissue (see also Jang et al. 2004). These findings are by no means evidence of a water or mineral nutrient transporting function of the respective AQP. However, they do infer that these or other root-specific functions are associated with a higher or exclusive expression of AQP isoforms in roots. In addition, AQP expression can vary along the main axis of roots, as demonstrated by Hachez et al. (2006) for maize (e.g. from tip to base ZmPIP1;5 and ZmPIP2;5 increased in expression, whereas ZmPIP2;1 decreased). Recently, Knipfer et al. (2011), studying a selected range of AQPs, showed for barley that expression of AQP isoforms differs between the main axis of seminal roots and their emerging lateral roots and between seminal and adventitious roots. *HvPIP2;2* and *HvTIP2;3* showed significant differences in expression between root zones, including lateral

roots. *HvTIP1;1* and *HvTIP2;3* were expressed lowest in the fully mature zone. Expression in the transition zone and lateral roots was 3–14 times higher. In comparison, the abundantly expressed *HvPIP2;5* was expressed evenly along seminal roots.

13.4.2 Tissue Localisation of Root AQPs

Tissue localisation of AQPs has been studied using mainly in situ hybridisation, which detects mRNA expression levels. In some cases, immmunocytochemical approaches have been used to detect the actual AQP protein (e.g. Hachez et al. 2006; Sakurai et al. 2005, 2008; Vandeleur et al. 2009). The most complete studies so far exist for rice (Sakurai et al. 2005, 2008), grapevine (*V. vinifera*, Vandeleur et al. 2009), maize (Hachez et al. 2006, 2012) and barley (Knipfer et al. 2011). For example, it was shown for maize that ZmPIP2;1 and ZmPIP1;2 protein were most abundant in stelar cells at the root tip; in more mature root regions, ZmPIP2;1 and ZmPIP1;2 were preferentially localised to the cortex and epidermis. ZmPIP2;5 was in particular found in cortex tissue at the root tip and in the endodermis in mature root tissue (Hachez et al. 2006). In rice, Sakurai et al. (2008) showed that OsPIP1s, OsPIP2;1, OsPIP2;3 and OsPIP2;5 were localised preferentially in the endodermis at the root tip. In other root regions, OsPIP2s were distributed rather evenly between tissues; OsTIP2;1 and OsTIP2;2 were localised preferentially in stelar cells. In grapevine, VvPIP1s and VvPIP2s were localised evenly in cortex tissue and vascular tissue at the root tip, but showed lower signals in the cortex of mature root regions (Vandeleur et al. 2009). In barley, tissue localisation of six AQP isoforms was tested (*HvPIP2;2*, *HvPIP2;5*, *HvPIP2;7*, *HvPIP1;2*, *HvTIP1;1*, *HvTIP2;3*) (Knipfer et al. 2011). There was generally high expression of AQP genes in the epidermis and protoxylem (Fig. 13.5). Expression in cortex tissue was evident in all root developmental zones and in both, seminal and adventitious roots. Expression in the endodermis and stele was observed particularly in adventitious roots, highlighting a potential role in regulating radial water transport. Of all barley AQPs tested, *HvTIP1;1* was the most ubiquitously expressed gene, while *HvPIP2;5* was expressed particularly in cortex tissue.

The percentage contribution of AQP isoform expression to total PIP/PIP2 expression can provide some information about possible pairs of PIP1s/PIP2s forming heterotetramers. Those heterotetramers can have a water-conducting channel activity that exceeds that of homotetramers of the respective PIP2 isoform (Fetter et al. 2004). Together with information about the contribution of different tissues to bulk root tissue volume, predictions can be made about the tissue localisation of PIP1/PIP2 pairs. For example, those PIPs which contribute 40 % or more to bulk tissue expression are likely to be localised in the cortex, making up the bulk of tissue volume in roots of many species. Of course, one cannot exclude the possibility that some PIPs, or AQPs in general, are expressed at a much higher

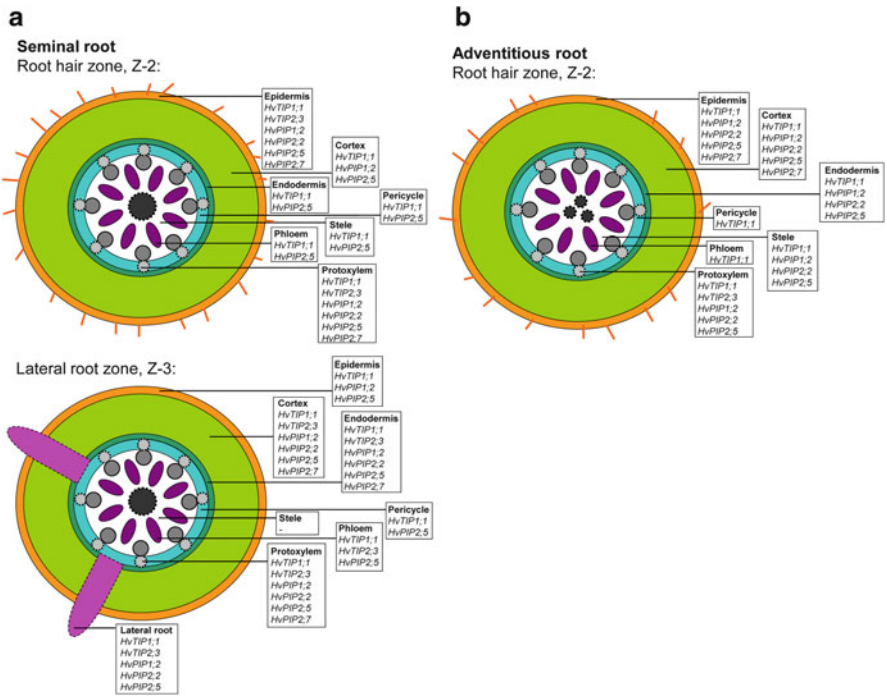

Fig. 13.5 Scheme of tissue distribution of gene expression of seven AQP isoforms in (**a**) seminal and (**b**) adventitious roots of barley. Expression was analysed by in situ hybridisation (Knipfer et al. 2011; Besse et al. 2011)

copy number per unit cell volume and plasma membrane surface area than other PIPs. The percentage contribution of PIP1/PIP2 isoform expression to total PIP1/PIP2 expression was determined here for barley, wheat, rice and *Arabidopsis* using published data of gene expression (Fig. 13.6). These analyses gave species-specific patterns, yet there were generally between 2 and 3 PIP isoforms that showed higher expression (Fig. 13.6). These included at least one PIP1 and PIP2, except for rice (Fig. 13.6). Those 'pairs' of PIP1 and PIP2 isoforms may form water-conducting heterotetramers in situ. The most abundantly expressed PIPs were *ZmPIP1;1* (45 %) and *ZmPIP2;5* (46 %) in maize, *AtPIP1;1* (50 %) and *AtPIP2;2* (52 %) in *Arabidopsis* and *HvPIP1;3* (62 %) and *HvPIP2;5* (64 %) in barley. In contrast, in rice, each PIP1 isoform contributed between 30 and 40 % to total expression of PIP1s, and each PIP2 isoform contributed between 10 and 20 % to total expression of PIP2s (Fig. 13.6).

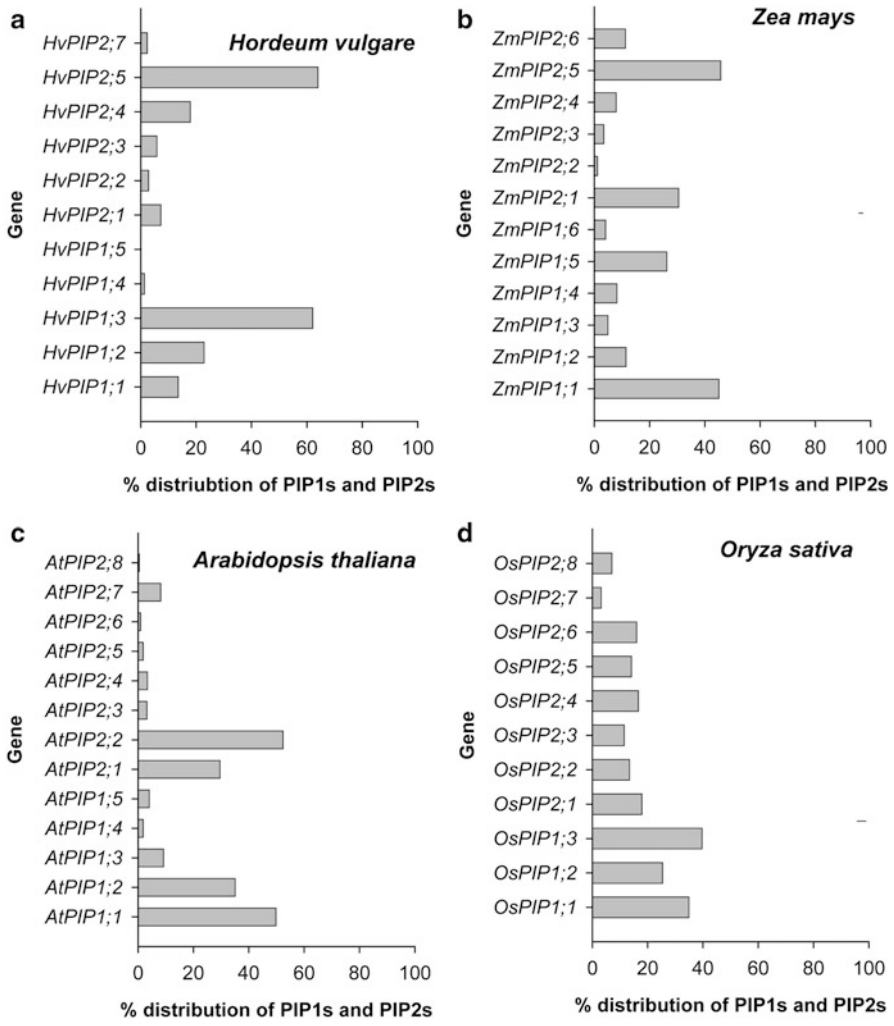

Fig. 13.6 Percent contribution of expression of PIP1 and PIP2 isoforms to the total expression of PIP1s and PIP2s in roots of four plant species. The percentage distribution of PIP1s and PIP2s was determined using data published for barley (*Hordeum vulgare*; Knipfer et al. 2011), maize (*Zea mays*; Hachez et al. 2006, taken from Fig. 1), *Arabidopsis* (*Arabidopsis thaliana*, Alexandersson et al. 2005, taken from Fig. 3A) and rice (*Oryza sativa*; Sakurai et al. 2005 taken from Fig. 2)

13.4.3 Comparing Particular Root AQP Isoforms Between Plant Species

When studying the function of a particular AQP in a particular plant species, one would like to compare their 'own' results with results obtained for that AQP in

other plant species. Which AQP isoform in species A corresponds to which isoform in species B?

The nomenclature of plant AQPs is based on their sequence homology to the respective *Arabidopsis* AQP (Johanson et al. 2001). For example, of all barley PIPs, HvPIP1;1 shows the highest homology to *Arabidopsis* AtPIP1;1. Similarly, of all maize AQPs, ZmPIP1;1 shows highest homology to AtPIP1;1. However, this does not mean that ZmPIP1;1 is among all maize PIPs that isoform which has the highest homology to barley HvPIP1;1. To test this relationship requires direct sequence comparison and phylogenetic analysis between barley and maize PIPs. This should be remembered when relating AQP isoform-specific data obtained for one species to data obtained for another species.

13.4.4 Effects of Environmental and Soil Conditions on AQP Expression

Changes in environmental conditions form part of the normal development of plants. The majority of such changes are gradual and longer term. 'Time' is a key factor in the stress response of plants. This should be remembered when drawing conclusions on AQP function from studies where shorter-term changes in root or shoot environment were imposed. Some general tendencies in changes in AQP expression can be observed. Firstly, the expression response is AQP isoform specific, that means there does not exist a general up- or downregulation in expression (e.g. Boursiac et al. 2005; Kawasaki et al. 2001; Aroca et al. 2003; Maathuis et al. 2003; Sakurai et al. 2005). Second, those treatments which lead to a lowered shorter-term availability of water in the root environment cause more AQPs to decrease than to increase in expression. Third, the proposed function or tissue localisation of an AQP isoform cannot be used to predict changes in expression of that isoform in response to, e.g. stress, without having information on accompanying changes in protein level. The protein level can, but does not have to, reflect changes in mRNA expression level, as recently demonstrated in the most comprehensive analysis so far, in maize (Hachez et al. 2012).

(i) Salinity. A downregulation of AQP expression in roots due to salt stress has been reported for, e.g. maize, rice and *Arabidopsis*. Decreases in expression were accompanied by decreases in root hydraulic conductivity (Kawasaki et al. 2001; Boursiac et al. 2005; Zhu et al. 2005).

(ii) Drought. A downregulation of AQP expression in response to drought stress for most of the tested AQPs was observed by, e.g. Alexanderssson et al. (2005) in *A. thaliana*. One AQP (AtPIP2;5) was upregulated in relatively immature root tissues. North et al. (2004) could relate changes in root hydraulic conductivity to AQP function in *Agave deserti* in response to changes in soil water content.

(iii) Temperature. Cold stress resulted in a downregulation of AQP expression in roots of, e.g. rice and *Arabidopsis*. This was paralleled by a decrease in root hydraulic conductivity (Jang et al. 2004; Sakurai et al. 2005). In rice, a subsequent increase in temperature resulted in an upregulation in AQP expression (Sakurai et al. 2005).

(iv) Diurnal changes. Diurnal changes in AQP expression have been reported for roots of, e.g. *Lotus japonicus* (Henzler et al. 1999), *Z. mays* (Lopez et al. 2003; Hachez et al. 2012) and *P. sativum* (Beaudette et al. 2007). Changes in expression were in the same direction (higher, lower) as accompanying changes in root hydraulic conductivity (Henzler et al. 1999; Lopez et al. 2003; Hachez et al. 2012). Diurnal up- and downregulations of HvPIP2;1 at the transcript and protein level in barley roots were linked to up- and downregulation of root hydraulic conductivity (Katsuhara et al. 2002). Diurnal changes in AQP expression have also been observed for shoot tissue, for example, in *Samanea saman* (Moshelion et al. 2002), *Z. mays* (Hachez et al. 2008; Kim and Steudle 2007; change in light intensity) and *Helianthus annuus* (Sarda et al. 2007).

13.4.5 Root AQPs Regulating Root Water Uptake and Whole-Plant Water Flow

13.4.5.1 Radial Water Uptake Across Roots

The main transport paths of water and minerals across roots are the apoplastic and cell-to-cell path. The cell-to-cell path consists of a symplastic path, through plasmodesmata, and a transmembrane path, either by simple diffusion through the lipid bilayer or facilitated diffusion through AQPs (for reviews, see Steudle and Peterson 1998; Steudle 2000). It is only in the latter case that AQPs can actually contribute significantly to water transport across the root cylinder. Therefore, prior to embarking on a detailed study of AQP localisation, expression and protein levels, one should first assess the contribution of an AQP-facilitated path to the total radial transport of water (or mineral nutrient). There exist several approaches to test this. All have their advantages and limitations. First of all, there does not exist an experimental approach through which we can distinguish currently between transport through plasmodesmata (symplast) and transport through membranes (transmembrane path; e.g. Hukin et al. 2002). Pioneering studies on leaf trichomes show that plasmodesmata can be pressure gated (Oparka and Prior 1992). The small channel (desmotubule) diameter of plasmodesmata makes laminar, hydrostatic pressure-driven bulk flow of water unlikely. Instead, transport of water and mineral nutrients will occur by diffusion or be facilitated through cytoplasmic streaming.

According to the composite model of water transport across roots (Steudle and Peterson 1998; Steudle 2000), movement of water along the apoplastic path is driven by hydrostatic pressure gradients (Steudle and Peterson 1998; Steudle 2000).

Therefore, when hydrostatic pressure gradients are applied during experiments on isolated roots or entire root systems/plants, the hydraulic conductivity measured is thought to reflect the hydraulic properties of the apoplastic path (e.g. Bramley et al. 2007). However, tensions as they are expected to build up during transpiration (about −0.5 MPa or more negative; Schneider et al. 1997) cannot be applied experimentally on detached roots. Instead, a positive pressure is applied either to the entire root system, using Passioura-type pressure chambers (Passioura and Munns 1984) or a high-pressure flowmeter (HPFM; Tyree et al. 1995) or pressure pulses (up and down) are applied, also to individual roots using the root pressure probe (Steudle 1993; Melcher et al. 1998). The disadvantage of these methods is that they all apply either unphysiological conditions (high hydrostatic positive pressures in root environment, decapitated roots), cause water to move at artificially high-volume flow rates through the plant or apply pressures not just to xylem vessels but to the entire cross-sectional area of cut roots. This can lead to the flooding of apoplast, with associated anoxia and low-resistance transport path and an effective axial 'short cutting' of the supposedly rate-limiting root tissue (e.g. endodermis).

Root water uptake can also be measured through application of osmotic forces, for example, by measuring exudation from decapitated roots or analysing roots with the root pressure probe through osmotic experiments (e.g. Knipfer and Fricke 2010, 2011). This is supposed to reflect transport along the cell-to-cell path, including AQPs. While this approach avoids the application of experimentally caused changes in pressures and allows water to move across roots in an undisturbed way, it relies on a driving force which is operative mainly during the night, but not day. Also, the volume flow rate associated with exudation is about one order of magnitude lower than daytime transpiration rates.

Additional approaches to distinguish between apoplastic and cell-to-cell path are the application of tracer dyes and determination of 'perfectness' of root to function as osmotic barrier [which it should do the less, the more transport occurs along the non-selective apoplast; root reflection coefficient; see Steudle (2000), Knipfer and Fricke (2010)]. Tracer dyes are suited for the study of movement of those solutes, which have physicochemical properties similar to the ones of the dye. Tracer dyes are not suited to deduce on transport path for water (for review, see Canny 1995). The movement of the two can be uncoupled from each other.

13.4.5.2 Role of AQPs in Root Water Flow

The contribution of AQPs to regulation of root water flow has been assessed through two basic approaches. Root water uptake and the hydraulic properties of cells have been determined in (i) absence or presence of conditions which inhibit AQPs and (ii) plants which have had altered expression levels of a particular AQP isoform. Both approaches suggest that AQPs play a role in root water uptake and plant water balance. Studies which have employed inhibitory treatments and focused on the root level have been referred to previously (see Sect. 13.3.3).

Here, we will look at those studies which involved plants with altered AQP expression levels and where whole-plant water flow has been analysed in response to AQP inhibitors.

Knipfer et al. (2011) showed for barley that application of root AQP inhibitors to the root medium of transpiring plants significantly reduced whole-plant water flow and transpiration. Similarly, in maize (corn), changes in root hydraulic conductivity associated with AQP function and mediated by ABA could be related to changes in whole-plant water flow (Ehlert et al. 2009; Parent et al. 2009). A reduction in transpiration was also observed in aspen following treatment of roots with $HgCl_2$. The reduction in transpiration could be reversed through application of the reducing agent mercaptoethanol (Wan and Zwiazek 1999). Changes in root hydraulic conductivity and transpiration could not always be explained through changes in root AQP activity, for example, in a study on diurnal changes in root AQP expression and transpiration rates in pea (*P. sativum* L.) (Beaudette et al. 2007).

There exist a rapidly increasing number of studies in which the expression of particular PIP or TIP isoforms has been altered through over-expression, knockout or antisense constructs of the respective gene/mRNA. Many of these studies have been carried out on *Arabidopsis* and maize (*Z. mays*) and have provided conflicting evidence, partly confirming and partly not confirming a role in root water transport of the respective MIP (Kaldenhoff et al. 1998; Javot et al. 2003; Ma et al. 2004; Schüssler et al. 2008; Beebo et al. 2009; Postaire et al. 2010). For example, in tobacco, an NtAQP1 antisense mutant showed reduced root hydraulic conductivity and plant transpirational water loss (Siefritz et al. 2002). Part of the discrepancy between these studies may be explained through the ability of plants to compensate. In particular, the complexity of root architecture and of alternative physiological means through which root water uptake is controlled (e.g. endodermis development (Schreiber et al. 1999) or root anatomy (Bramley et al. 2009)) has to be considered.

13.4.5.3 Root AQPs as Future Molecular Breeding Tools?

One important aspect that has been neglected so far in the study of root AQP is the possibility that manipulation of AQPs leads to an increase in root water uptake and plant yield under favourable growth conditions as part of 'normal' plant development. The question has to be asked: Do AQPs operate at their upper limit during normal plant development? Are AQPs suitable breeding tools to increase plant water uptake under favourable growth conditions?

To assess the hydraulic properties of roots, hydraulic conductivity (Lp, volume of water taken up per unit time, driving force and root surface; units: $m\ s^{-1}\ MPa^{-1}$) has to be measured. Instead, hydraulic conductance (L, volume of water taken up per unit time and driving force and root surface; units: $m^3\ s^{-1}\ MPa^{-1}$) is often measured. Hydraulic conductance 'L' does not provide information about the hydraulic property of a root, since L is a root surface-dependent size. For example, L can double through either doubling of root surface area or doubling of AQP activity. In contrast, a doubling of Lp means that the water uptake and transport

properties of root have doubled, possibly through a doubling of AQP activity. To highlight this point let us have a look at a barley plant during the first 4 weeks of development. The rate of shoot transpirational water loss ($m^3\ s^{-1}$) increases almost 15-fold during this period (Suku et al. 2014). The increase in transpirational water loss has to be matched by an equivalent increase in root water uptake. In the extreme case, this could be achieved through a 15-fold increase in root absorbing surface, at unchanged AQP activity per unit root surface, or a 15-fold increase in AQP activity per unit root surface (and plasma membrane) area of the water-conducting root tissues, at unchanged total root surface. There exist hardly any studies in which this question has been addressed. The most relevant study has been carried out by Fiscus and Markhart (1979) on bean (*P. vulgaris* L.) plants, during the pre-AQP area. These authors observed that root system Lp increased at the early stage of plant development and then remained at the same level, despite major increases in transpiration. In a recent study on barley, where root water uptake has been shown to occur along the cell-to-cell path involving AQPs (Knipfer and Fricke 2010; Knipfer et al. 2011), we obtained similar results, except that we did not observe the initial overshoot in Lp reported for bean (Suku et al. 2014). Early developmental increases in root Lp in 1- to 2-week-old barley plants were of the order 50 %, yet transpiration rates increased overall (by end of week four) by almost 1,000 %. In comparison, modelling of root water uptake in maize resulted in a continuous decrease in root Lp during plant development (Doussan et al. 1998), whereby this decrease was due mainly to a decrease in the portion of root system which was active in water uptake, an assumption that, as the authors pointed out, was not supported by experimental data. Martre et al. (2001) observed parallel increases in root hydraulic conductance (not, Lp) and leaf area during development of *Festuca arundinacea* plants. Root surface area will have increased too in that study, and it can only be speculated that resulting values of Lp will have increased less, or not at all, compared with values of hydraulic conductance. Rodriguez et al. (1997) observed no differences in root Lp between 3-day- and 9-day-old tomato plants. These data suggest that AQPs operate at near-maximum level early on during plant development. This makes AQPs unsuitable breeding tools to increase root water uptake and plant yield under optimum growth conditions.

13.5 Conclusions

Aquaporins belong to the family of major intrinsic proteins (MIPs). They are present in all organismal kingdoms and show the highest diversity in plants. Aquaporins were originally classified as ‘water channels’ due to their water transport function. However, in plants, aquaporins can also facilitate the diffusion of other small molecular weight molecules, for example, silicon, boron or urea. These additional transport functions, together with their known water transport capacity, have made aquaporins in recent years prime molecular targets for improving our understanding of the regulation of root function.

There can be no doubt that root AQPs are involved in facilitating the uptake of water and its transport across the root cylinder. Also, root AQPs have been shown to be involved in the uptake and transport of substances other than water, the best documented examples being silicon (Ma et al. 2006; Chiba et al. 2009; Mitani et al. 2009) and boron (Takano et al. 2006; Schnurbusch et al. 2010). What is not clear, though, is whether the abundance of root AQPs and organ-specific expression in comparison to shoot tissue reflects the necessity to transport large amounts of water through root cells and tissues. An alternative explanation could be that the flow rate across the plasma membrane (and tonoplast) of cells causes large perturbations in the local osmotic equilibrium and concentration of solutes involved in membrane transport processes beneath the plasma membrane. Abundance of AQPs would help to rapidly equilibrate any local perturbations and minimise downstream effects on, e.g. protein stability and conformation. The same argument could be used to explain abundance of AQPs in growing tissues [Wei et al. (2007); for reviews, see Fricke (2002) and Obroucheva and Sin'kevich (2010)], where the net flow of water into cells is significant.

The majority of studies, in which AQP inhibitory treatments were applied, have been concerned with shorter-term changes in root and root cell Lp and in response to sudden decreases in root medium water potential. Most studies which involved plants with altered expression levels of AQPs focused on the effect which downregulation of a candidate AQP had on Lp and in relation to increased tolerance to shorter- or longer-term decreases in root medium water potential (e.g. Clarkson et al. 2000; Javot and Maurel 2003; Vandeleur et al. 2009; Hachez et al. 2006, 2012). Although neither of these approaches has provided information about the possible role of AQPs in regulation of root Lp during 'normal' plant development, they have led to the idea of roots behaving as 'hydraulic rheostats' (Maurel et al. 2010). This idea is not supported by experiments which relate root hydraulics to longer-term, developmental increases in plant transpiration rate. Instead, it appears that early on during plant development, root AQPs provide a 'base' Lp in roots—a 'base' Lp which reflects fixed anatomical structures (Bramley et al. 2009) and maximum plasma membrane AQP activity per unit root surface.

In conclusion, future breeding strategies, which involve targeted AQP expression and are aimed at generating lines with a higher root Lp, water uptake capacity and yield under water-sufficient growth conditions are unlikely to succeed, at least in non-woody crops (Perrone et al. 2012). Rather, lines with intrinsically higher AQP expression levels should be considered for this purpose (e.g. see recent study on grapevine (*V. vinifera*) by Gambetta et al. 2012). It is hypothesised here that the physiological function of AQPs in roots is to provide a high 'base-level' hydraulic conductivity in cells to (i) enable rapid osmotic equilibration across membranes, (ii) sustain considerable transpirational water loss rates through root water uptake and (iii) allow for much 'play' to reduce root hydraulics and potential water loss when soil water potential decreases.

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Chapter 14
Root Strategies for Rill and Gully Erosion Control

S. De Baets, T.A. Quine, and J. Poesen

14.1 Introduction

14.1.1 Root Functions

The root system has to serve several functions simultaneously. First of all, the root system has to provide a stable platform for the plant to grow and function and for the soil in which it is growing, resisting water, wind and gravitational forces. In contrast to the shoots, which are essentially harvesting two resources, light and carbon dioxide, roots have to cope with a more challenging environment (Gregory 2006). Roots define the biologically and chemically most active zone of the soil profile. They transport water and nutrients, are a major source of organic material in soils, are the primary source of energy for many soil organisms, contribute to the weathering of minerals and modify soil structure (Schenk and Jackson 2005). Roots serve as carbon pumps that feed soil organisms and contribute to soil organic matter; storage organs; chemical factories that may change soil pH and poison competitors; a sensor network that helps in regulating plant growth; habitats for mycorrhizal fungi, rhizosphere and rhizoplane organisms; an absorptive network

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for limiting soil resources of water and nutrients; hydraulic conduits that reinforce soil water and nutrients; mechanical structures that support plants and strengthen soil, and they filter out toxins or can concentrate rare elements (Brundrett et al. 1996).

The mechanics by which plant roots influence shallow slope stability and the soil's resistance to erosion by concentrated flow may be broadly classified as either mechanical or hydrological/hydraulic in nature.

Mechanical functions

1. Roots provide additional strength to the soil due to the mobilisation of root tensile strength when shearing the soil. As a consequence, roots reinforce the soil, which is important for prevention of shallow mass movements. The shear strength increase depends on the tensile strength of the roots and the root area ratio (RAR), i.e. the area of shear surface occupied by roots per unit area (Greenway 1987).
2. Roots bind soil particles at the soil surface. This offers a protection to soil that is under pressure of detachment by run-off or concentrated flow (Greenway 1987).

Hydrological or hydraulic functions

1. The presence of roots increases the soil's roughness that may provide a greater capacity for infiltration and a reduction of surface run-off velocity (Greenway 1987). Higher infiltration rates, on the other hand, can also lead to a higher water table and to increased seepage pressures, which might in turn lead to higher landslide risk (Cammeraat et al. 2005). This is the case when dead roots decay. When thick roots decay, macro-pores are created which may be connected to the water table and allow bypass flow. When fine roots decay, they assist in keeping water in the upper soil layers (Archer et al. 2002).
2. On the other hand, living roots can reduce pore water pressure by consuming water which increases the apparent soil cohesion.

14.1.2 Effects of Roots on Slope Stability

The role of roots in improving slope stability has long been recognised. Plant roots increase soil shearing resistance both directly by mechanical reinforcing and indirectly through water removal by transpiration. Simon et al. (2006) indicate that in wet periods, the hydrological effects of roots on stream bank stability were smaller in magnitude than the mechanical effects. During rainy periods when soil matric potential can rise to zero irrespective of the presence of vegetation and soil slides often occur, the mechanical effect of roots is most important (Waldron 1977). One of the important mechanical characteristics of roots is that they are strong in tension. Soils on the other hand are strong in compression and weak in tension. A combined effect of soil and roots results in a reinforced soil. When shearing the soil,

roots mobilise their tensile strength whereby shear stresses that develop in the soil matrix are transferred into the tensile resistance of the root fibres via interface friction along the length of the fibres (Gray and Barker 2004). So, on sidewalls of gullies or on sloping land vulnerable to mass movements, roots can provide an increased stability. The magnitude in root reinforcement depends on morphological characteristics of the root system, root tensile strengths, root tensile modulus values, root tortuosity and the interface friction between the roots and the soil (Greenway 1987). The maximum effect on the resistance of the soil to failure occurs when the tensile strength of the roots is fully mobilised. The significance of mechanical slope stabilisation by roots also depends on the position of the roots to the depth of the potential slip surface, the failure mode, the steepness of the slope (Nilaweer and Nutalaya 1999), taproot length, the proportion of fine lateral roots and root topology (Burylo et al. 2009). In case the position of the slip surface coincides with the level of the water table or bedrock, most roots cannot effectively cross the potential slip surface and will not be able to prevent mass movement anymore (Reubens et al. 2007). Stokes et al. (2009) identified different root traits and their functions for slope stabilisation as follows (1) thick deep roots are important for soil anchorage and for determining the spatial arrangement of the associated thin roots and (2) thin roots act in tension during failure on slopes and, if they traverse the potential shear zone, provide a major contribution in protecting against landslides.

The effects of roots on the resistance of the topsoil to water erosion are much less studied and will be discussed in the next paragraphs.

14.1.3 Effects of Roots on Water Erosion Processes

Some experimental studies on the effects of roots on reducing water erosion rates have been conducted recently. Research by Bui and Box (1993) showed that roots had no stabilising effect during interrill soil erosion. In contrast, Ghidey and Alberts (1997) found that interrill erodibility decreased as dead root mass and dead root length increased. The decline in soil loss is even more pronounced in the case of rill and ephemeral gully erosion (Gyssels et al. 2005). Foster (2005) also indicates that soil biomass has a greater relative effect on rill erosion than on interrill erosion. Gyssels et al. (2005) developed a structural model, indicating the relative importance of above-ground vegetation cover and below-ground root density on different water erosion processes. When looking at splash and interrill erosion, the vegetation cover is the most important plant parameter controlling soil losses. When studying incisive processes such as rill and gully erosion, one should also consider the influence of plant roots. Gyssels and Poesen (2003) hypothesise that in the early plant growth stages, roots will be of more importance with respect to reducing soil loss by concentrated flow, because the above-ground biomass is still very limited at this stage of plant growth. During the growing season, the impact of above-ground biomass will become more important than the below-ground biomass. When crops,

grasses in grassed waterways or cover crops are harvested, vegetation cover returns to zero, and then again the roots may become very important in protecting the soil against erosion until they have disappeared. After harvesting or removing the above-ground biomass, fine roots are the first to disappear. Roots will lose their strength following a negative exponential relationship with time since cutting the above-ground biomass (Gray and Sotir 1996; Sidle et al. 2006).

The impacts of roots on water erosion rates might also become very important when the above-ground biomass disappears because of grazing, surface fire, drought or flooding. Especially in semi-arid environments, where vegetation cover can be restricted and shoots can temporally disappear, roots may play a crucial role (De Baets 2007).

Few studies report on the effects of roots on concentrated flow erosion rates (e.g. Li et al. 1991; Mamo and Bubenzer 2001a, b; Gyssels and Poesen 2003; Zhou and Shangguan 2005, 2008; Gyssels et al. 2006; Shit and Maiti 2012; Zhang et al. 2009; Wang et al. 2012). The fact that the below-ground biomass is hardly visible and difficult to sample may explain why the below-ground impacts of plants are much less studied and often neglected.

Mamo and Bubenzer (2001a, b) found an exponential decrease in rill erodibility with increasing root length density both for laboratory-grown grasses and for field-grown soybean and maize plants. Gyssels et al. (2006) report an exponential decline of relative soil detachment rates with increasing root density. A reduction exponent of 2.25 was established in their study. Li et al. (1991) found that soil erosion resistance increased exponentially with increasing root density and reported that the ability of plant roots to increase the soil erosion resistance of the soil mainly depends on the distribution of roots and on the number of fibrous roots less than 1 mm in diameter. Zhou and Shangguan (2005) observed a similar relation but with root surface area density (i.e. total root surface area or surface of all the roots placed on a horizontal plane divided by the soil volume, RSAD, $m^2\ m^{-3}$) as the root variable. Shit and Maiti (2012) also observed an exponential relationship between the root properties root density (RD, kg m^{-3}), root length density (RLD, km m^{-3}) and RSAD and the soil's anti-scourability during concentrated flow. Both Wang et al. (2012) and Zhang et al. (2013, Transactions of ASABE) detected a significant negative exponential relationship between erodibility factors (Kc) and root density (RD) when conducting concentrated flow erosion experiments with undisturbed subtropical Chinese Ultisols and loamy, grass root-permeated topsoils from a semi-arid field in the Chinese loess belt, respectively.

Overviews of the parameters for different relationships between root variables and erosion variables are reported by Gyssels et al. (2005) and Duran Zuazo et al. (2008). A comparison between different studies is difficult, because different root and erosion variables were used. De Baets et al. (2006, 2007) were the first to calculate the reduction in soil loss due to the presence of roots as compared to equivalent rootless topsoils. Although roots from different plant species were studied, most studies did not consider the effects of different root architectures on soil erosion rates. However, Wischmeier (1975) and Dissmeyer and Foster (1985) already assumed that species with contrasting root architectures have a different

erosion-reducing effect. These authors assumed that fibrous root systems are more effective in reducing erosion rates as compared to species having a taproot system.

Few field studies confirmed the role of roots on reducing the erodibility of soils. Kiley and Schneider (2005) found a good correspondence between the spatial variability in root densities of riparian vegetation along riversides and the vulnerability of those areas to erosion. Prosser et al. (1995) performed concentrated flow experiments in the field and observed a reduction of the critical flow shear stress relative to that under natural conditions (undisturbed grassland) for sediment transport by concentrated flow after complete clipping of the vegetation. They attributed this effect to the strong cohesion provided by the dense grass root mat.

14.2 Effects of Roots on the Resistance of Soils to Concentrated Flow Erosion

In order to assess concentrated flow erosion rates, an accurate prediction of the soil erosion resistance is very important. The soil's resistance originates from the bonding forces that hold together the soil particles and other materials in the soil matrix, such as organic matter, plant roots and rock fragments (Knapen et al. 2007a). Soil erosion resistance must be regarded as the summation of a highly complex response pattern, strongly influenced by intrinsic soil characteristics and extrinsic macro-environmental variables (Bryan 2000). The most universal and widely used soil erosion parameters are concentrated flow soil erodibility and critical flow shear stress (Knapen et al. 2007a). Soil shear strength, aggregate stability, infiltration capacity, bulk density, texture, organic matter and chemical composition are the most important soil parameters controlling soil erodibility and critical flow shear stress (Gyssels et al. 2005):

(a) Soil shear strength. The maximum resistance a soil can sustain to shearing forces is directly related to the cohesive forces between soil particles. A positive linear relationship has been reported in previous studies between saturated soil shear strength and critical flow shear velocity, needed to detach soil particles (e.g. Rauws and Govers 1988; Léonard and Richards 2004). One of the important mechanical characteristics of roots is that they are capable of increasing soil shear strength. Roots are strong in tension. Soils on the other hand are strong in compression and weak in tension. A combined effect of soil and roots results in an increase in soil cohesion (Gray and Sotir 1996). Prosser et al. (1995) report shear stresses of 25–43 Pa, required for channel incision, to overcome the resistance of an organic clay loam permeated with grass roots. The magnitude of root reinforcement depends on morphological characteristics of the root system, root tensile strengths, root tensile modulus values, root tortuosity, the interface friction between roots and the soil and the orientation of roots to the principal direction of strain (Greenway 1987). Quang and Oumeraci (2012) stress the importance of the decay rate of grass root density

with soil depth. In their model simulations, they show that soil detachment rates by overtopping waves on grassed inner sea-dike slopes are rapidly accelerated when the grass strength reduces rapidly with soil depth.

(b) Aggregate stability. Together with shear strength, soil aggregate stability is often been used as a first indicator for the erosion resistance of a soil (Knapen et al. 2007a). In general it is found that plant roots significantly increase the soil's structural stability and thus decrease the soil's erodibility (Li et al. 1992a; Turkelboom et al. 1997; Morgan 2005). Plant roots can be seen to enmesh soil particles by acting as a "sticky string bag" or bonding agent (Oades 1993). Fragments of roots can also act as nuclei for formation of small aggregates (Oades and Waters 1991; Bronick and Lal 2005). Pohl et al. (2009) found that RD, together with sand content and the number of plant species, explained a large portion of the variance in aggregate stability measured on disturbed and undisturbed soils in the Swiss Alps.

(c) Infiltration capacity. Plant roots create macro-pores in the soil that improve water movement and enhance the infiltration capacity of the soil (Glinski and Lipiec 1990), although there is little experimental evidence to show how infiltration changes as the roots of specific vegetation type develop over time (Quinton et al. 1997). An increased infiltration capacity reduces run-off and consequently water erosion.

(d) Soil bulk density. Compaction reduces detachment by concentrated run-off when the soil is sufficiently wet (Govers et al. 1990). The effect of roots on soil bulk density depends on the root diameter and the nature of the soil. Large roots push the soil near the roots aside, and therefore, the bulk density near the roots increases (Glinski and Lipiec 1990). In contrast, fine roots decrease the bulk density of the soil and increase soil porosity, hydraulic conductivity and permeability (Li et al. 1992b; Cavalieri et al. 2009).

(e) Soil texture, organic matter content and chemical composition. These soil properties are important because of their influence on soil aggregate stability (Morgan 2005). The influence of plant roots on texture must be seen through intensified weathering of the soil materials in the vicinity of roots. The most important effect is the supply of organic residues (root exudates) to the soil by roots. The effectiveness of organic matter in reducing concentrated flow erodibility depends on the clay percentage and the soil water content (Knapen et al. 2007a). Roots also release or absorb polyvalent cations and increase or decrease the concentration of ions in solution (Amezketa 1999).

Knapen et al. (2007b) studied soil properties controlling the spatial and temporal variability of the erosion resistance of loess-derived topsoils during concentrated flow and showed that the dry mass of the organic material in the topsoil (i.e. roots and crop residue) is an important variable to predict channel erodibility. In the following paragraphs, the erosion-reducing potential of a topsoil reinforced with plant roots as compared to a bare soil with similar soil conditions, the effects of different types of roots, the impacts of soil and flow conditions on the erosion-reducing potential of plant roots and the effects of roots sown in different spatial

configurations will be discussed. At the end of this section, the root effects during concentrated flow erosion are summarised under one dynamic soil property, namely, soil cohesion under the presence of plant roots.

14.2.1 Root Variables Determining the Erosion-Reducing Potential of Roots

A standard methodology to evaluate the effect of roots of different plant species on the erosion resistance of topsoils to concentrated flow erosion is lacking. Studies investigating the impacts of roots on the resistance of the topsoil to concentrated flow erosion use different root variables to predict their water erosion-reducing effect. Most studies use root density or root length density to predict the effects of roots on soil erosion rates by concentrated flow. Mamo and Bubenzer (2001a, b), Gyssels and Poesen (2003), Gyssels et al. (2006) and De Baets et al. (2006) reported an exponential decline of rill erodibility and soil detachment rates with increasing root length densities or root densities. Du et al. (2010) report an increase in soil stability with increasing RLD for both native vegetation and *T. ascendens* or *S. babylonica* planted communities on coastal shelterbelts along the south coast of Shanghai. Few studies report an effect of root diameter on the erosion resistance of the topsoil to concentrated flow erosion (Li et al. 1991; Zhou and Shangguan 2005). Zhou and Shangguan (2005) agree with Li et al. (1991) that RLD is a good parameter for expressing the root-soil contact area but state that the root effect cannot be precisely predicted if only the number of roots <1 mm is used. Zhou and Shangguan (2005) state that root diameter distribution has to be taken into account as well when predicting the erosion reduction by roots during concentrated flow and therefore propose to use root surface area density (RSAD, $m^2\ m^{-3}$) for predicting the soil anti-scourability (i.e. soil erosion resistance). In this overview, the effect of roots on erosion rates are expressed by root density and mean root diameter, as these variables are quite easy to measure and address major root type differences.

14.2.1.1 Root Density

De Baets et al. (2006) showed that grass root-permeated topsoils are much more resistant during concentrated flow erosion compared to rootless topsoils and that the resistance to erosion increases with increasing root density. Plant roots are capable to reduce concentrated flow erosion rates drastically. This underlines the great importance of plant roots in resisting concentrated flow erosion. Even at a root density (RD, $kg\ m^{-3}$) of 2 $kg\ m^{-3}$, erosion rates are reduced to almost zero (i.e. RSD (relative soil detachment rate) values of 0.001 for grass roots, 0.03 for carrot roots tested on a silt loam and 0.25 for carrots and 0.17 for grasses tested on a sandy loam soil) as compared to rootless topsoils with similar soil conditions and

tested under the same circumstances. The grass root densities at which relative soil detachment rate (RSD, dimensionless) was reduced by 50 % were already achieved approximately 30 days after sowing, whereas for taprooted plants such as carrots, it took about 50–60 days to achieve this RD in the appropriate growing season (De Baets and Poesen 2010). This indicates that RD needed to increase the topsoil resistance against concentrated flow erosion to a great extent is already achieved after 30–60 days of growth, depending on the growth conditions and on plant species. So, in the early growth stages, root reinforcement can contribute significantly to the soil's resistance to erosion. Zhou and Shangguan (2008) confirm this significant effect of growth time on run-off and erosion reduction, with ryegrass found to decrease run-off by 25 % after 12 weeks of growth and by 70 % after 27 weeks and sediment yield decreased by 80 % and 98 % after 12 and 27 weeks, respectively.

A good model, explaining the reduction in erosion rates, was obtained using an exponential relationship with root density [Eq. (14.1)]. This model clearly indicates that the farther you are from the minimum value of RSD, the more effective an increase in root density is in reducing erosion rates. Whereas Zhou and Shangguan (2007) reported a linear decrease of soil loss with increasing RD for interrill erosion processes, De Baets et al. (2006, 2007), De Baets and Poesen (2010) clearly show that during concentrated flow, roots reduce erosion rates exponentially with increasing RD.

$$\mathrm{RSD} = \mathrm{e}^{-0.93\mathrm{RD}} \quad \left(\mathrm{adj}.R^2 = 0.63, \mathrm{ME} = 0.69, n = 280\right) \tag{14.1}$$

where RSD (dimensionless) is the relative soil detachment rate of the root-permeated topsoil sample relative to a bare topsoil sample with similar soil conditions and RD (kg m^{-3}) is the root density. Equation (14.1) (De Baets and Poesen 2010) can be used to predict the erosion-reducing potential of root-permeated loamy topsoils when only root density information is available.

Zhang et al. (2013) found a much lower (−0.41) reduction exponent when plotting relative soil detachment rates against RD of versatile switch grass for a loamy soil from the Chinese loess plateau. They attribute the differences to differences in plant species, mass of dead roots or residue in soils, and experimental conditions and procedures.

14.2.1.2 Root Type

When adding root diameter to the negative exponential relationship between RD and RSD, the relationship between RSD and RD is much improved and the adjusted R^2 value increases to 0.67 [Eq. (14.2)]. This reveals that 67 % of the variance in relative erosion rates can be explained by root architectural properties only.

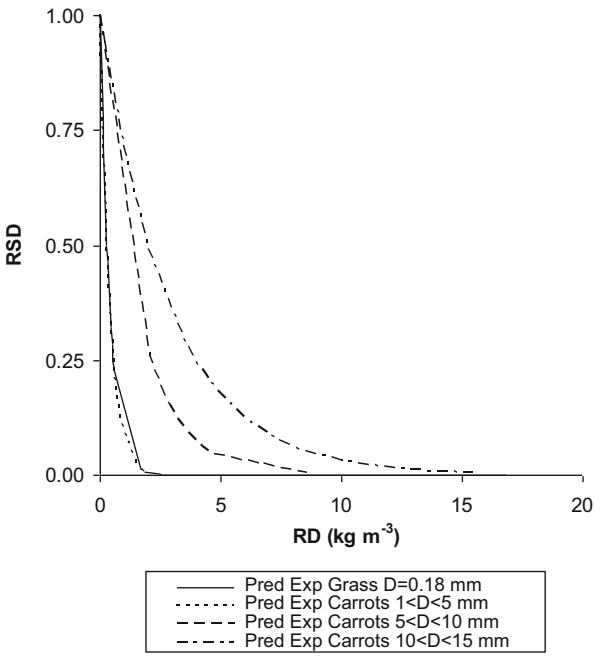

Fig. 14.1 The erosion-reducing potential of plant roots having different mean root diameters (D, mm). RSD (fraction, dimensionless) is the relative soil detachment rate and RD (kg m^{-3}) is the root density

$$\mathrm{RSD} = \mathrm{e}^{-1.25\mathrm{RD}+0.06\mathrm{RD}^{*}D} \quad \left(\text{adj. } R^2 = 0.67, \mathrm{ME} = 0.74, n = 268\right) \qquad (14.2)$$

where D (mm) is the mean root diameter. Equation (14.2) (De Baets and Poesen 2010) can be used to predict the erosion-reducing potential of root-permeated loamy topsoils with roots having all similar root diameters (e.g. grasses, wheat, barley, etc.).

While previous studies never investigated the impact of root type on the erosion-reducing potential of plants roots, these results reveal that roots reduce erosion rates differently depending on their root architecture. Grass roots are most effective in reducing concentrated flow erosion rates. RSD decreases to very low values (from 1 to 0.001) for grass roots grown in a silt loam topsoil with increasing RD from 0 to 2 kg m^{-3} only. The parameter in Eq. (14.2), predicting the interaction between root density and root diameter, is positive, indicating a lower erosion-reducing potential of root systems having thicker roots. These results confirm the hypothesis made by Wischmeier (1975), assuming that grasses having a fibrous root system are more effective in reducing rill erosion than taprooted plants such as broadleaf weeds. For taprooted species, the erosion reduction with increasing RD is thus less pronounced (Fig. 14.1). RSD decreases to very low values (from 1 to 0.03) for fine carrot roots grown in a silt loam topsoil with increasing RD from 0 to 2 kg m^{-3}. The erosion-reducing potential becomes less when carrot's root diameter increases. RSD values are only reduced to 0.50 for root densities of 2 kg m^{-3} consisting of roots with a mean diameter up to 15 mm (Fig. 14.1).

For predicting the erosion-reducing potential of root systems having roots of different root diameters (e.g. shrubs), the following relationship was established by De Baets et al. (2007):

$$\mathrm{RSD} = \mathrm{e}^{-1.45\mathrm{RD}_1}\mathrm{e}^{-0.47\mathrm{RD}} \quad \left(\text{adj. } R^2 = 0.46, \ n = 27\right) \tag{14.3}$$

where RD_1 is the density (kg m^{-3}) of roots with $1 < D < 5$ mm and RD_2 is the density (kg m^{-3}) of roots with $5 < D < 15$ mm.

14.2.2 Environmental Factors Controlling the Erosion-Reducing Potential of Root-Permeated Topsoils During Concentrated Flow

14.2.2.1 Impacts of Soil and Overland Flow Characteristics on the Erosion-Reducing Potential of Roots During Concentrated Flow

Apart from root density and root diameter, soil and flow conditions also affect the erosion-reducing potential of plant roots during concentrated flow erosion. Here, we only report on the significant effects.

Soil texture has an effect on the erosion-reducing potential of grass roots. With increasing sand content, the erosion-reducing potential of grass roots is less pronounced (De Baets et al. 2007; De Baets and Poesen 2010). Differences in soil texture did not affect the erosion-reducing potential of taproot systems.

Soil moisture content prior to saturation has a significant effect on the erosion-reducing potential of root-permeated topsoils. With increasing soil moisture content prior to saturation, the erosion-reducing potential of plant roots becomes more pronounced (De Baets and Poesen 2010).

Flow shear stress (tau) affects the erosion reduction by plant roots as well. At high flow shear stresses (ca. 40–60 Pa), the erosion-reducing potential of root systems decreases (De Baets and Poesen 2010) as a result of upstream hydraulic turbulences of the roots sticking out at the soil surface.

The impacts of the soil and flow variables affecting the erosion-reducing potential of two types of root systems are expressed in Eqs. (14.4, 14.5, 14.6, 14.7 and 14.8), established by empirically modelling the data gathered during laboratory experiments.

For *both fibrous root systems* and *taproot systems*, following relationships could be established (De Baets and Poesen 2010):

$$\mathrm{RSD} = \mathrm{e}^{-1.12\ \mathrm{RD}+0.05\mathrm{RD}^{*}D+0.02\mathrm{RD}^{*}\tau-4.12\mathrm{RD}^{*}\mathrm{SM}} \quad \left(\text{adj. } R^2 = 0.69, \mathrm{ME} = 0.75, n = 266\right) \tag{14.4}$$

where τ is the mean bottom flow shear stress (Pa) and SM is the soil moisture content prior to saturation (g g^{-1}). Equation (14.4) indicates that the erosion-reducing effect of plant roots decreases with increasing diameter and with mean bottom flow shear stress and increases with increasing root density and soil moisture content prior to saturation.

For *fibrous root systems* such as grasses, the erosion-reducing potential by roots can be best expressed as follows (De Baets and Poesen 2010):

$$\mathrm{RSD} = \mathrm{e}^{-2.45\mathrm{RD}+0.03\mathrm{RD}^{*}\mathrm{sand}\%} \quad \left(\text{adj. } R^2 = 0.79, \mathrm{ME} = 0.93, n = 16\right) \tag{14.5}$$

where sand% is the percentage ($21 < \text{sand}\% < 56$) of sand particles in the soil sample. Equation (14.5) indicates that the erosion-reducing potential of fibrous root systems is less pronounced for sandy loam soils as compared to silt loam soils, as the erosion-reducing potential of fibrous root systems decreases with increasing sand content.

For *taproot systems* such as carrots, the erosion-reducing potential during concentrated flow can be best expressed using root architectural information only [Eq. (14.6), De Baets and Poesen (2010)]. The studied soil and flow variables did not have a significant impact on the erosion-reducing potential of taproots.

$$\mathrm{RSD} = \mathrm{e}^{-1.01\mathrm{RD}+0.05\mathrm{RD}^{*}D} \quad \left(\text{adj. } R^2 = 0.50, \mathrm{ME} = 0.42, n = 104\right) \tag{14.6}$$

Equation (14.6) indicates that the erosion-reducing potential of taproot systems is improved by increasing root density and reduced by increasing mean root diameter.

For fibrous root systems, 79 % of the variance of relative erosion rates (RSD) is explained by Eq. (14.5). Still, there is a part of the variance that cannot be explained by the root, soil and flow variables investigated in this study. The effects of algae or moss cover on reducing erosion rates were not accounted for in this study. This soil property can probably have caused scatter on the data. Non-specific monitoring of soil bulk density of the root-permeated topsoil samples may also explain the nonsignificant contribution of this variable to the model. More accurate and detailed measurements of the flow and soil properties investigated in this study are recommended in order to improve the impacts of these conditions on the erosion-reducing potential of plant roots during concentrated flow.

For taproot systems, only 50 % of the variance of RSD values is explained by Eq. (14.6). Part of the explanation for this large scatter on predicted RSD values for taprooted species can be attributed to the fact that the effect of taproots on soil erosion rates is not so straightforward. Although it is clear that, on average, taproots reduce erosion rates, it is not illogical that RSD models predicting a reduction (with RD negatively related to RSD) do not perform better than the mean RSD value, due

to the presence of RSD values higher than 1. Hence, more insights into the processes generating accelerated erosion or reduced erosion in the presence of taproots are needed.

To predict the reduction in erosion rates due to the presence of roots in the topsoil, it is recommended to use Eq. (14.1) if only information on RD is available. If information on root diameter (D) is available as well, Eq. (14.2) or (14.6) can be used in case all roots of the root system have similar root diameters. In case the root system consists of roots of different diameters, Eq. (14.3) can be used (De Baets et al. 2007). If information on soil texture or SM and τ is available, Eq. (14.4) or (14.5) can be used.

14.2.2.2 Spatial Organisation of Plants

For fibrous root systems, such as grasses, the erosion-reducing effects during concentrated flow of grasses sown randomly were significantly higher than the erosion-reducing potential of grass roots established in rows perpendicular to the dominant flow direction, but the adjusted R^2 values for the established relationships predicting the erosion-reducing potential of roots for different spatial configurations were very low, indicating the low predictability of the established parameters. Moreover, a comparison with larger topsoil samples (length $= 0.7$ m, width $= 0.2$ m) did not allow us to draw strong conclusions and to identify the most suitable plant orientation for increasing the topsoil resistance against concentrated flow erosion by roots. Both a random orientation and rows perpendicular to the dominant flow direction are effective in reducing concentrated flow erosion rates by roots. Planting or sowing species in rows parallel to the flow direction does not offer a good protection to erosion by concentrated flow (Fig. 14.2).

14.2.3 Soil Cohesion

The effects of roots on the resistance of the topsoil to concentrated flow erosion can be modelled with EUROSEM by adjusting the soil cohesion value and the corresponding soil detachment efficiency coefficient β. More information on the methodology used to calculate soil cohesion for root-permeated topsoils can be found in De Baets et al. (2008a).

A combination of an exponential and a power relationship was established to predict the increase in soil cohesion on wet silt loamy soils due to the presence of fibrous grass roots and taproots such as carrots, respectively, using root density information (De Baets et al. 2008a).

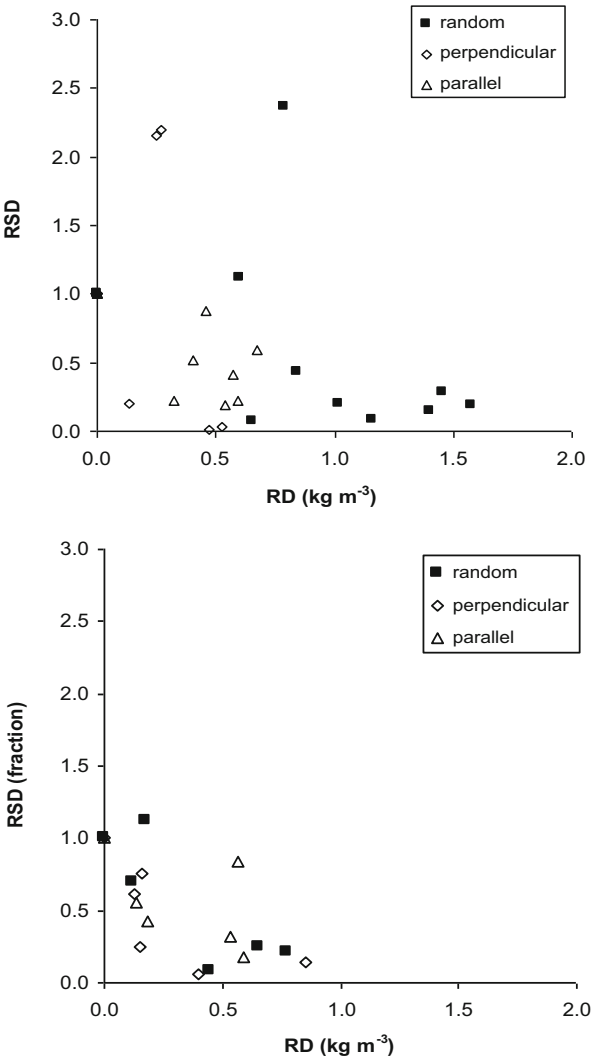

Fig. 14.2 Relative soil detachment rate (RSD, fraction) as a function of root density (RD, kg m^{-3}) for different sowing treatments (sown random, perpendicular and parallel to the flow direction) tested on (**a**) small-scale samples (length = 0.356 m, width = 0.087 m) and (**b**) larger-scale samples (length = 0.7 m, width = 0.2 m)

$$C_r = 6.53 - (6.53 + 0.76)^{*}\exp\left(-0.67^{*}\mathrm{RD}^{0.66}\right) \quad \left(\text{adj. } R^2 = 0.70, n = 32\right) \quad (14.7)$$

$$C_r = 5.21 - (5.21 + 0.10)^{*}\exp\left(-0.29^{*}\mathrm{RD}^{0.55}\right) \quad \left(\text{adj. } R^2 = 0.43, n = 33\right) \quad (14.8)$$

where C_r (kPa) is the increase in soil cohesion due to the presence of roots and RD (kg m) is the root density.

Roots significantly increase soil cohesion up to 3.41 ± 2.36 kPa and 1.66 ± 1.66 kPa for fibrous root and taproot systems, respectively. Taproot systems thus increase soil cohesion to a lesser extent. This can be explained by their smaller

root-soil contact area. With increasing root density, the soil reinforcement effect increases. From a certain root density on, i.e. 2–5 kg m^3, the increase in soil cohesion reaches a limit (De Baets et al. 2008a). So, in the early growth stages, root reinforcement can contribute significantly to the soil's resistance to erosion.

14.3 Effects of Roots on Flow Erosivity

Soil properties affect both the soil's erosion resistance as the erosivity of the flow. In the previous paragraphs, the effects of roots on soil erosion resistance were unravelled. The effects of root area ratio and root type on the flow hydraulics will be discussed in this section.

For covered soils, part of the total bottom flow shear stress will be dissipated on the vegetation or roughness elements, and hence, flow shear stress responsible for soil detachment will be less and needs to be corrected for. Giménez and Govers (2008) developed a method to address the effects of straw mulch on rill erosion and hydraulics, and based on this, Knapen et al. (2008) developed a method to account for the flow-retarding effects of mulches, above-ground plant components and geotextiles on soil detachment during concentrated flow erosion (Knapen et al. 2009). Until now, no studies have reported on flow-retarding effects by plant roots. Knapen (2007) suggests that fine roots will only affect soil cohesion as they act as a binding network. As fine roots do not stick out at the soil surface, it is assumed that they do not directly affect flow velocity and flow shear stress. However, it has been shown that this root mat protects the soil surface from substantial erosion up to very high flow shear stresses (Prosser et al. 1995; De Baets et al. 2006), thereby preventing the roots from outcropping. In contrast, thicker taproots are assumed to act in a similar way as above-ground vegetation elements once concentrated flow incision has started, as thick roots sticking out at the soil surface will also act as soil obstacles, retarding flow velocity. However, it was observed by De Baets et al. (2007) that flow velocity and soil detachment rate could also be increased upstream of roots sticking out at the soil surface. This phenomenon is described as horseshoe vortex erosion and was observed on stony soils (Bunte and Poesen 1994) and on carrot root-permeated loamy topsoils (De Baets et al. 2007). Provided that horseshoe vortex erosion does not become a dominant soil detachment trigger, the method developed by Knapen et al. (2008) to calculate effective flow shear stress as soil detachment predictor is expected to be successful.

Quantifying the effects of different types and densities of roots on flow velocity and soil detachment capacity will allow us to separate the effects of roots on flow erosivity from the effects on soil erodibility. In the following paragraphs, the results of this analysis are presented.

14.3.1 Correcting for the Flow-Retarding Effects of Roots

The methodology to calculate and use effective flow shear stress as a better soil detachment predictor for covered soil surfaces is explained in detail in Knapen et al. (2008). The determination of effective flow shear stress is based on recalculation of the hydraulic radius for covered soil surfaces using flow hydraulics on uncovered surfaces. This method was tested in a laboratory flume by comparing soil detachment rates of identical pairs of soil samples that only differ in the presence or absence of crop residues (Knapen et al. 2008).

Effective flow shear stress can be calculated as follows (Knapen et al. 2008):

$$\tau_{\text{eff}} = \rho_{\text{w}} g R' S \tag{14.9}$$

where $R' = 0.007\ v^{1.32}$, v (m s^{-1}) is the mean bottom flow velocity and 0.007 and 1.32 are the parameters from a relationship between hydraulic radius and flow velocity for bare soil surfaces.

Since part of the soil surface is covered by roughness elements (i.e. geotextiles, crop residues, vegetation) and thus cannot contribute to soil detachment, mean soil detachment rates are corrected as follows:

$$\text{Dr}_{\text{corr}} = 100 \times \frac{\text{Dr}}{100 - C} \tag{14.10}$$

where Dr_{corr} is the corrected mean soil detachment rate (kg m^{-2} s^{-1}), Dr is the measured mean soil detachment rate (kg m^{-2} s^{-1}) and C is the soil surface covered by roughness elements (%). In this study, C is the root area ratio (RAR, −), which is the surface occupied by plant roots divided by unit surface area (i.e. the sample surface). RAR is calculated as follows:

$$\text{RAR} = \frac{\sum_i^n n_i \cdot a_i}{A} \tag{14.11}$$

where n_i is the number of roots, a_i is the mean root cross-sectional area (m^2) and A is the sample box area (m^2). For grasses n_i is the number of individual plants and a_i is the mean area of a plant base and the root-shoot interface.

To test this methodology for roots as roughness elements, a series of bare, grass root-permeated (*Lolium perenne* or ryegrass) and taproot-permeated (*Sinapis alba* or yellow mustard) silt loam topsoil samples were created and grown in a greenhouse. The samples were made and treated identically in terms of soil compaction and irrigation.

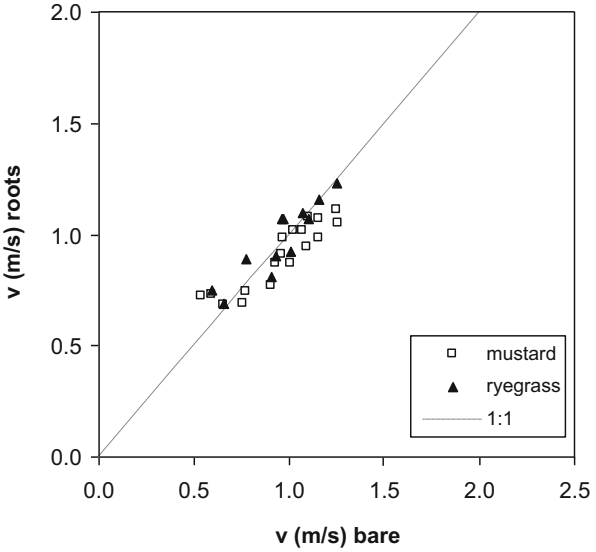

Fig. 14.3 Mean flow velocity (v, m/s) for bare and root-permeated topsoils

14.3.2 Do Plant Roots Influence Flow Erosity?

Figure 14.3 shows that grass roots do not show a flow-retarding effect, whereas flow velocities on mustard root-permeated samples were lower as compared to bare topsoils tested at a same discharge/slope. However, this difference remains very small. Since the method is based on average flow characteristics, flow turbulences are not accounted for. At high flow shear stresses, vortex erosion due to flow turbulence is reported for the thickest roots. Hence, in this case, the small observed flow-retarding effect might be underestimated for taproots.

Figure 14.4a shows that soil erodibility (i.e. slope of regression line) differs significantly between bare and vegetated treatments but not between grass and mustard. Moreover, critical flow shear stress (i.e. intercept with X-axis) for soil detachment is significantly higher for the vegetation treatments as compared to the bare treatments. On Fig. 14.4b, corrected detachment rates were also plotted against effective flow shear stress, calculated with Eqs. (14.9) and (14.10), respectively. This figure shows that grass roots do not have a flow-retarding effect, and hence, effective flow shear stress values do not differ much from total flow shear stress values. This is also a consequence of the very low fraction of soil that is occupied by fine-branched roots (less than 1 %). For mustard roots, the regression line moves a little bit towards the regression line of the bare samples after performing the corrections following Eqs. (14.9) and (14.10) (Fig. 14.4b). This indicates that there is a small flow-retarding effect of taproots sticking out at the soil surface, which is in accordance with our hypothesis.

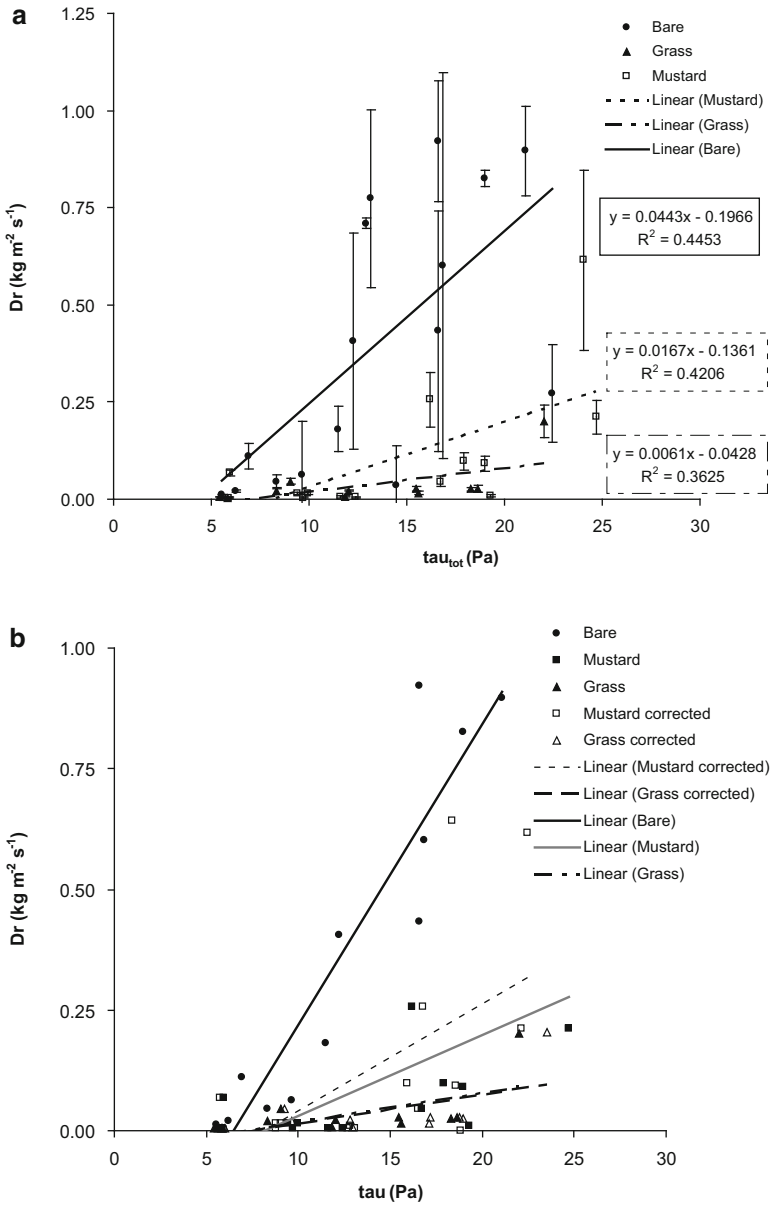

Fig. 14.4 Testing the flow shear stress partitioning method described by Knapen et al. (2008) for soil detachment during experiments with root-permeated topsoils in comparison with bare soil surfaces. (**a**) Measured soil detachment rate (Dr, kg m^{-2} s^{-1}) versus total flow shear stress (tau_{tot}, Pa) and (**b**) measured and corrected soil detachment rate versus total and effective flow shear stress (tau, Pa). Error bars represent standard errors

Hence, we can conclude that the improving effect of roots on soil structural stability (cf. Sect. 14.2) explains the majority of the observed differences in Kc-tau relationship for bare and root-permeated topsoils and that plant roots mainly affect soil erodibility.

14.4 The Relative Contribution of Roots Versus Shoots on Soil Erosion by Concentrated Flow

It is generally accepted that soil loss reduction by vegetation is a result of the combined effects of roots and shoots. Zhou and Shangguan (2007) report that grass roots contribute more to soil loss reduction by interrill erosion than shoots, whereas the shoot effect on run-off reduction was relatively greater than the root effect. Following Zhou and Shangguan (2007), roots reduced soil loss up to 90 %, especially in the late growing stage (i.e. 27 weeks of growth). The results of a scenario analysis performed with EUROSEM, presented in De Baets et al. (2008a), also show that roots are responsible for the largest part (i.e. 98 %) of the reduction in soil loss during concentrated flow. These model predictions were validated by conducting laboratory flume experiments with topsoil samples containing both roots and shoots, containing only roots and bare ones. These results confirm the model predictions as they show that grass roots are responsible for 90–98 % of soil loss reduction, whereas grass stems were only responsible for 2–7 % of the reduction in soil loss as compared to the erosion rates measured on the bare samples, tested under the same circumstances. Neglecting the effects of roots in soil erosion models will therefore result in unrealistic predictions of soil loss and run-off.

14.5 An Integrated Methodology to Evaluate the Effects of Plant Shoots and Roots for Rill and Gully Erosion Control

Many studies measure single plant specimen characteristics to assess the effects of vegetation on soil erosion processes (e.g. the effects of vegetation cover on water erosion rates, the effects of roots on soil shear strength). Moreover, the suitability of plants for water erosion control is often only attributed to the effects of the above-ground biomass on reducing erosion rates (e.g. Bochet et al. 2006), whereas the role of the below-ground biomass is neglected (Gyssels et al. 2005). A standard methodology to evaluate entire plants, including roots, for erosion control strategies is needed. A theoretical attempt to select species for rehabilitation of degraded soils by water erosion in semi-arid environments has been made by Albaladejo et al. (1996). In their study, the capacity of plant species to germinate and establish, the degree of suitability to satisfy the rehabilitation objectives (e.g. improving soil

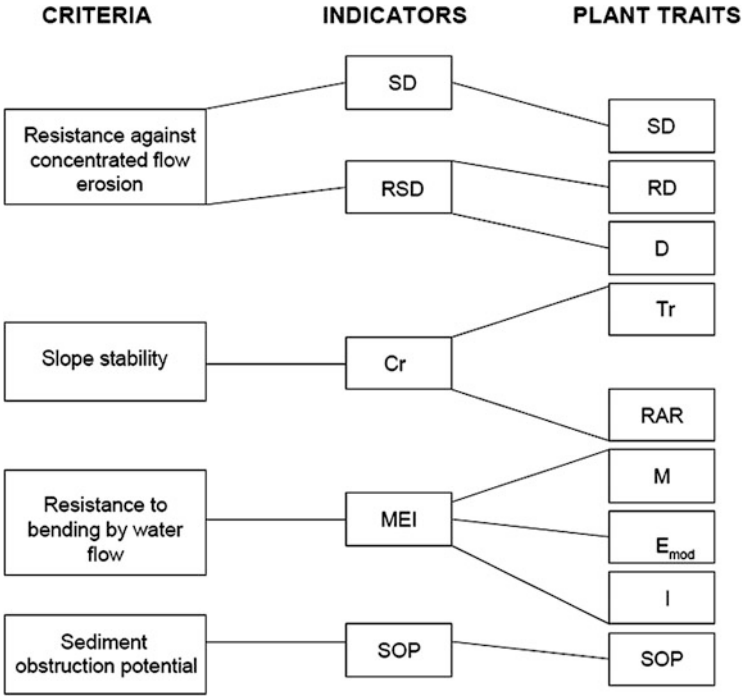

Fig. 14.5 Multi-criteria approach for selecting plant species to control rill and gully erosion. C_r (kPa) is the root cohesion at 0.3–0.4 m soil depth, MEI (N) is the index of stiffness, SD ($m^2\ m^{-2}$) is the stem density, RSD (dimensionless) is the topsoil erosion-reducing potential of plant roots during concentrated flow erosion, SOP ($m\ m^{-1}$) is the sediment obstruction potential, T_r (kPa) is the mean root tensile strength, RAR ($m^2\ m^{-2}$) is the root area ratio, M (m^{-2}) is the stem density, E_{mod} (Pa) is the modulus of elasticity, I (m^4) is the second moment of inertia, RD ($kg\ m^{-3}$) is the root density is and D (m) is the mean root diameter

stability and fertility), the ecological conditions and the considerations concerning landscape aesthetics are proposed to evaluate and select suitable plant species. Other studies focus on environmental conditions of the rehabilitation site and its effects on successful germination and growth rate (e.g. Bochet et al. 2007; García-Fayos et al. 2000). Quinton et al. (2002), on the other hand, made a listing of advantageous ecological and bioengineering properties of potentially useful Mediterranean plant species for revegetation of abandoned lands, but all information presented is descriptive. Burylo et al. (2009) linked plant morphological traits to uprooting resistance in eroded marly lands (Southern Alps, France) and found that taproot length, the proportion of fine lateral roots and root topology were the best predictors of anchorage strength and uprooting resistance. Hence, these plant traits can be measured and evaluated when considering bioengineering works for the prevention and stabilisation of mass movements on steep degraded slopes.

This section presents an integrated methodology developed by De Baets et al. (2009) to assess the suitability of plants for the prevention of rill and gully

initiation and development. In this study, determination of suitable plants for controlling concentrated flow erosion is based on a multi-criteria analysis (Fig. 14.5). First, four main criteria were determined, i.e. (1) the potential of plants to prevent incision by concentrated flow erosion, (2) the potential of plants to improve slope stability, (3) the resistance of plants to bending by water flow and (4) the ability of plants to trap sediments and organic debris. Then, five indicators were selected to assess the scores for the four criteria, i.e. additional root cohesion, plant stiffness, stem density, the erosion-reducing potential during concentrated flow and the sediment and organic debris obstruction potential. The above- and below-ground plant traits that were taken into account and measured to assess the scores for the five indicators are discussed below. Several plant traits were then combined to assess the score for the five indicators (Fig. 14.5).

14.5.1 Above-Ground Plant Traits

Stem density (SD, $m^2\ m^{-2}$, i.e. the number of roughness elements per unit of area) is needed to determine the resistance of vegetation to flow shear stress and is determined as follows (De Baets et al. 2009):

$$\mathrm{SD} = \frac{\sum_i^n \pi (d_{\mathrm{s},i}/2)^2}{A_\mathrm{r}} \tag{14.12}$$

where $d_{\mathrm{s},i}$ (m) is the diameter of each stem at the plant base and A_r (m^2) is the total surface occupied by the vertical projection of the above-ground biomass.

A *Sediment and organic debris obstruction potential* (SOP, $m\ m^{-1}$) was determined to assess the ability of plants to trap sediments and organic debris. This is a one-dimensional variable as plant roundness is inversely correlated with sediment trapping and mound formation (Isselin-Nondedeu and Bédécarrats 2007). SOP is the ratio of the sum of the diameters at the plant base ($d_{\mathrm{s},i}$, m) of the horizontally projected stems on a line perpendicular to the dominant flow direction and the maximum length of this perpendicular line (L_tot, m) defined by the vertical projection of the above-ground biomass, i.e.

$$\mathrm{SOP} = \frac{\sum d_{\mathrm{s},i}}{L_\mathrm{tot}} \tag{14.13}$$

where SOP ($m\ m^{-1}$) is the sediment obstruction potential, $d_{\mathrm{s},i}$ (m) is the stem diameter at the plant base and L_tot (m) is the length determined by the projection of the above-ground biomass, in a direction perpendicular to the dominant flow direction (assessed topographically) (De Baets et al. 2009).

14.5.1.1 Plant Stiffness

To assess the resistance of individual plants to bending, an MEI index can be calculated based on a formula reported by Kouwen et al. (1980), i.e.

$$\mathrm{MEI} = \frac{\sum_i^n I_i E_{\mathrm{mod}}}{A_{\mathrm{r}}} \tag{14.14}$$

where I_i (m^4) is the second moment of inertia of each individual stem, E_{mod} (Pa) is the mean bending modulus of the stems (assessed by performing laboratory stem bending tests) and A_{r} (m^2) is the area occupied by the vertical projection of the above-ground vegetation elements.

14.5.2 Below-Ground Plant Traits

14.5.2.1 The Erosion-Reducing Potential of Plant Roots During Concentrated Flow Erosion

As previous sections of this chapter indicate that plant roots increase soil's resistance to concentrated flow erosion to a large extent, plant root characteristics should be taken into account when assessing the resistance of plants to rill and gully erosion. Therefore, Eqs. (14.3) and (14.4) are used to determine the root erosion-reducing potential of plants.

14.5.2.2 Root Reinforcement at Deeper Soil Layers to Resist Shallow Mass Movements

To assess the effects of plants on slope stability (of gully walls or terrace slopes), root area ratio (RAR, dimensionless) and root tensile strength (T_{r}, MPa) information are combined to obtain root cohesion values (C_{r}, kPa) following Wu's model (Wu et al. 1979), expressing the root reinforcement effect on slope stability. For more information, reference is made to De Baets et al. (2008b). This root-reinforcing effect was assessed for a soil depth of 0.3–0.4 m, because this depth can be regarded as a common depth at which overhanging gully walls become unstable.

In order to compare species, the numerical data were non-linearly rescaled to scores ranging from 0 to 4. The minimum and maximum interval values were determined by the minimum and maximum values measured, and the class borders were defined when there was a gap in the data series. For each set of continuous data (i.e. for each indicator), five groups were made, i.e. 0 = very low value, 1 = low

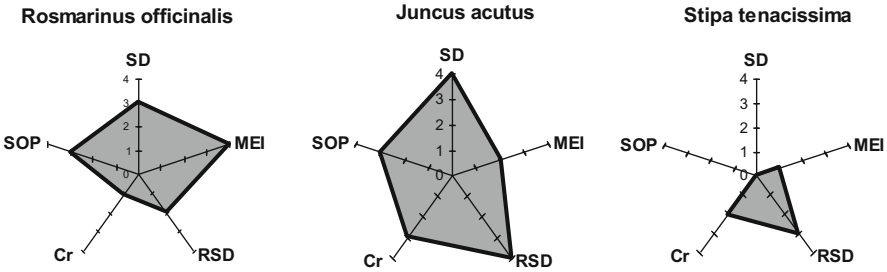

Fig. 14.6 Amoeba diagrams presenting the suitability of three Mediterranean plant species for rill and gully erosion control. C_r (kPa) is the root cohesion at 0.3–0.4 m soil depth, MEI (N) is the index of stiffness, SD (m^2 m^{-2}) is the stem density, RSD (dimensionless) is the topsoil erosion-reducing potential of plant roots during concentrated flow erosion and SOP (m m^{-1}) is the sediment obstruction potential

value, 2 = medium value, 3 = high value and 4 = very high value. The higher the score, the more effective the plant is for controlling rill and gully erosion.

14.5.3 Outcome

The scores for each indicator can then be plotted on amoeba diagrams, with the five indicators forming the axes. The amoeba diagrams enable visual interpretation of the suitability of a plant species for rill and gully erosion control, indicating the beneficial and the weak plant traits. An example of such an evaluation was given for some Mediterranean plants in Fig. 14.6. This figure shows that *Rosmarinus officinalis* L. scores well for the above-ground indicators, whereas *Stipa tenacissima* L. scores best for the below-ground indicators and *Juncus acutus* L. scores well for all indicators. When evaluating a set of species, a cluster analysis can be performed on the scores for the five indicators, in order to form groups of equally scoring species. In addition, suitable species can be grouped according to their main habitat and possible application position within the landscape. As determination of suitable species depends on the process of interest and the main aim of the restoration project, species can also be ranked according to their scores for the four main criteria. This way, either suitable species for preventing initiation by concentrated flow erosion or species for preventing gully wall retreat can be selected and allocated to the corresponding positions in the landscape where prevention is needed. Application of this methodology to a series of representative Mediterranean plant species (De Baets et al. 2009) revealed that a combination of species (e.g. on the one hand a grass having a high potential to resist concentrated flow erosion and a high ability to trap sediments and on the other hand a shrub with a high resistance to bending by water flow and a high potential to improve slope stability) or the allocation of species to specific target areas (e.g. grasses in

concentrated flow zones and on terrace walls, deep-rooted species to stabilise gully walls) is recommended.

14.6 The Ideal Root Architecture for Erosion Control

Determining the ideal root architecture for erosion control is difficult. The ideal root characteristics will depend on the process of study and on the achievements that have to be made. According to Reubens et al. (2007), woody shrub species with a large, deep and strong central part of the root system, having some rigid vertical roots penetrating deeply into the soil and anchoring into firm strata, as well as a large number of finer roots numerously branching from the main lateral roots would be most effective to increase shallow slope stability. An asymmetrical distribution of the laterals parallel to the contours may also be beneficial, as they can possibly form a barrier for run-off and sediment. According to De Baets et al. (2009), a combination of species having an extended network of fine roots in the topsoil to prevent concentrated flow erosion and taprooted species attached more deeply into the substratum, having a high resistance to removal and a better potential to prevent shallow mass movements, is recommended to control rill and gully erosion. Also Körner and Spehn (2002) and Pohl et al. (2009) have suggested that plant communities with diverse growth forms are more effective at stabilising slopes.

14.7 Conclusions

Many studies attribute the effects of vegetation in reducing water erosion rates to the effects of the above-ground biomass. The effects of the below-ground biomass on flow erosivity and topsoil resistance to concentrated flow erosion are much less studied. However, roots play an important role in controlling soil erosion rates, especially when the above-ground biomass disappears (e.g. due to fire, drought, harvest, grazing) and particularly when incisive processes are concerned. Roots affect properties of the soil, such as soil roughness, infiltration rate, aggregate stability, moisture content, soil cohesion and organic matter content, all of which control soil erodibility to various degrees.

Plant roots increase soil cohesion and the resistance of the soil to concentrated flow erosion to a large extent. The erosion-reducing effect of topsoils permeated with fibrous roots is more pronounced and can be predicted very well, whereas predicting relative erosion rates for taproot-permeated topsoils still remains difficult. The overall model shows that the erosion-reducing potential of plant roots reduces with increasing plant root diameter. Plant roots have little effect on flow erosivity, although a small flow-retarding effect of taproots sticking out at the soil surface was observed. Furthermore, it is shown that roots are responsible for the largest part (i.e. 98 %) of the reduction in soil loss during concentrated flow.

An integrated methodology where both above- and below-ground plant traits (i.e. stem density, sediment and organic debris obstruction potential, modulus of elasticity of the stems, moment of inertia of the stems, root density, root diameter distribution, root area ratio and root tensile strength) are taken into account was presented. Amoeba diagrams can then indicate the beneficial and the weak plant traits, regarding to erosion control. The ideal root architecture for erosion control will depend on the process of study and on the achievements that have to be made. To control rill and gully erosion, a species mixture of plants having fine roots to prevent incision by concentrated flow erosion and plants having a more deeply rooted structure in order to prevent shallow mass movements is recommended.

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Chapter 15
Root System Response to Drought and Salinity: Root Distribution and Water Transport

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15.1 Introduction

The greatest challenge faced by agriculture for the near years will be to increase food production using less water, especially in countries with limited water and land resources. Many studies have been conducted to improve the water-use efficiency in different crops by applying new strategies which decrease water consumption with a minimum impact on yield. In this sense, regulated deficit irrigation (RDI) techniques have been applied in deciduous orchards (Ruiz-Sánchez et al. 2010); it is based on that plant sensitivity to water stress is not constant throughout the growth season and that water stress during a specific period may be beneficial in terms of water saving and improvement of water-use efficiency. Another effective water saving method is partial-root drying (PRD), whereby half of the roots are well watered, while the other half are left to dry. The chemical signals produced in the drying roots reduce stomata conductance and control vegetative vigour, while the fully hydrated roots maintain a favourable water status (Dodd 2009). Wetting the soil may encourage the initiation and growth of secondary roots and recover the roots sensitivity to soil drying. In addition, a more evenly distributed root system in the soil as a result of alternate drying and wetting may also lead to a better use of soil nutrients in the whole root zone (Kang and Zhang 2004). An additional benefit of such techniques is that they would also reduce the

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volume of water used in nurseries and improve commercial plant quality by reducing excessive growth (Cameron et al. 2006; Álvarez et al. 2009).

In order to overcome water shortages and to satisfy the increasing water demand for agricultural development, the use of water of low quality (brackish, reclaimed, drainage) is becoming important in many countries (Chartzoulakis 2005; Yermiyahu et al. 2008). However, reclaimed water contains high concentrations of salt, which can be an important problem when it is used for irrigation. Recent research into the salt tolerance of various crops, water, soil and crop management and irrigation and drainage methods will enhance and increase the use of low-quality water for irrigation with minimum adverse impacts on yield, soil productivity and the environment (Pedrero et al. 2010; Bañón et al. 2011).

Plant responses to water stress and salinity have been discussed over some decades (Flowers et al. 1977; Greenway and Munns 1980; Franco et al. 2006; Chaves et al. 2011), and substantial research has been carried out in these areas. Most reports have focused on the alterations of the growth parameters of aerial parts (i.e. plant height, shoot length, leaf area, stem number and diameter, dry weight) (Navarro et al. 2009; Álvarez et al. 2011). Typical plant responses to salinity include reduced shoot growth and reduced whole plant size (Munns 2002). As salinity stress becomes more severe, foliar damage such as leaf burn, scorch, necrosis and premature defoliation may occur (Niu et al. 2010). However, the growth of stressed plants is often limited by the ability of roots to extract water from the soil and transport it to the shoot (Navarro et al. 2008). Also, the quantity of water moving from the root to the shoot and its speed determine the concentration of substances reaching the shoot (Navarro et al. 2007). In spite of this, little work has been done on roots with regard to salt or water stress. Roots might seem to be the part of the plant most vulnerable to such stress as they are directly exposed to salt or to drying soil; however, they are surprisingly robust. Their growth rate is not so affected as that of shoots (Munns 2002), sometimes provoking a decrease in the root to shoot ratio in plants submitted to water stress (Sánchez-Blanco et al. 2004) and to salinity (Navarro et al. 2008). As a result, the water-use efficiency of the plants under both stresses increases.

In recent years, progress has been made in understanding the chemical signalling related to the response of roots to stress conditions (changes in xylem sap pH and ABA concentrations, ethylene, reactive oxygen species) (Hsiao and Xu 2000; Liao et al. 2011).

In general, the size, morphology and architecture of the root system will determine the ability of plants to acquire water and nutrients (Passioura 1988) and influence the relative size and growth rate of the shoot (Vamerali et al. 2003). Optimum root systems can ensure optimum shoot growth and development since they serve as the interface between the plant and the soil. It is known that a prolific root system is more advantageous to the plant for acquiring water and nutrients. Moreover, root length is an indicator of the ability of a plant to absorb water from deeper layers of the soil (Franco et al. 2011a). However, recent studies have shown that species with small root systems may be more effective than others with larger root systems (Ma et al. 2010).

Normally, root hydraulic conductance is expressed with regard to the whole root dry weight, without taking account the role of root architecture on the capacity of water uptake. For any given value of root dry weight, the amount of fine roots which determine the root length and surface area may vary greatly, thus affecting to water absorption (Zobel et al. 2007). Therefore, the architectural features of the root as well as the amount of fine roots should be considered in any attempt to improve plant water uptake (Jonathan et al. 2006). Other root characteristics such as the width of the root cortex, number and diameter of xylem vessels, number of root hairs and suberin deposition, in both the root exodermis and endodermis, will also determine the permeability of the roots to water (Steudle 2000; Ranathunge et al. 2010) and are of great interest (Franco et al. 2008).

Research into plant root systems of woody trees under field conditions is difficult because the soils limit accessibility for observation. Reviews on the methods used for studying root systems (Smit et al. 2000; Milchunas 2009) run from traditional excavation methods, which are labour intensive and destructive, offering limited quantification and repeatability of measurements over time, to glass wall methods, rhizotron and minirhizotrons, non-destructive tools for observing root growth in situ.

The water entering the plant from the soil encounters significant resistance (Naor 2006). In soils with high clay content, lower stem water potential than observed in soils with low clay content, even under full irrigation, indicates limitation to the water absorption capacity of the roots system. Such limitations may be related with lower oxygen fluxes into the root zones or with resistance to root growth or low soil hydraulic conductivity (Naor 2006). Also, substrate temperature is a crucial factor in root development and function. For example, above or below the range 15–27 °C, root growth decreases, water and nutrient uptake is affected, and root morphology may be altered (Adam et al. 2003). In addition, inappropriate fertilisation, heavy metal toxicity, high atmospheric CO_2 or salinity may limit root growth.

Especially, in the case of woody trees, the choice of an appropriate rootstock adapted to local soil and climatic conditions can improve the water absorption capacity (Atkinson 1980). The overall water balance of the shoot can be affected by limitations of water supply to the plant imposed by the rootstock (Syvertsen and Graham 1985). In fact, studies have demonstrated that root hydraulic conductivity varies between rootstocks (Rodríguez-Gamir et al. 2010; Tombesi et al. 2010).

In the nursery, the cultivation of plants in pots or containers is a very common practice, because of the many advantages compared with ground cultivation. These advantages include lower stress for plants during transport and manipulation, space reduction in the nursery, the increased possibility of mechanisation, a longer supply period and greater transplantation success (Franco et al. 2006). However, container cultivation is strongly affected by environmental conditions in the substrate-root complex, where extreme temperatures can negatively influence root development. The climatic season will determine thermal stress through cold or heat. Container characteristics (material, colour, form, drainage holes, etc.) also influence temperature in the root system. Container size can affect the growth and development of plants; for example, lateral branching and leaf expansion are suppressed by root

restriction. It has been seen that using larger container sizes affects seedling establishment and survival in semi-arid environments. Also, containers of different types, e.g. rigid plastic, pot in pot, above-ground and root pruning containers (Miralles et al. 2012), reduce the number of roots in different ways and show different degrees of effectiveness in stopping root growth, thus affecting shoot and root growth (Franco et al. 2006).

This chapter reviews recent works on root system responses in ornamental species and woody trees growing in saline and water-deficit conditions. It includes aspects related with deficit irrigation management and the use of low-quality water in plants cultivated in pots or containers and in trees growing in soil. The aim is to extend our understanding of how the above-mentioned conditions may affect to the characteristics and activity of roots, especially the water absorption capacity.

15.2 Root System of Ornamental Species

15.2.1 Root Dry Weight

Root development and dry matter accumulation are strongly influenced by growing conditions such as moisture deficit. Growth reductions as a result of water deficit have been reported widely in many ornamental species (Sánchez-Blanco et al. 2002; Franco et al. 2006). Nevertheless, the absolute root biomass in drying soil may increase compared with the values observed in well-watered soils (Santos et al. 2007).

Water deficit has been seen to significantly reduce *Rhamnus alaternus* growth (Álvarez et al. 2012a). Drought induced a significant decrease in root dry weight of 48 %. *C. albidus* plants from the water-deficit treatment also showed a reduction in root dry weights, although these differences disappeared after re-watering (Sánchez-Blanco et al. 2002). The opposite was found in *Pelargonium hortorum* (Sánchez-Blanco et al. 2009) and in *Asteriscus maritimus* (Rodríguez et al. 2005) grown with different levels of irrigation, where there were no significant differences in the root dry weight.

However, it has been documented that the intensity of the root system response can also vary, according to the level and duration of the stress (Cameron et al. 1999). Differences in the water-deficit stress levels applied led to substantial differences in the root growth of myrtle plants; while moderate water stress produced no significant changes in root development, a severe water deficit clearly reduced that parameter (Navarro et al. 2009). This finding may be important for growers of ornamental plants because plants are often exposed to drought treatments during nursery production to reduce excessive growth. However, it goes without saying that it is first necessary to know the level of drought at which a species can maintain healthy growth and acceptable quality (Henson et al. 2006).

Different water stress levels applied also induced different growth responses in *Callistemon citrinus* in a greenhouse, suggesting that the severity of the water stress is an important aspect when used as an irrigation strategy to save water without reducing quality in ornamental species. *C. citrinus* plants submitted to severe deficit irrigation showed a reduction in the relative growth rate of root biomass production compared with a control treatment, while plants submitted to moderate deficit irrigation did not (Álvarez 2011).

Significant differences have also been seen in the growth of the other ornamental species under different levels of irrigation. For example, Álvarez et al. (2009) found that deficit irrigation reduced root dry weight proportionally to the imposed drought level in carnation plants and similar behaviour was recorded for *Phlomis purpurea* (Álvarez et al. 2012b). This response was maintained when *Phlomis purpurea* plants were exposed to salt stress. Both salinity and drought stress affected the root growth of the Phlomis plants, and root dry weight was similarly reduced in saline and both water-deficit treatments.

Although the response to water and salt stress was similar, ornamental plants have demonstrated wide variability in their reaction to environmental stresses. Previous research results have indicated that the salt tolerance of ornamental plants varies widely among species and that drought-tolerant native plants are not necessarily salt tolerant. This is the case with *Phillyrea angustifolia* and *Evonimus* plants, whose root system was less affected by drought than salt stress (Castillo 2011; Gómez-Bellot et al. 2013). Plant irrigated with saline water (100 % of crop needs; 4 dS m^{-1}) showed a reduced root dry weight, while plants that had received 50 % of the crop needs (1 dS m^{-1}) did not.

In contrast, the root system of *Pistacia lentiscus* and *Callistemon citrinus* plants was less affected by salt than water stress. Plant watered to 25 % water-holding capacity reduced their root dry weight, while plants that had been irrigated with saline water (4 dS m^{-1}) did not. In this case, then, drought had a more marked effect than salinity (Castillo 2011).

Irrigation water salinity has different effects on root growth. In general, root growth is inhibited by exposure to high salinity as a result of osmotic and toxic effects (Bañón et al. 2012), although this response depends on the species and salinity level. The responses of plant species to salinity and osmotic stress in terms of growth are the ultimate expression of several interacting physiological and biochemical parameters (Sidari et al. 2008).

Regarding the differences between species, *Phlomis* plants irrigated with water of 4 dS m^{-1} reduced root DW, although *C. citrinus*, *Evonimus* and *Pistacia lentiscus* plants irrigated with the same salt level did not (Castillo 2011; Álvarez et al. 2012b; Gómez-Bellot et al. 2013). This last species remained unresponsive to salinity even when plants were irrigated with much more saline NaCl solutions, 15 dS m^{-1}.

Furthermore, it has been documented that the degree of response to salt stress may vary considerably within a family, within a genus and even within a species. High NaCl had a negative effect on the DW of roots in *Fragaria x ananassa* cv. Selva (Khayyat et al. 2009), but Turhan and Eris (2005) reported that NaCl

increased root DW in the strawberry cv. Camarosa. This was also the case with *Callistemon*, in which irrigating with 4 dS m^{-1} had a negative effect on the DW of roots in *C. laevis*, while this parameter was not modified in *C. citrinus*.

When the combined effects of deficit irrigation and salinity were studied, the resulting plants had lower root dry weight than when the individual effects of each stress were studied, as in *Evonimus* and *Callistemon laevis*, whose respective root dry weights were greatly reduced.

Another important aspect to evaluate is the response of the root system to irrigation with reclaimed water since the use of low-quality water is becoming commonplace in many ornamental species. The toxic effect due to salt accumulation from reclaimed wastewater is generally assumed to be similar to that resulting from saline conditions, although this kind of water has different chemical properties with respect to salt water (NaCl solutions) and may contain high B, K^+ and S^- concentrations.

Irrigating with reclaimed wastewater induced a decrease in root growth in *Viburnum tinus* plants, even with irrigation water lower than 2 dS m^{-1}, while in *Eugenia myrtifolia, Pistacia lentiscus* and *Evonimus* plants irrigated with wastewater of 4 dS m^{-1}, root growth was not affected (Castillo 2011; Gómez-Bellot et al. 2013). However, in *Myrtus communis* a higher root dry weight was observed when plants were irrigated with wastewater (Acosta, J.R. personal communication).

15.2.2 Root to Shoot Ratio

Exposure to deficit irrigation usually has a pronounced effect on plant growth although this reduction does not always affect all the parts of the plant similarly. The effect of drought stress is usually greater on shoot growth than on root growth (Franco et al. 2011a). This lower sensitivity of roots appears to be a consequence of the rapid osmotic adjustment of roots in response to a decrease in the soil water content, thus maintaining water uptake, and of the enhanced loosening ability of root cell walls (Sharp et al. 2004).

Thus, an increase in the root to shoot ratio under drought stress is a frequently observed phenomenon and is caused either by a relatively great decrease in shoot growth than in root growth (Franco et al. 2006) or by an increase in root growth. However, according to other authors this parameter is not modified under deficit irrigation, or even decreases, depending on the species and variety, genotype, irrigation frequency and stress degree.

The distribution of assimilates from the aerial part to the root system in water stress situations has been observed by several authors in different species, such as *Capsicum annuum* (Davies et al. 2002; Kulkarni and Phalke 2009; Shao et al. 2010), *Lonicera implexa* (Navarro et al. 2008), *Lotus creticus* (Franco et al. 2001; Bañón et al. 2004), *Lupinus havardii* (Niu et al. 2007), *Myrtus communis* (Bañón et al. 2002), *Nerium oleander* (Bañón et al. 2006; Niu et al. 2008), *Opuntia ficus-indica* and *Opuntia robusta* (Snyman 2004), *Rhamnus*

alaternus (Bañón et al. 2003), two rose (*Rosa multiflora* and *Rosa odorata*) rootstocks (Niu and Rodriguez 2009), *Rosmarinus officinalis* (Sánchez-Blanco et al. 2004), *Sambucus mexicana* (Feser et al. 2005), *Silene vulgaris* (Arreola et al. 2006; Franco et al. 2008), *Limonium cossonianum* (Franco et al. 2002) and *Argyranthemum coronopifolium* (De Herralde et al. 1998).

Plant growth, especially the aerial part, is usually limited when soil water availability decreases. In contrast, the root to shoot ratio usually increases as a result of deficit irrigation treatments, largely because the reductions in shoot growth are not matched by an equivalent loss of root development (Sánchez-Blanco et al. 2004). This response could promote a more rapid establishment of ornamental plants in gardening or landscaping (Franco et al. 2006, 2011a).

These changes have been described in several ornamental species by Jaleel et al. (2008) in *C. roseus*, by Henson et al. (2006) and by Hassanein and Dorion (2006) in *P. hortorum*, by Andersson (2001) in *P. zonale* and by Andersson (2011) in *I. walleriana* and *Petunia x hybrid*.

In potted geranium plants, exposure to deficit irrigation caused a significant decrease in aerial dry mass, but had no significant effect on root mass. This was confirmed by the root to shoot ratio which increased in plants under water-deficit conditions. But following re-watering after the drought treatment, plants presented similar values to the control (Sánchez-Blanco et al. 2009).

The same responses were found by Álvarez et al. (2013) when geranium plants were exposed to regulated deficit irrigation in different phenological stages. Water deficit also had a significant effect on biomass accumulation in geranium plants. Aerial dry weight decreased with deficit irrigation. However, root dry weight was not modified and the root to shoot ratio increased in the plants grown under deficit irrigation conditions, regardless of the time when the reduction was applied (during the vegetative growth phase or during the flowering development phase).

The root to shoot ratio of the carnation plants stressed throughout the experiment was higher than in control plants and those exposed to deficit irrigation only during the first and third growth phases (outside blooming) (Álvarez et al. 2009).

In *Callistemon citrinus* plants grown under greenhouse conditions, exposure to deficit irrigation had a less pronounced effect on root mass. Thus, the root to shoot ratio also increased proportionally to the imposed drought level (Álvarez 2011).

This was confirmed in *Callistemon laevis* plants grown in a controlled environment, where water deficit was seen to have significantly altered plant growth (the total dry matter of drought-treated plants was 47 % of the control values) although the extent of the changes depended on the plant organ studied. The greatest accumulation of dry matter in relation to total plant dry matter was seen in the leaves of the control plants and in the roots of stressed plants. The application of a water deficit to the plant substrate led a decrease in aerial dry matter accumulation while the root to shoot ratio increased in the plants grown under drought conditions (Álvarez et al. 2009).

Nevertheless, the root to shoot ratio is not always modified as a result of drought stress. Although *Rhamnus alaternus* plants exposed to deficit irrigation showed lower biomass accumulation, the root to shoot ratio was not modified (Álvarez

et al. 2012a). As regards biomass partitioning in each part of the plant (leaves, stem and root), no differences between the control and stressed plants were observed.

The same response was observed in *Pistacia lentiscus*, where the root to shoot ratio was not modified as a result of drought stress. Deficit irrigation decreased the aerial and root dry weight to a similar extent, and so no differences were found between control and deficit irrigation treatments as regards biomass distribution in the root, stems and leaves.

The root to shoot ratio was not affected in *Phillyrea angustifolia* either in this case, because deficit irrigation did not reduce aerial or root growth.

The water stress level must be considered an important aspect since if the stress is too severe, the effect may be the opposite. In *Myrtus communis* plants, the root to shoot ratio decreased in the plants exposed to severe water deficit (Navarro et al. 2009). After 5 months since beginning of treatments, the severe water-deficit treatment was seen to have reduced both shoot and root DWs and the root to shoot ratios of the *M. communis* plants compared with the controls and those exposed to moderate water deficit.

The degree of response to water stress may vary considerably within a family, within a genus and even within a species. *C. monspeliensis* plants submitted to water deficit had a lower total biomass than control plants due to the significant reduction in shoot dry weight, but the root dry weight was not affected by the water deficit. Both *Cistus* species plants submitted to drought stress had lower total biomass (10 %) at the end of the water shortage periods than the control plants. However, as regards the allocation of the biomass reductions, both species behaved differently. For example, *C. monspeliensis* reduced its leaf dry weight, whereas *C. albidus* reduced its leaf, stem and root dry weights (Sánchez-Blanco et al. 2002).

Ornamental shrubs in general have demonstrated wide variability in their reaction to water stress and salinity (Cassaniti et al. 2009). The different distribution of biomass induced by both stress situations may be due to the need to maintain a higher root surface area under drought conditions and the need to reduce root volume in plants exposed to salinity, which may be a favourable trait limiting their capacity to accumulate toxic ions in the shoot (Munns 2002; Alarcón et al. 2006). The effect of salt stress and water stress on plant growth and dry matter accumulation has been described in several crops species (Shannon and Grieve 1999; Sánchez-Blanco et al. 2002; Rodríguez et al. 2005).

The different stresses applied to *Phlomis purpurea* plants induced different growth responses: both soil drying and salinity that reduced plant growth, especially in drought-exposed plants, which showed the lowest aerial dry weight values, resulting in an increased root to shoot dry weight ratio. This data suggest that shoots are more sensitive to water stress than roots. This latter response was not maintained when plants were exposed to salinity. As regards biomass partitioning with respect to total biomass production, no differences between the control and saline treatment were observed (Álvarez et al. 2012b). These data suggest that Phlomis plants are more sensitive to water stress than to salinity.

In *P. lentiscus*, salinity did not reduce plant growth, but water stress reduced aerial and root growth to a similar extent. The opposite was found in *P. angustifolia*

where the growth was not affected by deficit irrigation, but salinity reduced both aerial and root growth strongly, also to a similar extent (Castillo 2011).

The root to shoot ratio increased in Callistemon plants under greenhouse conditions and irrigated with saline water, just as was found with deficit irrigation (Álvarez et al. 2011).

Watering with reclaimed wastewater did not modify this ratio in *Evonimus* plants despite the growth reduction observed. However, plants irrigated with a NaCl solution with the same EC (4 dS m^{-1}) increased the ratio due to a marked reduction in leaf DW. After a recovery period, this effect was reversed when plants were irrigated with normal water. The root to shoot ratio of *Viburnum tinus* plants tended to increase in plants irrigated with the water normally used in the area (reclaimed wastewater blended 50 % with well water, EC 1.5 dS m^{-1}), but when wastewater of higher EC (4 dS m^{-1}) was used, this parameter was not modified and tended to decrease when plants were irrigated with NaCl solution of the same EC (Gómez-Bellot et al. 2013).

Eugenia myrtifolia is more tolerant to irrigation with reclaimed water with a high EC (1.5, 4, 8 dS m^{-1}). The root to shoot ratio increased proportionally with the level of electrical conductivity of the reclaimed water, due to aerial growth reduction.

15.2.3 Root Morphology

The root system is an important factor for successful transplanting and establishment in the field, where root anatomy and structure may be decisive for plant survival (Steudle and Peterson 1998; Bañón et al. 2004).

Root length is a critical factor in influencing the ability of a plant to absorb water from the soil under water stress conditions. In this sense, previous studies in some ornamental species have indicated that plants subjected to low-moisture regimes can develop a more extensive root system. This was the case with *Phaseolus vulgaris*, as reported by De Sousa and Lima (2010), who found an increase in root length under drought stress. Drought also caused longer roots in geranium and impatiens plants growing in soil with 30 % water content compared with 80 % (Chyliński et al. 2007). Similarly, *Viburnum odoratissimum* plants growing under drought conditions exhibited greater root growth (Shober et al. 2009).

Nevertheless, in a variety of species, the opposite effect has been found in response to deficit irrigation, as reported in *Nerium oleander* (Bañón et al. 2006) and *Rhamnus alaternus* (Bañón et al. 2003; Álvarez et al. 2012a). Álvarez et al. (2011) reported that meeting 50 % of plant needs had a significant effect on root morphology in Callistemon plants grown in a growth chamber. Total root length decreased by 27 % with water stress, a reduction observed in all root sizes. Besides, this reduction was proportional to the imposed drought level when Callistemon plants in a greenhouse were submitted to two water-deficit levels (Álvarez 2011).

Independently of root length, the change in root distribution for each root diameter can also be modified by the irrigation. Thinner roots, compared with non-stressed controls, were reported for drought-stressed *S. vulgaris* (Franco et al. 2008) and *L. esculentum* (Kulkarni and Deshpande 2007). This behaviour has also been described in Callistemon by Álvarez et al. (2011), who observed that water deficit increased the percentage of fine roots and decreased those with a diameter higher than 0.5 mm. A greater percentage of fine roots, capable of penetrating smaller soil pores, presumably optimises the exploratory capabilities of the root system as a whole and may have an important role in the survival of plants faced with adverse edaphic factors (Koike et al. 2003). In contrast, deficit irrigation increased the percentage of thick roots and reduced the percentage of medium and fine roots in *M. communis* and *N. oleander* plants (Bañón et al. 2002, 2006). Similarly, the shift in root diameters in moderately stressed Callistemon plants was due to both the greater production of medium-sized roots and the lower production of fine roots. This reduction was observed especially in roots *R. alaternus* with a diameter of less than 0.5 mm, because roots with a diameter greater than 1 mm increased their length significantly with water stress effects (Bañón et al. 2003). The root system morphology was modified by water stress in an attempt to improve plant physical support. The plants subjected to deficit irrigation showed morphological changes in their root system, being shorter, less ramified and thicker.

Likewise, the root surface area of *S. vulgaris* plants increased under moderate drought stress (Franco et al. 2008). This can minimise localised water depletion around the roots, thus minimising resistance to water transport to the root system (Franco et al. 2006).

In general, in stressed plants, root volume is reduced more than dry weight, with the result that root density increases under deficit irrigation. This was the case with *Rhamnus alaternus* (Bañón et al. 2003) and *Callistemon* (Álvarez et al. 2011). The greater root density of these plants suggests greater robustness and, presumably, a higher accumulation of reserves (Cameron et al. 2006; Franco et al. 2006; Álvarez et al. 2011). This may also be interpreted as an accumulation of solutes by the plants in order to maintain the water gradient necessary for absorbing water even when in short supply in the soil. Processes of osmotic adjustment in roots through the active accumulation of organic solutes from the aerial part would alter the physical support, strengthen the roots and lessen the possibility of breaking during transplanting (Bañón et al. 2006). In this sense, moderate deficit irrigation in nursery conditions would improve plant resistance to water-deficit situations in plants grown in field conditions after transplanting (Franco et al. 2001). This behaviour was also seen to be related to the reordering of the assimilate gradient as the flow of solutes towards the roots intensified.

The hardening of roots, as revealed by an increased percentage of brown roots at lower irrigation rates in *Lotus creticus* (Franco et al. 2001) and *Limonium cossonianum* (Franco et al. 2002), is of great interest for producing seedlings that are better adapted to drought stress after transplantation. The change in colour from white to brown is associated with suberisation of the exodermis and may reflect a

metacutisation process, a process of lignification and suberisation that results in a resting root being protected against significant fluctuations in environmental conditions, including drought, and renders it capable of regrowth when conditions improve.

As regards root growth under saline conditions, root length decreased with irrigation solutions of increasing EC in experiments with three varieties of *Solanum melongena* (Akinci et al. 2004) and *P. oleracea* (Franco et al. 2011b). Croser et al. (2001) and Franco et al. (2011b) also found an increase in root diameter (hypertrophy) in response to salinity.

In Callistemon grown under greenhouse conditions, the effect of irrigation with saline water was very similar to that of water stress, a decrease in total root length, which was observed in all root sizes, an increase in the percentage of thick roots and a decreased percentage of fine roots. Salinity decreased root volume, although root dry weight was not modified, with the result that root density increased in these plants.

Likewise, salt stress can cause profound modifications to root architecture. NaCl treatment of *L. esculentum* resulted in a more branched root system, compared to the untreated controls, with the roots being shorter and each main root having more lateral roots. These modifications gave rise to a larger root system (Karni et al. 2010). In addition, Rose et al. (2010) reported shallower root systems for plants grown under saline conditions than in plants grown with adequate rainfall.

A significant decrease in total root length was observed in *Evonimus* plants irrigated with NaCl solution and reclaimed water, more specifically in thin ($Ø \leq 0.5$ mm) and medium thickness ($0.5 < Ø \leq 2.0$ mm) roots. Neither the dry weight nor the total length of the roots of those plants recovered after being irrigated with normal water (Gómez-Bellot et al. 2013).

15.2.4 Root Hydraulic Conductivity

One indicator of the capacity of a plant to absorb and transport water is the root hydraulic conductivity or its inverse, root hydraulic resistance. This parameter depends on several factors such as root surface area, the density of vessels and the length and the diameter of root vessels, which can be modified under deficit irrigation conditions. Thus, an increase in root surface area under drought stress can minimise localised water depletion around the roots, thus reducing the resistance to water transport to the root system (Franco et al. 2006). A high density of vessels would improve resistance to water-deficit situations. This was the case with *L. creticus* in a study by Bañón et al. (2004), who reported that plants subjected to deficit irrigation showed a higher density of xylem vessels in their roots than well-watered plants. Drought also caused a decrease in the diameter of root metaxylem vessels, increasing water flow resistance in *C. annuum* (Kulkarni and Phalke 2009), although a combination of larger and smaller xylem vessels provided better stress tolerance and biomass production under water-deficit conditions.

Increased water flow resistance from the substratum to the plant in water stress conditions has been observed in many species (De Herralde et al. 1998; Sánchez-Blanco et al. 2002), while no significant differences in root hydraulic resistance were observed in geranium plants submitted to a variety of different irrigation strategies (Álvarez et al. 2013).

The water deficit applied in Callistemon produced increases in root hydraulic resistance, with values of 1.02 and 3.20 g MPa s mg^{-1} recorded for the control and drought treatments, respectively (Álvarez et al. 2011).

Deficit irrigation applied in myrtle plants led to increases in root hydraulic resistance proportional to the level of drought imposed, with values of 1.4, 3.3 and 5.4 g MPa s mg^{-1} recorded for the control, moderate, and severe water-deficit treatments, respectively. This could have affected the decrease in values of leaf water potential at predawn (Navarro et al. 2009). The higher root hydraulic resistance in stressed plants may have reduced water transport towards the leaves. Such a response would cause the leaf water and leaf turgor potentials in water-deficit treatments to decrease, especially at dawn, which would cause a substantial fall in stomatal conductance (Pereira and Chaves 1993; Munné-Bosch et al. 1999).

On the other hand, root hydraulic conductivity may also be affected by salinity, although the plant response can vary between water and salt stress. This is the case with *Phlomis purpurea*, as reported by Álvarez et al. (2012b), who found a decrease in root hydraulic conductance in the severe water-stressed plants, while no significant differences were observed between saline, moderate water stress and control treatments.

On the other hand, the response of root hydraulic conductance was seen to differ between control and saline treatments in *Arbutus unedo* plants, where root hydraulic conductivity decreased proportionally to the saline concentration applied (Navarro et al. 2007). Some researchers have found that long-term exposure to sodium chloride affects root permeability (O'Leary 1969). Such variations in root hydraulic conductivity as a result of saline stress affected the water flow through the root system as could be seen from the soil water content. In the saline conditions, the water from the irrigation remained in the substrate and a significant increase in the volumetric water content in the substrate of the pots was observed as the external salinity increased.

Salt stress also reduced the facility to take up water from the substrate in *Pistacia lentiscus* plants as can be seen from the values recorded for root hydraulic conductivity. This parameter decreased with irrigation solutions of increasing electrical conductivity (Castillo 2011).

A similar response was found in *Eugenia myrtifolia* plants irrigated with different treated municipal wastewaters. In general, this parameter fell as the irrigation water electrical conductivity increased, even after a 2-month recovery period, when all plants were irrigated with the control water. The species under observation showed no recovery of this parameter during relief from salinity due to the salt accumulated in the substrate. Soil salinity management is recommended to ensure the successful use of recycled water, paying particular attention to the specific salt level in recycled water for each species.

15.2.5 Rates of Ions Absorption by Roots

Because NaCl is the most soluble and widespread salt, it is not surprising that all plants have evolved mechanisms to regulate its accumulation and to select against it in favour of other nutrients commonly present in low concentrations. In this sense, plant tolerance has been widely correlated with the root system ability to limit uptake and/or transport of Na^+ and Cl^- to aerial parts, retaining these ions in the root and lower stem (Murillo-Amador et al. 2006) and limiting to transport to the shoots (Colmer et al. 2005).

In general, an increase in external NaCl concentrations induces an increase of Na^+ and Cl^- in roots and leaves of different ornamental species. This is the case with *Phlomis purpurea* plants, as reported by Álvarez et al. (2012b), who found that the concentrations of both ions increased with salinity (Na^+ especially in leaves, while the increase in the Cl^- concentration was similar for roots and leaves); however, no accumulation of Cl^- and Na^+ was observed in the plants subjected to water stress. An analogous response was recorded for Bougainvillaea (Cassaniti et al. 2009).

Viburnum tinus was neither able to limit transport Na^+ and Cl^- from roots to the shoots, as both ions accumulated in the leaves of the plants irrigated with irrigation water of 4 dS m^{-1}. As regards their distribution, Na^+ concentration in plants irrigated with water with EC lower than 2 dS m^{-1} was higher in roots than in leaves. Nevertheless, when EC increased to 4 dS m^{-1} the distribution changed, and the concentration was higher in the leaves. A similar response was also found for Cl^- accumulation. These results agree with a work by Bañón et al. (2012), which classified this species as salt-sensitive, since salinity produced a significant growth reduction even in the plants irrigated with water of EC lower than 2 dS m^{-1}.

However, the opposite behaviour has been found in some tolerant species, such as *Eugenia myrtifolia*, in which substantial increases in external NaCl concentrations (up to 8 dS m^{-1}) only induce slight increases of Na^+ and Cl^- in roots and leaves. Besides, both ion concentrations increased similarly and plants showed similar Na^+ and Cl^- concentrations in roots and leaves. Eugenia plants irrigated with different treated municipal wastewaters only increased leaf Na^+ concentration when irrigated with water of 4 dS m^{-1} or more, while the increase in leaf Cl^- concentration only occurred above 8 dS m^{-1}.

Another important mechanism that allows plants to survive and grow in saline conditions is the restricted entry of ions through the roots. Thus, in halophytes, the natural flora of highly saline soils, Na^+ and Cl^- are effectively excluded by roots, while water is taken up from the soil, even at very high salinity levels. For example, sea barley grass, *Hordeum marinum*, excludes both Na^+ and Cl^- up to at least 450 mM NaCl. A similar response has been found in field-grown *Atriplex* irrigated with reclaimed wastewater, in which the Cl^- concentration did not increase in any of the parts of the salt-stressed plants, and Na^- was withheld so effectively in the woody roots and stems that little reached the leaves. According to Munns and

Tester (2008), *Atriplex* spp. continues to grow well at salinity levels greater than that of seawater.

15.3 Root System of Woody Trees

15.3.1 Roots and Drought

15.3.1.1 Root Distribution

Although root development is genetically determined in different plant species, climate, together with the physical, chemical and biological characteristics of the soil, can change this pattern (Atkinson 1980; Baker et al. 1992). Soil temperature and shoot growth affect root growth in woody plants (Bevington and Castle 1985), although it is generally accepted that soil water availability, among other edaphic factors, has the greatest importance (Fernández et al. 1992). For these reasons, in irrigated conditions the root system is largely restricted to the wetted zone, as established by the studies in apple (Levin et al. 1979), apricot (Pérez-Pastor et al. 2014; Ruiz-Sánchez et al. 2005), almond (Abrisqueta et al. 1994), citrus (Bielorai 1982), olive (Fernández et al. 1991) and peach (Abrisqueta et al. 2008) trees, among others; overall, these studies conclude that almost the whole fruit tree root system is located in the first 0.75 m of soil depth. This is a common feature of drip irrigation systems, where hydrotropism dominates over geotropism.

Root depth penetration and root length density (RLD) determine a plant's ability to withstand water stress (Smucker and Aiken 1992); high RLD values and plant vigour have been associated with a high efficiency of water and nutrient assimilation. A large root system may improve a plant's ability to continue growth during drought, and it has been found that the genotypes with larger root systems can support accelerated plant growth, maintaining relatively high transpiration efficiency under water stress conditions (Puangbut et al. 2009).

Deficit irrigation, both continuous and regulated, induced higher root length density values in the wet zone of apricot trees compared with the well-irrigated trees (Pérez-Pastor et al. 2014), and the same was found in subsurface deficit-irrigated almond trees (Romero et al. 2004), mainly due to the smaller size of the wet bulbs in the deficit irrigation. This effect would facilitate the rapid depletion and recharging of the soil water content in the periods of application and recovery from deficit irrigation (Lampinen et al. 1995). Nevertheless, total root growth was lower, and root system extent was reduced under deficit irrigation conditions (Pérez-Pastor et al. 2014; Abrisqueta 2010) (Fig. 15.1).

Water deficit affects vegetative growth and often leads to restrictions in root growth (Chalmers et al. 1984), but in contrast drought has been shown to stimulate root growth in the deeper soil layers. Comas et al. (2005) showed that nonirrigated vines produced more roots at greater depths in dry years than vines receiving

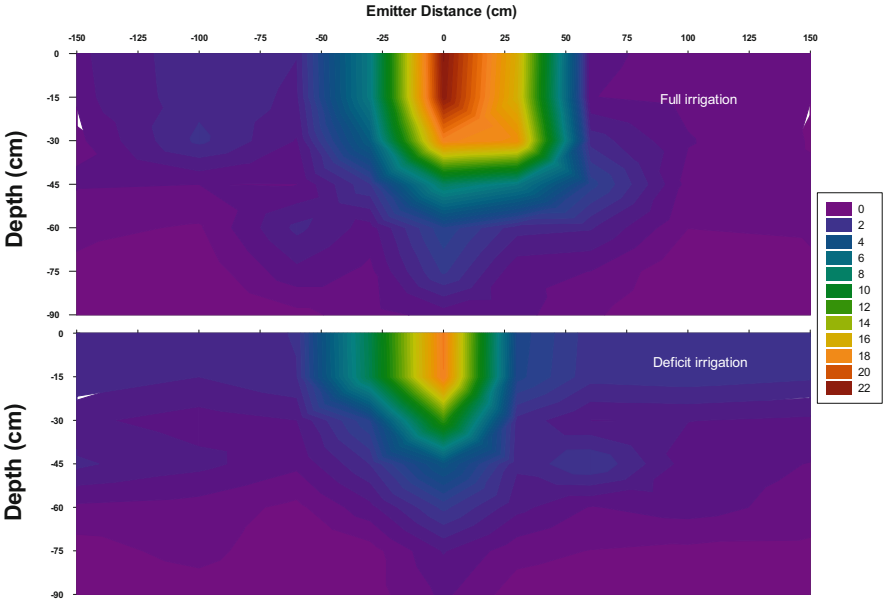

Fig. 15.1 Root length density of drip irrigated peach trees under full irrigation (*upper graph*) and continuous deficit irrigation (*lower graph*) [from Abrisqueta (2010)]

irrigation, because roots could extend to greater soil depths to absorb water. The promotion of root biomass allows the root system to access resources that would otherwise be unavailable to well-irrigated plant. The stimulation of root development during severe soil water deficits may play an important role in drought resistance (McCully 1999). In this sense, if deep rooting genetic materials can be combined with high yielding ones, the result could have a beneficial impact on production in areas where deep soil water is available. However, if additional photosynthates are necessary to form and maintain deeper root systems, the trade-off for higher yields may not be possible. For these reasons, Ritchie (1981) concluded that there have been no clear field demonstrations of the benefits derived from breeding plants for deeper root water extraction.

In field-grown experiments, there was no clear evidence of a greater capacity for deeper soil exploration in deficit-irrigated peach trees as compared with well-irrigated trees, although, root length density data revealed that alternate irrigation in partial-root drying treatment stimulated root production along the season, compared with the continuous deficit-irrigated treatment (Abrisqueta et al. 2008). In this sense, Green and Clothier (1999) observed that apple root system can modify their water uptake strategy in a very short period, by adjusting uptake to match the current soil moisture pattern of seemingly inactive roots after irrigation.

15.3.1.2 Root to Shoot Ratio

Vegetative growth has long been known to be highly sensitive to water stress, root growth being more resistant than leaf growth (Hsiao and Xu 2000). Gowing et al. (1990) showed that the inhibition of leaf growth by drying part of the root system in apple trees was alleviated by the excision of this part of the roots, which was taken as evidence of a positive inhibitory effect, produced by drying roots and which influences shoot growth. Fort et al. (1997) suggested that plants with a well-established root system can utilise localised supplies of available soil water to maintain leaf gas exchange despite appreciable portions of the root system being in dry soil. However, Nicolás et al. (2005) found a similar decrease in shoot and root growth in peach trees submitted to different partial-root drying treatments, although root growth decrease was higher in the trees with drought applied in two compartments than in those where drought was applied to only one compartment, probably because of compensatory root growth in the well-watered compartment. Thus, it was concluded that hydraulic and non-chemical signals in young peach trees are responsible for the shoot response in a water-deficit situation.

Also, it must be taken into account that any modification in growth partitioning in favour of the root system may be accompanied by an improvement in the tree water status and so that the increase in above-below ground biomass ratio could be related with the gradual decline in the water status of well-irrigated deciduous trees during the season (López et al. 2008).

15.3.1.3 Root Dynamics

The root system dynamics throughout the season, as measured by minirhizotron, indicated no quiescent period in field-grown peach and almond trees growing under Mediterranean conditions (Fernández et al. 1992; Franco et al. 1995; Abrisqueta et al. 1994, 2008), demonstrating that soil temperature and humidity were not limiting as they are in colder geographic zones or in dry farming (Westwood 1982). Growth of the root system in woody trees corresponded to non-suberised very thin roots with diameters of $\leq$0.5 mm (Abrisqueta et al. 2008), which are the most active in water uptake (Zhang et al. 1996).

In general, the root growth rate declined during the fruit growth period and increased after harvest to reaching a peak in summer. Alternate growth patterns between aerial parts (buds, trunks and fruits) and roots have also been observed in other tree species, including apricot (Pérez-Pastor et al. 2004), almond (Ross and Catlin 1978) and citrus (Bevington and Castle 1985) trees. These authors indicated that the alternating growth pattern might be due to the competition for assimilates as well as to the inhibitory effect of the auxins produced during active shoot growth periods, which cause low root growth rates. Other authors reported a decrease in root growth during summer, especially when evapotranspiration demands are high (Fernández et al. 1992).

The higher root growth observed after harvest (Abrisqueta et al. 2008) could be an additional cause of the decrease in peach productivity recorded by Girona et al. (2003) when severe postharvest water deficits were applied.

15.3.2 Roots and Salinity

15.3.2.1 Root Growth and Architecture

Salinity is a very common problem in Mediterranean regions where high rates of evaporation and insufficient leaching occur. Moreover, field crops are often irrigated with low-quality water. Recently, the use of reclaimed water in agriculture has become an important management strategy in areas with limited freshwater resources (Al-Absi et al. 2009; Pedrero et al. 2013; Mounzer et al. 2012). However, this kind of water generally tends to have a higher salt content than the irrigation water from other sources.

Plants irrigated with low-quality water may develop mechanisms to adapt saline stress, allowing better development under these conditions. Such mechanisms can be functional and/or structural and include root architectural changes. In this sense, Rewald et al. (2012) described the phenological and physiological plasticity of *Citrus volkameriana* rootstocks under severe salt stress, identifying a rapid and major modification of the root system involving a decrease in the surface root area and the number of lateral roots. This was indicative of a decrease in functionality in terms of uptake as several studies on woody species have remarked (Eshel and Waisel 1996; Croser et al. 2001; Korn 2004).

Also, salts often promote suberisation of the hypodermis and endodermis in woody tree roots, resulting in the formation of a well-developed Casparian strip closer to the root apex than is found in non-salinised roots (Walker et al. 1984). The walls of root cells of salinised plants are often unevenly thickened and convoluted (Shannon et al. 1994).

Furthermore, the morphology of some plants makes them sensitive to salinity. In the case of avocado trees, for example, the fact that the root system is quite superficial and presents low ramification, thus reducing water and nutrient absorption capacity (Whiley et al. 2002), results in heightened sensitivity to soil salinity (Bernstein et al. 2004). These morphological features limit this crop to areas where irrigation water is of good quality.

15.3.2.2 Root to Shoot Ratio

High salt concentrations in the irrigation water result in lower plant growth (Munns and Tester 2008), limiting leaf expansion (Cramer 2002) and changing the relationship between aerial and root parts (Tattini et al. 1995). These mechanisms are the result of metabolic changes (synthesis of abscisic acid and osmoprotectant

solutes, such as proline or betaine) and physiological changes (altered membrane permeability to ions in the water, stomatal closure, reduced transpiration and photosynthesis, etc.).

Although, in most woody trees, shoot growth is more sensitive to salt stress than root growth, increasing the root to shoot ratio, the few reports available demonstrate an inhibitory effect of salinity on root development (Bernstein et al. 2004). The inhibition of root growth, and hence root surface area, is also suggested by the reduced capacity of trees under salt stress to take up water (Lo Gullo et al. 2007).

In situations of moderate and high salinity, many studies have shown that growth of olive trees (i.e. shoot length, total leaf area, dry weight, root length and rooting ability) is inhibited (Marin et al. 1995; Tattini et al. 1995; Tattini and Traversi 2008). Under high salinity, dry root mass was higher than shoot dry mass resulting in an increased root to shoot ratio (Chartzoulakis et al. 2002), which is thought to improve the source/sink ratio for water and nutrients in such conditions (Zekri and Parsons 1989). Also, in Loquat plants, salinity resulted in the redistribution of dry matter, favouring the development of roots at the expense of shoots (García-Legaz et al. 2008), which could be considered as an adaptive response to maintain the shoot/root equilibrium when the uptake capacity of the roots to give water and nutrients to the leaves is reduced (Engels and Marschner 1992).

15.3.2.3 Root Hydraulic Conductivity

Hydraulic conductivity determines the capacity of water transport affecting plant water relations as well as the water and nutrient absorption efficiency. It is considered one of the main factors that controls the movement of water through the soil-plant system (Kriedmann and Barrs 1981) and has an important influence on plant transpiration and on other physiological processes related with transpiration. According to Passioura (1988), differences in root hydraulic conductance may cause differences in water transport to the aerial part of the plant, influencing leaf water status and therefore the growth and other physiological responses of the plant (Lo Gullo et al. 2007). Furthermore, it was proposed that the hydraulic conductance of apple (Cohen and Naor 2002; Cohen et al. 2007) and peach (Solari et al. 2006) rootstocks may be involved in the mechanisms controlling plant size.

Root hydraulic conductivity in field crops may vary in response to the salt content of the irrigation water applied (Steudle 2000). In general, plants irrigated with poor quality irrigation water tend to decrease root hydraulic conductivity. In fact, one of the main factors affecting the sustainability of a reclaimed water irrigation system is the hydraulic conductivity of the root. Salinity reduces plant growth through osmotic and toxic effect, and high sodium adsorption ratio values cause sodicity which increases soil resistance, reduces root growth and reduces water movement through the root with a reduction in hydraulic conductivity (Rengasamy and Olsson 1993).

These changes in root hydraulic conductivity could depend on plant morphology and the anatomical characteristics of the xylem tissues (Moya et al. 2003).

Rodríguez-Gamir et al. (2010) demonstrated that the main anatomical difference between citrus rootstocks in non-salinised soil may be related with their hydraulic conductivity. Roots with xylem vessels of a larger diameter had greater hydraulic conductivity (Gonçalves et al. 2007). Zekri and Parsons (1989) showed that the decrease in citrus root hydraulic conductivity under saline conditions was associated with an increase in specific root weight, i.e. a higher ratio of root mass to root length. Tombesi et al. (2010) reported that the anatomical characteristics of the xylem of peach rootstocks appear to influence their hydraulic conductivity.

15.3.2.4 Ion Uptake and Partitioning of Cl^- and Na^+ in Plant Roots

It is well established that salt can impair the performance of many agricultural plants. Salts present in the soil and irrigation water are serious problems for commercial agriculture, particularly in arid and semi-arid regions. For example, reclaimed water typically contains high levels of nutrients (mostly NH_4^+, PO_4^{2-} and organic nutrients), salts (particularly Na^+, K^+, Cl^-), bacteria, viruses and parasites, and there is also a possibility of high boron and heavy metal levels. Boron loads may result in B toxicity for sensitive crops, while the loads of heavy metals are not likely to cause any immediate problems, although they may accumulate in the soil following long-term irrigation and become a threat to animals through food chain transfer. However, the most common problem in using reclaimed water is the strong tendency for them to have a higher salinity.

Salinity increases ion concentrations differently between plant parts and in different ways, depending on the species, rootstocks and environmental conditions. In general, root and leaf concentrations of sodium and chloride in stone-fruit trees increased when low-quality irrigation water was applied (Chartzoulakis et al. 2002; Melgar et al. 2008).

Usually, sodium is retained in the roots and lower trunk. An example of this is the higher Na^+ concentrations in citrus root than in the leaves of plants irrigated with highly saline water (Melgar et al. 2008). This agrees with observations made in peach trees (Boland et al. 1996). However, both citrus and peach trees accumulated Cl^- in the leaves and an excess can be toxic for the plant (Ferguson and Grattan 2005; Fornes et al. 2007). Chloride moves readily with the soil water and is taken up by the roots. It is then transported to the stems and leaves (Grattan 2002). In woody trees, the total amount of Cl^- that the leaves take up may be limited by the ability of these trees to retain this ion in woody tissues (Ziska et al. 1991).

Roots play an important role in the mechanisms involved in tolerance to salinity. Salt tolerance in most woody trees is associated with an effective mechanism of ion exclusion. In peach (Boland et al. 1996), olive (Cimato et al. 2010), plum (Bolat et al. 2006) and citrus (García-Sánchez et al. 2006) trees, among others, this mechanism aimed at limiting the flux of harmful ions to sensitive shoot organs mainly involves a reduced water mass flow and inherently low relative growth rates (Loreto et al. 2003; Remorini et al. 2009). Relatively low fluxes of potentially toxic ions to the leaves are usually associated with an early osmotic imbalance in the leaf

(Tattini and Traversi 2008). However, the salt-exclusion mechanism in some crops only works effectively below a threshold level of salinity. For example, it works effectively at low and moderate levels of salinity in olive trees, while, at high levels, salt ions accumulate in the roots and leaves, resulting in toxicity symptoms (Tattini et al. 1995; Chartzoulakis et al. 2002).

Harmful ions accumulation in a given scion may be greatly modified by the rootstock. The choice of a salt-exclusion rootstock for a scion is important in areas with highly saline water since differences in resistance to salinity of the plant depend largely on the ability of roots to exclude salts. The variations observed among citrus (García-Sánchez et al. 2006), avocado (Castro et al. 2008), grapevines (Walker et al. 2002) and stone-fruit tree (Beckman 2003) rootstocks in their resistance to salinity are attributable to variations in their salt-exclusion attributes.

15.4 Conclusion

In conclusion, this revision provides insight into the importance of understanding the limitations in root activity resulting from deficit irrigation and salinity conditions in different species. It describes research into understanding how changes in hydraulic conductivity, morphology and root function influence plant water-use and nutrient efficiency.

Plant responses to water stress and salinity have been widely discussed in many species. The growth of stressed plants is often limited by the ability of roots to extract water from the soil and transport it to the shoot, which determines the concentration of substances reaching the aerial part. In spite of this, little work has been done in connection with roots as regards salt or water stress. Although roots might seem the most vulnerable part of the plant, since they are directly exposed to salt or to drying soil, the intensity of the root system response may vary according to the species, the level and duration of the stress and cultivation conditions. For example, roots growing in pots are more affected by environmental conditions in the substrate-root complex than they would be with ground cultivation. This work reviews the recent literature on root system responses in ornamental species grown in containers and woody trees growing in soil exposed to deficit irrigation strategies and alternative irrigation sources (brackish, reclaimed). The aim is to extend our understanding of how these conditions may affect the characteristics and activity of roots, especially the water absorption capacity.

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Chapter 16
Cluster Roots

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16.1 Introduction

16.1.1 Cluster Roots and Plant Nutrition

Phosphorus (P) is one of the most important plant nutrients that significantly affect growth and plant metabolism (Yan et al. 2002; Richardson et al. 2004; Vitousek et al. 2010). Although the amount of total P in soil may be high, it is often present in unavailable forms such as phytic acid (Richardson 1994) or Ca, Fe, and Al phosphates (Holford 1997). Frequently P is the second mineral nutrient limiting plant growth, after nitrogen. Modern agriculture requires annual fertilizers rich in P, since most of it (80–90 %) may be adsorbed to soil colloids and hence be unavailable to plants (Jones and Brassington 1998). The high prices of fertilizers and problems associated with their application (such as groundwater eutrophication) as well as the depletion of global P reserves (nonrenewable resources) (Cordell et al. 2009) make it relevant to study the mechanisms used by plants that are more efficient in P acquisition. This is the case of cluster-bearing species.

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Mycorrhizal fungi, nitrogen-fixing bacteria, and cluster roots (CR) have been considered the primary strategies used by plants to increase nutrient uptake (Skene 1998). Cluster roots have been described as dense clusters of fine rootlets around a main axis (Purnell 1960) and are found in most species belonging to the Proteaceae family, with the exception of *Persoonia* (Lamont 1982; Skene 2000). Other species that show CR have been found in nine other families such as Betulaceae, Casuarinaceae, Cyperaceae, Elaeagnaceae, Fabaceae, Myricaceae, and Restionaceae (Lambers and Shane 2007) and are distributed in both the Northern and Southern Hemispheres in regions with significant biodiversity (Skene 2000). Cluster roots are composed of determinate rootlets that grow for a limited time but remain physiologically active for some days (Skene et al. 1998).

Cluster roots increase the surface area available to absorb nutrients and exude large amounts of carboxylates (Lambers et al. 2002, 2006; Lamont 2003; Sousa et al. 2007), acid phosphatase (Reddell et al. 1997; Gilbert et al. 1999; Neumann et al. 1999; Delgado et al. 2013), and/or proteases (Schmidt et al. 2003; Paungfoo-Lonhienne et al. 2008) compared with non-cluster roots.

The carboxylates exuded from roots are important for promoting P mobilization, because they form stable complexes with cations that are bound to phosphates (e.g., Al^{3+}, Fe^{3+}, and Ca^{2+}) or displace phosphate from the soil matrix by ligand exchange (Jones and Brassington 1998; Shane and Lambers 2005). Also, acid phosphatase can hydrolyze organic P compounds to Pi, which is the form available to plants (Bieleski 1973; Duff et al. 1994; Ryan et al. 2001). This is particularly important in P-deficient soils as described by Hopper (2009) (old climatically buffered, infertile landscapes, OCBIL) and also in young soils of volcanic origin with high total P but low availability of southern South America (Lambers et al. 2012).

16.2 Cluster Root Morphology

Cluster roots consist of dense clusters of rootlets of determinate development, arising endogenously from the pericycle of lateral roots, opposite protoxylem poles (Purnell 1960). Two types have been defined: simple and compound (see Lambers and Shane, from Spiertz et al. 2007). Simple cluster roots have a distinct bottlebrush-like appearance (e.g., *Leucadendron meridianum*), and there are several morphologies of this type among Proteaceae species and in other families, e.g., Fabaceae species (rooibos (*Aspalathus linearis*)) (Lambers and Shane 2007). Cluster roots from Chilean Proteaceae such as *Gevuina avellana*, *Embothrium coccineum*, and *Lomatia ferruginea* have been shown to be simple (Ramírez et al. 2005; Zúñiga-Feest et al. 2010; Lambers et al. 2012; Delgado et al. 2013).

Few genera of Proteaceae family produce (alone or in combination with the simple type) a compound type of CR, which are essentially branched simple CR; this type is found in some Australian (e.g., *Banksia sp*.) and several South African genera (e.g., *Leucadendron* and *Protea*) (Lamont 1982). Lamont (1983) suggests

that compound CR could be ontogenetically more advanced than simple CR; however, this idea has not yet been tested.

Proliferation of rootlets in a cluster creates a high root surface area, increasing contact with soil. For example, in *Hakea obliqua*, each cluster has a surface area (excluding root hairs) 25 times greater than that of an equivalent mass of axial root (Watt and Evans 1999). CR are short-lived structures (ephemeral), which exhibit similar formation in several Proteaceae species of different geographical and phylogenetical origins. In fact, their development from rootlet emergence to senescence occurs in around 25–30 days (Shane et al. 2004a; Delgado et al. 2013) and takes place in different parts of the root system, mainly in the upper soil horizons (Australian Proteaceae) or at least in one meter deeper (Chilean Proteaceae), depending of the total soil profile. Senescent CR are replaced by new young CR that start their development in other points of the root system. For example, in CR of *Grevillea robusta*, development occurs in predetermined areas at set distances along lateral roots (Skene et al. 1998) as occurs also on *L. ferruginea* and *L. dentata* (Avila 2013).

The morphology, physiology, and functions of cluster roots have been studied in a wide range of species, with *Lupinus albus* L. (Gardner et al. 1983; Johnson et al. 1994, 1996; Neumann et al. 1999; Neumann and Römheld 1999; Kihara et al. 2003) along with some Australian and South African Proteaceae being the main species studied (Lamont 2003; Shane et al. 2004a, b, c; Lambers et al. 2006).

Some anatomical descriptions of CR in *G. avellana* (South American species) have been reported by Grinbergs et al. (1987) and Ramírez et al. (2005). These reports show CR with claviform structures of unknown function at the end of each rootlet (Fig. 16.1 a, b, c). Other Proteaceae from South America did not exhibit this kind of root.

Environmental factors such as soil texture affect CR morphology. In fact, in *H. obliqua* (Dell et al. 1980), *G. robusta* (Skene 1998), and *L. albus* (Watt and Evans 1999), rootlet length is shorter when plants are grown in hydroponics compared with seedlings grown in vermiculite or soil. In addition, root hairs are absent when *H. obliqua* or *G. robusta* are grown in hydroponics. Also, significant differences in root hair length have been observed in Chilean Proteaceae growing at similar greenhouse conditions, with root hairs of *Orites myrtoidea* (an endemic shrub that grows in the Andes Mountains) growing three times longer than those observed in *L. ferruginea* (an evergreen tree from the South American rainforest) (Avila 2013; Díaz 2012). Differences could be related to the nutrient distribution of the soil profile, although this hypothesis has not yet been evaluated.

Other kinds of cluster roots have been described in monocotyledonous plant families, such as Cyperaceae (sedges) and Restionaceae (rushes). Cyperaceae species have a dauciform root cluster that resembles a carrot shape (Lamont 1974). These structures were first reported by Selivanov and Utemova (1969) and were then found in many other members of the family around the world (Lamont 1982; Shane et al. 2006; Lambers et al. 2006). The most remarkable external feature is the very dense formation of long root hairs over the carrot-shaped axes. Restionaceae (the “Southern Hemisphere rushes”) are abundant in Australia and

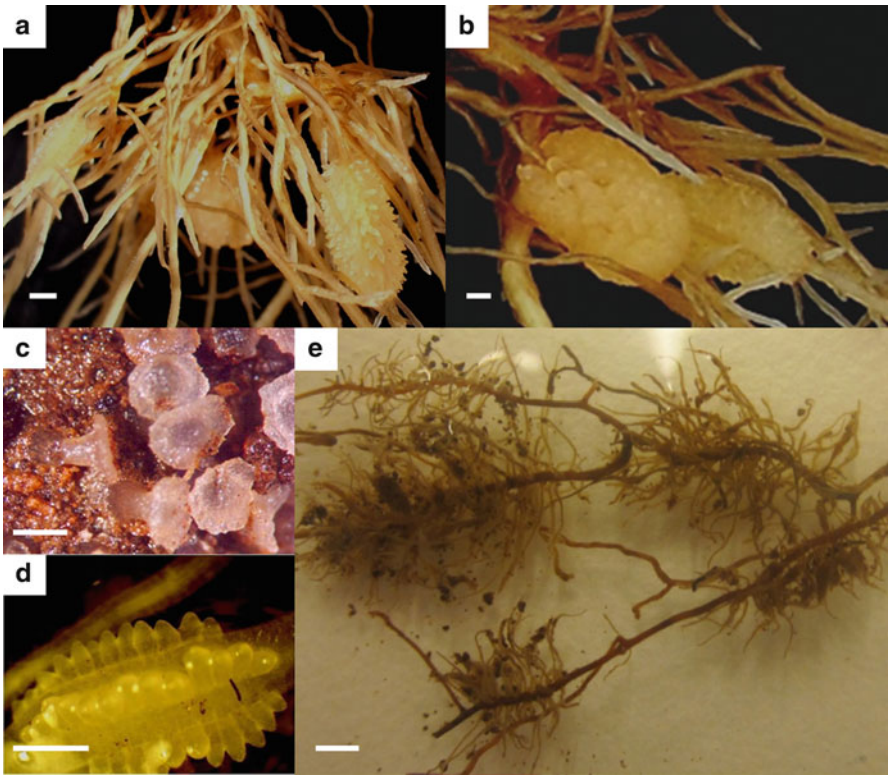

Fig. 16.1 Cluster root in Chilean Proteaceae. (**a**) Root system in *Gevuina avellana* and (**b**) two cluster roots of *G. avellana* grown on hydroponics (*white bar* represent 5 mm); (**c**) cluster root of *G. avellana* showing a detail of "claviform" structures at the end of each rootlet, seedling grown on clay soil (see particles between "claviform"); (**d**) young cluster root of *Embothrium coccineum* grown on sand (see the growing rootlets and it distribution); (**e**) cluster roots of *Lomatia dentata* collected on the field (39°S), grown on clay/organic soil (see small particles of the soil on each structure) (each *white bar* represents 5 mm)

South Africa, and in this family, CR have been termed "capillaroid" and are characterized by high numbers of rootlets densely covered with long root hairs. The term "capillaroid" comes from their sponge-like properties of holding soil water (Lamont 1982). In plants that exhibit CR or dauciform roots, these structures are stimulated by low P supply, as occurs with carboxylate exudation (Lamont 1974; Shane et al. 2005; Playsted et al. 2006).

16.3 Key Factors in CR Formation

The main factor inducing cluster root formation and its concomitant carboxylate and acid phosphatase exudation is P deficiency, but its formation is also influenced by N and Fe deficiency (Shane and Lambers 2005). In *Casuarina* species, Fe deficiency produces CR rather than P deficiency (Zaid et al. 2003). Recently, Delgado et al. (2013) showed that CR formation in *E. coccineum* was suppressed when plants were cultured in hydroponics with over 10 μM P. Additionally, different modulations of CR formation (without P supply) have been observed when comparing seedlings of *E. coccineum* of different origins. In an experiment under controlled greenhouse conditions, a southern ecotype (Coyhaique 46°S) showed no CR suppression under full nutrient solution, unlike that which was observed in temperate origins (Ochoa 2010). Under common controlled conditions, seedlings of *E. coccineum* from contrasting environments (35–51°S) showed significant differences in the number of cluster roots, relative CR biomass allocation, and frequency of plants with CR. Seedlings from the coldest environments (Chilean Patagonia) were smaller but exhibited a higher allocation of CR and higher frequency of plants with CR, compared with those of temperate origins (Bustos 2011; Zúñiga-Feest et al. 2010).

Cluster root biomass allocation in several Proteaceae species values ranges from 25 to 5 %. The South American Proteaceae *E. coccineum* exhibits relatively few resources for cluster root formation (8–10 % plant biomass), with many small CR with a higher carboxylate exudation rate (Zúñiga-Feest et al. 2010) compared with *G. avellana* (25–30 %) and Australian Proteaceae, as well as *Hakea prostrata* (Shane et al. 2004b, c) and *Grevillea crithmifolia* R. Br. (Shane and Lambers 2006) (25 % of total plant biomass). In the field in the coldest environment (Punta Arenas, Chile 51°S), we observed that 2-year-old seedlings growing on young rocky soils showed the highest CR allocation, which was observed in *E. coccineum* (25 % of total plant biomass) (Fig. 16.2).

Other factors that influence CR allocation have been reported in *Myrica gale* and *Gymnostoma papuanum*, where nitrogen forms influence size, number, and biomass distribution (Crocker and Schwintzer 1993; Racette et al. 1990). In small seedlings of *E. coccineum*, it has also been observed that N content of the soil is negatively related to CR number and CR/root biomass, with no significant relation with Olsen P from the soil in a fertility gradient in Chilean Patagonia (Piper et al. 2013).

16.4 Cluster Root Metabolism and Rhizosphere Interactions

A remarkable feature of CR is their ability to strongly acidify the rhizosphere. For example, in calcareous soil (20 % $CaCO_3$), CR of *L. albus* acidified the rhizosphere from pH 7.5 to 4.8 (Dinkelaker et al. 1989). In some cases, rhizosphere soil pH can

Fig. 16.2 *Embothrium coccineum* from Chilean Patagonia, collected at field. (**a**) Several cluster roots of 2-year-old seedlings (see some of these structures attached to the metamorphic rock) (*white bar* represents 1 cm); (**b**) Part of the root system of the same seedling (see the distribution of CR) (*white bar* represents 5 cm); (**c**) 2-year-old seedling of *E. coccineum* growing on rocky young soil in Chilean Patagonia (51ºS–72ºO)

even be decreased by CR activity to as low as 3.6 (Li et al. 1997). This acidification is produced by a huge amount of organic acids, predominantly citric and malic acids (Gardner et al. 1983; Neumann et al. 1999). The carbon cost associated with acid exudation has been evaluated in *H. prostrata* (Shane et al. 2004a), which shows increases of around three times in respiration rate before exudation, which occurs at 4 days after CR initiation. These results reveal important metabolic changes that occur in CR during the exudative burst in coordination with their development.

Exudation of malate and citrate have been mainly reported in CR of different species, such as *H. protrata*, *L. albus* (Kihara et al. 2003; Shane et al. 2004a; Peñaloza et al. 2005), and recently in *E. coccineum* (Delgado et al. 2013).

A release rate of organic acids in the range of 2.4 to 7.4 mol h g fresh weight has been reported for CR of *L. albus* (Keerthisinghe et al. 1998; Neumann et al. 1999). Because the rootlets are closely arranged, the released organic acids can accumulate at high concentrations in the rhizosphere of CR (Yan et al. 2002). Results showed that 0.1 mmol citric acid per g soil has been produced by CR activity (Gerke et al. 1994; Li et al. 1997), a concentration sufficient to release P from sparingly soluble Fe and Al phosphate by a mechanism of ligand exchange or chelation of metal ions (Hinsinger 1998). Citrate is one of the most powerful P-extracting acids from an inorganic P source (Jones 1998; Ryan et al. 2001).

Plasticity of carboxylate exudation has been observed in *Banksia grandis*, which has different carboxylate compositions according to whether aluminum phosphate or iron phosphate was supplied (Lambers et al. 2002). Factors that control the exudation of citrate are important targets of research; citrate efflux by CR has been studied in P-starved *L. albus*, showing that it may account for up to 23 % of plant dry weight (Dinkelaker et al. 1989). The efflux occurs in CR sections where citrate accumulates (Peñaloza et al. 2002). This exudation begins shortly after CR rootlets reach their final length (Watt and Evans 1999) and follows a transient pattern that lasts from 3 to 9 days. The highest exudation activity occurs in the mature CR stage, while young and old CR release only a limited amount of acid (Neumann et al. 1999; Watt and Evans 1999). Rhizosphere acidification also has been observed in mature CR from *L. ferruginea*, *L. dentata*, and in young and mature CR of *O. myrtoidea* (Avila 2013) (southern South American Proteaceae); however, carboxylate exudation has not yet been identified.

The source of organic acids exuded by roots is assumed to be a combination of the glycolytic pathway (from sucrose to the phloem) and the tricarboxylic cycle (TCA), operating in the root tissue (Massonneau et al. 2001). During active accumulation of carboxylates before the exudative burst, a metabolic bypass occurs in TCA that leads to an accumulation of citrate and/or malate (Cramer et al. 2005). Metabolic changes in CR leading to citrate accumulation and efflux have been associated with an increase in phosphoenolpyruvate carboxylase (PEPC) activity (Neumann et al. 1999; Shane et al. 2004a). Kihara et al. (2003) discuss that changes in metabolism of CR of *L. albus* (P-deprived) involve an increase in the rate of citrate synthesis by enhancement of the supply of substrates (pyruvate, oxaloacetic acid) and blocking citrate catabolism in the cytosol. These authors report that PEP phosphatase increases its activity threefold in P-deprived root apexes; this feature could be important for maintaining the supply of acetyl-CoA to the mitochondria by pyruvate production. During CR development, there is also an increase in alternative oxidase (AOX) activity (Shane et al. 2004a). The authors have suggested that during CR development, this enzyme sustains electrochemical homeostasis at the mitochondrial level before the exudative burst occurs.

These metabolic changes lead to higher exudation rates in CR than in non-cluster roots or root systems from other species that do not have this adaptation, such as *Lupinus* species (Roelofs et al. 2001; Ryan et al. 2001).

About 30 % of carbon released in the form of organic acids originates from dark CO_2 fixation by PEPC in roots of P-deficient *L. albus* (Johnson et al. 1996), while the other portion of the carbon used on carboxylate exudation could be carbon assimilated through photosynthesis and then transported to the roots. A large amount of protons produced during the synthesis of organic acids may be removed from the cell by the plasma membrane H^+ ATPase. This enzyme responds to a number of environmental factors, such as saline stress (Niu et al. 1993), nutrient supply (Schubert and Yan 1997), Fe deficiency (Dellórto et al. 2000), and P starvation (Yan et al. 2002). In the case of the PEPC, the activity of this enzyme is about three times greater in CR of *L. albus* compared with P-supplemented plants. Yan et al. (2002) showed that in active cluster root cells, H^+ and organic anions are exported separately and that modification of the plasma membrane H^+ is essential for enhanced rhizosphere acidification by active CR. Transgenic plants that overexpress citrate synthase in *Arabidopsis thaliana* (Koyama et al. 2000) or malate dehydrogenase in *Medicago sativa* (Tesfaye et al. 2001) improve growth under low P supply and tolerance to aluminum, respectively; however, citrate excretion is limited or in some cases negligible (Delhaize et al. 2001).

Low P availability also increases acid phosphatase (APase) secretion to the rhizosphere in several crop plant species such as *Zea mays*, *Oryza sativa*, and *Lycopersicum esculentum* (Yan et al. 2002). CR of *L. albus* showed high rates of acid phosphate activity compared with other species and non-cluster roots (Yan et al. 2001). Recently, higher APase activity has been detected in CR compared with non-cluster roots in *E. coccineum* seedlings growing under controlled conditions (Delgado et al. 2013) and CR of *O. myrtoidea* and *E. coccineum* growing in volcanic soil deposits (pumicite) at greenhouse conditions (Avila 2013). In both cases, APase activity was six times higher in CR compared with non-CR and four times higher in volcanic soil (pumicite) than in organic soils.

The source of organic acids exuded by roots is assumed to be a combination of the glycolytic pathway and the tricarboxylic cycle (TCA), operating in the root tissue with the PEPC activity (Massonneau et al. 2001). During active accumulation of carboxylates before the exudative burst, a metabolic bypass occurs in TCA that leads to an accumulation of citrate and/or malate (Cramer et al. 2005).

16.5 Seasonal Variation in CR Production

In Proteaceae species from Mediterranean climates, CR formation occurs in the wet winter season (Lamont 1976, 1983; Shane and Lambers 2005). However, in temperate cold areas in South America, *E. coccineum* exhibits higher CR formation in spring and summer than in winter. This variation is concomitant with the longest period of growth and relative water availability in the soil (Zúñiga-Feest et al. 2009;

Donoso et al. 2010). Results obtained in our laboratory, using *E. coccineum* seedlings under nursery conditions and high water availability, showed that there is a seasonal variation in CR formation and phosphatase activity, which is higher (around 60 %) in CR than in non-cluster roots, during summer and autumn (Delgado et al. 2011). A variation in acid exudation, detected by bromocresol purple as a pH indicator, exhibited a higher amount in autumn (Zúñiga-Feest et al. 2009). The number of CR during summer is three times higher (60 mean per plant) than during the winter, and CR/root biomass is also higher during spring and summer compared with winter (Zúñiga-Feest et al. 2009).

Donoso et al. (2010) suggest that the seasonal variation in cluster root formation of *E. coccineum* seedlings in the field in temperate areas (40°S) could depend on the organic matter content of soil. These authors explain that high organic matter content favors the retention of moisture in summer, but in winter, it may cause low drainage and lead to flooding. Furthermore, low organic matter content could keep soil moisture optimal for cluster root formation in the rainy season but would be unable to keep moisture in the dry summer, resulting in unsuitable conditions for cluster root formation.

Water availability is essential for root growth, a requisite for cluster root formation (Lamont 2003). Lamont (1976) reported that CR and non-cluster roots in Australia are produced only during winter-spring, although dormant roots can be induced to form new root structures in summer, when sufficient water is applied to that part of the root system. Jeschke and Pate (1995) have reported that slow growth rates, absorption, and storage of internal phosphorus in *Banksia prionotes* occur mainly in the winter season for remobilization and use during spring-summer, when leaf expansion occurs. Also, P concentration of different organs and its content showed seasonal variation in *E. coccineum* seedlings. It accumulated in the stem in winter, before spring growth actively begins. *E. coccineum* exhibits a high growth relative rate (over 500 %), and the highest rhizhosphere acidification (evaluated by a pH indicator) is coincident with the lowest P concentration values in the stem. It is likely that during the active growth of seedlings, there is a high nutrient demand, and *E. coccineum* uses its abundant P reserves. This hypothesis is in agreement with the low remobilization of P from senescent leaves to mature leaves observed in South American Proteaceae (Lambers et al. 2012; Avila 2013), in which the stem is the storage organ.

16.6 CR in an Ecophysiological Context

The Proteaceae family has several species that are common colonizers of volcanic deposits in Chile and Argentina, such as *E. coccineum* and *L. hirsuta* (Alberdi et al. 2009; Segura-Uauy 1999). In part this capacity is sustained by high photosynthetic rates and highly efficient dissipation of excess light as well as likely CR activity that can acquire P from young volcanic soils. Recently, Lambers et al. (2012) compared Proteaceae species from SW Australia (OCBIS) and

young, volcanic, P-rich soils, but with low availability, from Chile. These Proteaceae species differ in P mobilization and photosynthetical P use efficiency, with both features higher in SW Australian Proteaceae species. Lambers et al. (2012) proposed that the role for species with cluster roots that grow on young P-rich soil with low P availability (Chilean Proteaceae) could access strongly sorbed P and then act as ecosystem engineers, providing P in leaf litter for neighboring plants without cluster roots. This capacity is an ecologically important trait especially in young volcanic soils and is not restricted to CR-forming species and in fact is an important trait of carboxylate-exuding species such as several *Lupinus* spp. that do not form CR (Lambers et al. 2013).

On the other hand, Proteaceae species from Chile such as *L. hirsuta* and *O. myrtoidea* showed low P mobilization from old leaves to mature leaves, compared with Australian species. Saplings of *L. hirsuta* growing close to Antuco (a volcanic area, 38°S) showed higher mobilization than *O. myrtoidea*, which did not show significant differences in P content between leaves, with around 3–4 mg P/g DW. This concentration was also higher than which was reported for Australian Proteaceae.

Recently, Piper et al. (2013) showed that *E. coccineum* seedlings growing in a gradient of soil fertility in Chilean Patagonia have a negative logarithmic relationship between CR number and soil N and between CR relative mass and soil N, but not with P available (Olsen P), as expected. This relationship is stronger in small seedlings ($\leq$1 year) than in old seedlings (>1 year), suggesting that in young soils or cold areas, where mineralization is low, CR could be involved not only in P acquisition but also in N organic uptake.

In this sense, it is known that CR exudes proteases (enzymes that use protein compounds from the soil) (Schimidt and Steward 1997; Schmidt et al. 2003). Also, plants take up organic nitrogen compounds of low molecular mass, including amino acids and possibly di- and tripeptides, via membrane transporters into root cells (Rentsch et al. 2007). The role of CR in nutrient acquisition in low-temperature environments has been highlighted by Paungfoo-Lonhienne et al. (2008), who studied *Hakea actities* (Australian Proteaceae) from wet heathland growing in a protein-rich and inorganic nitrogen-poor soil habitat. The CR of *H. actities* exuded proteolytic enzymes that digest protein at the root surface and intact protein also was taken up into cells, most likely via endocytosis. The same authors found that the expression of putative peptide transporters (di- and tripeptide) was regulated in response to N supply (Paungfoo-Lonhienne et al. 2009). The possible exudation of proteases by other members of the family has not yet been evaluated and could be relevant in Proteaceae from the coldest areas in South America.

Zúñiga-Feest et al. (2013) in a greenhouse experiment showed that CR formation, acid phosphatase activity, and acid exudation (using pH indicator bromocresol purple) were higher when *E. coccineum* plants were grown in volcanic material (pumicite) compared with organic soil, and these differences were higher in seedlings of southern origins (Punta Arenas 51°S) than those of temperate origins (Puerto Montt 40°S) (Avila 2013).

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Chapter 17
The Role of Roots in Plant Defense Responses to Aboveground Herbivores

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17.1 Introduction

Roots are integral to a plant's growth and survival. In addition to anchoring plants to the soil, they absorb water and nutrients from the surrounding soil, serve as sites of storage for valuable photoassimilates, and also act as sites of synthesis for several plant hormones and secondary metabolites. As a consequence, plants invest heavily in the development and establishment of their root system with some plants investing substantially more than 50 % of their body weight in roots (Taiz and Zeiger 2002). Belowground herbivores (BGH) such as insects and nematodes have evolved to take advantage of the tremendous amount of organic matter that the roots represent (Blossey and Hunt-Joshi 2003; Raaijmakers et al. 2009). To protect themselves against BGH, roots also utilize both direct and indirect defenses similar to those found in the aboveground parts of a plant (van Dam 2009; Kandoth and Mitchum 2013). However, the experimental limitations posed by the general inaccessibility of roots and the "hidden" nature of belowground herbivores have resulted in a paucity of data. Given the importance of roots to any plant, interest in the role of roots in plant defenses has surged, and literature comparing root and shoot defense has been on the rise in the past decade (Bezemer and van Dam 2005; Kaplan et al. 2008; Rasmann and Agrawal 2008; van Dam 2009; Soler et al. 2012b).

Previously, plant–insect interactions have been largely studied by only focusing on the response in the aboveground tissue, i.e., the leaves of plants, leading to a skewed and unbalanced understanding. Plants counter insect herbivory by utilizing a wide variety of strategies comprising of constitutive and inducible defenses (Howe and Jander 2008; Walling 2008; Fürstenberg-Hägg et al. 2013). In some cases, the plants' traits affect insect preferences, such as morphological features that affect host plant selection and feeding behavior. Preformed barriers such as

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waxes on leaf surface, leaf toughness, trichomes, accumulation of chemicals in reproductive tissues, etc., are hardwired into developmental programs and are constitutively present (Howe and Jander 2008; Walling 2009). In other cases, the plants' traits influence insect performance, such as the production of secondary metabolites in response to herbivory that either deters or kills the insect. Inducible defenses are initiated at the site of attack and also transported systemically through the entire plant (Karban and Baldwin 1997). These highly dynamic induced defenses not only offer the advantage of reduced cost in terms of resource allocation but also increase the phenotypic variability in the plant phenotype resulting in higher efficiency (Karban et al. 1997; Karban and Baldwin 1997; Cipollini et al. 2004).

In this chapter, we would like focus on the contribution of roots to aboveground herbivory (Fig. 17.1). A growing body of evidence supports an integral role for roots in aboveground plant defense strategies. Roots serve as (1) sites of synthesis for several secondary metabolites that are not only actively synthesized to protect roots against BGH but are also transported aboveground to increase foliar resistance to AGH, (2) act as dynamic storage organs for valuable photoassimilates which can be reallocated aboveground after the threat has passed, and, finally, (3) also actively recruit beneficial soil microbes to help deal with aboveground herbivory.

17.2 Root-Derived Defenses to Aboveground Herbivory

17.2.1 AGH Induced Changes in Root Transcriptome

Plants resist insect herbivory by rapidly and accurately inducing defenses. Large-scale gene expression profiling has provided researchers with an insight into the transcriptional changes that occur in response to herbivory (Smith 2005; Chen 2008). The induction of defenses occurs at the site of insect attack and also systemically in undamaged tissue (Bostock 2005). In recent years, the physiological and transcriptional changes occurring in roots due to AGH is also being investigated (Erb 2009; Erb et al. 2009b; Ankala et al. 2013; Tytgat et al. 2013). Erb (2009) found that in maize (*Zea mays*), AGH by cotton leafworm (*Spodoptera littoralis*) resulted in the differential regulation of a larger proportion of genes in roots as compared to changes in the shoot tissue. In shoots, genes involved in plant defense were upregulated after *S. littoralis* attack including those involved in the biosynthesis and signaling of two critical plant defense hormones, jasmonic acid (JA) and salicylic acid (SA). Functional analyses of transcripts showing differential regulation in roots, however, did not display any overlap with changes that occurred in the shoot tissue. Further, transcripts implicated in protein metabolism were found to be preferentially upregulated in roots suggesting a specific function of root metabolism in response to shoot attack. Ankala et al. (2013) studied transcriptional

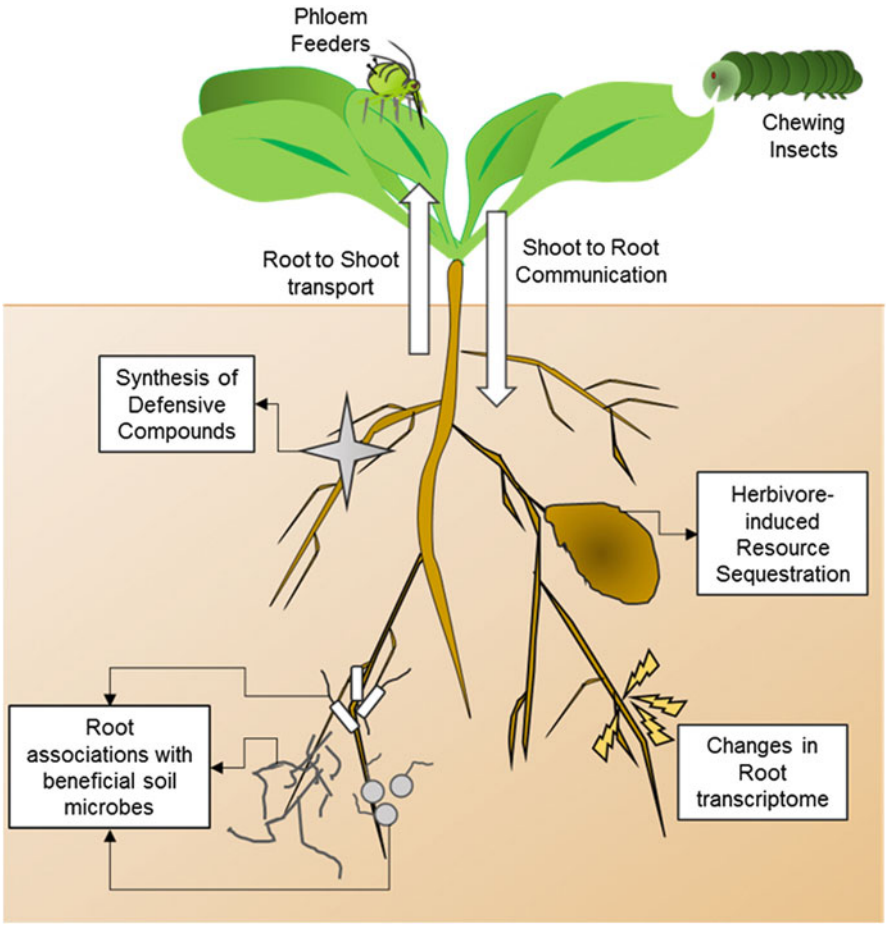

Fig. 17.1 The contribution of roots in plant defense strategies against aboveground herbivory

changes occurring in roots and shoots of a maize inbred line resistant to the fall armyworm (*S. frugiperda*). Interestingly, in this case, although genes involved in JA biosynthesis and signaling were upregulated in the roots, genes involved in the signaling and perception of another plant defense hormone, ethylene (ET), were also upregulated in roots in response to AGH. Further, transcript levels of genes encoding proteins involved in direct defenses were enhanced in roots. However, whether these transcriptional changes that occur in roots in response to AGH have an impact on the performance of the insect herbivore is not clear and warrants further research. Tytgat et al. (2013) studied the transcriptional responses that occur in shoots and roots of *Brassica oleracea* plants by mimicking herbivory by JA application. Specific responses in primary metabolism, development, and defense were observed depending on whether JA was applied to the shoots or the roots. These differential responses observed in these studies suggest that plants are able to

respond differently to signals coming from the shoots or from the roots. As a result plants are able to fine-tune their responses specifically to only the plant part that is under attack. Taken together, these studies indicate that distinct transcriptional changes occur in roots during AGH and further highlight the need to include roots to develop a comprehensive understanding of plant response to AGH.

17.2.2 Synthesis of Shoot Defensive Compounds

Plants have evolved a wide variety of defensive compounds to ward off, inhibit, or kill insect herbivores. These defensive compounds are usually secondary metabolites or plant proteins that are either constitutively present or induced in response to insect herbivory. A large body of evidence supports the role of secondary metabolites such as alkaloids, terpenoids, glucosinolates, furanocoumarins, etc., in plant defense, and several of these are synthesized in the roots of plants and transported to the shoots where they act on AGH [reviewed in Van der Putten et al. (2001), Kaplan et al. (2008), Rasmann and Agrawal (2008), Erb et al. (2009a), Erb (2012) and Nalam et al. (2013)]. The reasons why plants have adopted to synthesize defensive compounds in roots are uncertain. One reason may be that by separating the site of synthesis and the site of action, plants can guarantee delivery of the defensive compounds in situations where extensive defoliation occurs making roots safer areas for synthesis. The earliest example for a root-derived secondary metabolite providing resistance against AGH comes from studies in tobacco (*Nicotiana sp*.) plants (Dawson 1941). Nicotine, an alkaloid that is constitutively present in the leaves of plants belonging to the genus *Nicotiana*, is synthesized in the roots of the plant. Simulated insect herbivory by the application of methyl jasmonate (a derivate of jasmonic acid) on the leaves of the plant results in an increase in root nicotine synthesis and transport to the shoots providing increased protection against the herbivore (Baldwin et al. 1994; Morita et al. 2009). Another class of alkaloids, the tropane alkaloids are also synthesized in the roots of plants of the genus *Solanaceae* and transported to the shoots (Bais et al. 2001; Ziegler and Facchini 2008).

Glucosinolates are a class of secondary metabolites that are mainly found in plants belonging to the genus *Brassicaceae*. Their role in plant defense against not only insect herbivores but also pathogens has been well documented (Bednarek et al. 2009; Hopkins et al. 2009). An increase in the levels of glucosinolates is observed in the shoots and roots of plants under attack by an AGH (Ludwig-Müller et al. 1997; Soler et al. 2009). Although the site of synthesis of these compounds has not been identified, the presence of these compounds in roots of plants under attack by AGH raises the possibility that they may be synthesized in roots. In certain other instances, the final version of a bioactive secondary metabolite is not completely synthesized in the roots; rather precursors are synthesized and transported to the shoots where they undergo further modifications. A prime example comes for a group of secondary metabolites known as furanocoumarins. Umbelliferone is a precursor for several bioactive furanocoumarins that act as feeding deterrents and

also have antifungal and antibacterial properties (Berenbaum 1978; Yamane et al. 2010). The site of synthesis for this precursor has been localized to the roots in bishop-weed (*Ammi majus*) plants from where it is transported to the rest of the plant for further modifications (Sidwa-Gorycka et al. 2003). In addition to secondary metabolites, plants also produce defensive proteins (arthropod-inducible proteins, AIPs) that protect the plant against insect herbivory by interfering with the insect digestive system [reviewed in Zhu-Salzman and Liu (2011)]. In maize lines that are genetically resistant to a wide variety of insect pathogens, a unique AIP termed Mir1-CP accumulates in the foliar tissue during insect herbivory (Lopez et al. 2007). The site of synthesis of this protein was found to be in the root and in maize plants, where the roots were removed just prior to insect feeding, and the same accumulation was not observed suggesting strongly that Mir1-CP is synthesized in roots. With the advent of genomics and an increased interest in root biochemistry, it is quite plausible that other examples of AIPs synthesized in roots will be uncovered.

Secondary metabolites and AIPs synthesized in the roots for shoot defenses are probably transported to the roots via the vasculature of the plant. Evidence for transport through both the xylem and phloem exists. For instance, the immunohistochemical localization of enzymes involved in nicotine biosynthesis indicates that the synthesis of nicotine occurs in the cortex and endodermis of roots from where it diffuses into the upward-flowing stream of the xylem (Shoji et al. 2000). Although the site(s) of synthesis of tropane alkaloids within the roots is unknown, the xylem serves as the mode of transport for several of them (Ziegler and Facchini 2008). Mir1-CP synthesis was also shown to occur in roots specifically in xylem parenchyma and from where it moves to the xylem for upward transport to the foliar tissue. Interestingly, in the foliar tissue, Mir1-CP is also found in the phloem of both minor and intermediate veins (Lopez et al. 2007). The transport of secondary metabolites may also occur through the phloem. Pyrrolizidine alkaloids are another class of secondary metabolites that provide protection against insect herbivores (Lindigkeit et al. 1997). The transport to shoots of the precursor of pyrrolizidine alkaloids, senecione N-oxide, which is synthesized in roots has also been shown to occur via the phloem. The use of girdling experiments and the localization of biosynthetic enzymes for senecione N-oxide in root cortex parenchyma and endodermis across the phloem confirm that roots are the sites for synthesis and the phloem is the mode of transport (Moll et al. 2002; Ober and Kaltenegger 2009).

17.2.3 Signal(s) in Shoot-to-Root Communication

The synthesis of shoot defensive compounds in the roots suggests that upon perception of an AGH, a signal is sent to the roots resulting in the synthesis and subsequent transport of the compounds aboveground (Fig. 17.1). This shoot–root–shoot communication and transport likely follows the internal vascular network of the plant, i.e., phloem and xylem. Plant- and insect-derived molecules have the

potential of acting as signal molecules. However, to be considered as signal, the molecule must be found at the site of attack and be capable of inducing plant responses both locally and systemically.

Among the plant-derived candidates, the defense hormone, jasmonic acid (JA), is a likely candidate for a mobile element. JA and associated compounds, collectively termed jasmonates, are critical for long-distance wound signaling and the activation of defenses against necrotrophic pathogens and chewing insects (Heil and Ton 2008; Wu and Baldwin 2009; Woldemariam et al. 2011). There is strong evidence that methyl jasmonate moves in the phloem along with photoassimilates and also in the xylem as a result of vigorous exchange allowing the movement to regions which are the sources of the photoassimilates (Thorpe et al. 2007). Support for jasmonates as mobile elements comes from studies in several plants. Simulated foliar herbivory of tobacco (*N. sylvestris*) results in an increase in JA concentration immediately in the leaves and subsequently in the roots stimulating the synthesis of nicotine (Baldwin et al. 1994; Winz and Baldwin 2001). A similar pattern is observed in poplar (*Populus nigra*), where mechanical wounding or foliar JA application results in the induction of defense marker genes in the roots (Major and Constabel 2007). In maize plants, Mir1-CP accumulation is observed in response to foliar methyl jasmonate treatment (Ankala et al. 2009). Evidence for the role of other plant hormones involved in defenses, SA, ethylene (ET), and ABA as mobile elements in shoot-to-root communication, is however less clear and warrants further research (Soler et al. 2012a; Nalam et al. 2013). For instance, SA is critical for the activation of systemic acquired resistance that occurs in response to pathogen attack. Although it is clear that SA itself is not the mobile element, the activation of SA signaling at the site of attack results in the transmission of long-distance signal(s) through the whole plant (Shah 2009; Dempsey and Klessig 2012). With respect to insect herbivory, feeding by phloem-feeders such as aphids and whiteflies induces the activation of SA-mediated signaling and defenses (Moran and Thompson 2001; Zarate et al. 2007) which raises the possibility of a SA-derived signal being a mobile element involved in shoot-to-root communication. In several plant species, small RNAs molecules such as short-interfering RNAs and micro-RNAs play an important role in plant defense (Padmanabhan et al. 2009). These small RNAs have been identified in the phloem sap of several plant species and could quite possibly function as mobile elements (Kehr and Buhtz 2008, 2012). However, evidence supporting their role as mobile elements performing shoot-to-root communication during AGH is lacking and requires further research.

Insect-derived candidates can also potentially function as mobile elements in shoot-to-root communication. The saliva of phloem-feeders and chewing insects contains effector proteins that are introduced into plants during feeding. These effector proteins modulate plant defenses by interfering with or eliciting plant defense responses (Tjallingii 2006; Will et al. 2007; Harmel et al. 2008; Mutti et al. 2008; Carolan et al. 2009; Bos et al. 2010). For instance, a peptide in the 3–10 kDa range in the saliva of the green peach aphid (*Myzus persicae*) induces defense responses in *Arabidopsis* resulting in reduced aphid performance (De Vos

and Jander 2009). On the other hand, a secreted protein product of the salivary gland gene, *C002*, is delivered into plant tissue and aids in the enhancement of aphid fecundity (Mutti et al. 2008; Bos et al. 2010). Furthermore, foliar feeding by *M. persicae* results in the induction of *LOX5* expression in roots of *Arabidopsis* (Nalam et al. 2012). Although the identity of the component responsible for root-specific induction is unknown, it is conceivable that aphid salivary proteins secreted into the phloem are capable of eliciting plant responses in the roots.

17.3 Herbivore Induced Resource Sequestration

Roots in many plants are modified to serve as storage organs for water and nutrients. Many of these modified roots like cassava, sweet potato, ginger, etc., are edible and form an important part of the human diet. In some perennial species, photoassimilates are often reallocated to roots to help tide over unfavorable periods serving as buffers against abiotic and biotic stresses (Palacio et al. 2007; Kobe et al. 2010). It is however clear that in several species, roots can aid in the ability of plants to tolerate AGH (Orians et al. 2011; Schultz et al. 2013). This phenomenon termed induced resource sequestration involves the rapid transport of nutrients/photoassimilates from the aboveground parts to the roots, making them temporarily inaccessible to the aboveground attacker. These nutrients/photoassimilates can then be reallocated back aboveground for regrowth after the herbivore has passed (Mauricio et al. 1997). In several herbaceous species, this reallocation from roots to shoots is the most common mechanism of tolerance (Welter and Steggall 1993; De Jong and Van Der Meijden 2000).

Herbivore induced short-term resource allocation has been shown to occur in several species. AGH by a generalist grasshopper (*Romalea guttata*) on maize plants growing in a $^{14}CO_2$-rich environment showed that a significant amount of ^{14}C is recovered from not only the roots but also root exudates (Holland et al. 1996). In poplar, leaf feeding by gypsy moth (*Lymantria dispar*) larvae resulted in an immediate increase in the export speed of carbon to the stem and roots (Babst et al. 2008). Similarly, AGH by tobacco hornworm (*Manduca sexta*) caterpillars on tobacco plant growing a $^{13}CO_2$-enriched chamber resulted in increased ^{13}C allocation to roots (Kaplan et al. 2008). In common milkweed (*Asclepias syriaca*), AGH by monarch caterpillars (*Danaus plexippus*) induced significant changes in the allocation of carbon to the roots (Tao and Hunter 2013). In addition to feeding by an insect herbivore, simulated herbivory by either mechanical damage, application of defense hormones, or insect salivary regurgitant has also been shown to result in resource reallocation. For instance, in poplar, tomato (*Solanum lycopersicum*), and *Arabidopsis*, the application of JA also resulted in resource allocation to the roots (Babst et al. 2005; Gómez et al. 2010; Ferrieri et al. 2013). Interestingly, in addition to mechanical damage caused due to insect feeding, the presence of insect-derived elicitors present in the insect salivary regurgitant is required to induce resource allocation. In tobacco and tomato plants, regurgitant from *M. sexta* larvae applied to

damaged leaves but not mechanical damage alone resulted in a 75 % increase in carbon transport to roots (Schwachtje et al. 2006; Gómez et al. 2012). Taken together, these studies show that plants can transport some resources away from the AGH. However, relatively little is known about the long-term consequence of induced resource sequestration. In wild tobacco, herbivore induced sequestration results in an extended flowering time and increase in seed production suggesting an increase in fitness (Schwachtje et al. 2006) although whether this occurs due to resource remobilization from the roots was not demonstrated.

The mechanisms that initiate and control reallocation to the roots are not fully understood. Photoassimilates are normally transported in the plant via the phloem. It is therefore plausible that by either increasing the loading into the phloem in the leaves or increasing unloading from the phloem at the roots can help achieve the rapid sequestration that is observed (Turgeon and Wolf 2009). The observation that the activity of sugar cleaving enzymes such as invertases increases in tobacco roots after herbivory (*M. sexta*) (Kaplan et al. 2008) or simulated herbivory (*M. sexta* regurgitant) (Hermsmeier et al. 2001) suggests that during AGH, the "sink" strength of roots increases. The molecular basis of this phenomenon has been studied in tobacco plants where it was found that sucrose non-fermenting-related kinase1 (SnRK1) plays an important role in this process. SnRK1 plays a central role in the energy metabolism of the cell (Halford and Hey 2009). The transcripts of SnRK1 are downregulated in the leaves within hours of simulated herbivory by *M. sexta* resulting in an increase of 10 % more photoassimilate allocation to the roots (Schwachtje et al. 2006). The initiation of sequestration by the application of JA suggests that this plant defense hormone is involved in the process. And indeed, leaf-derived jasmonates are major regulators of this process. However, tobacco plants which lack a fully functional JA pathway still exhibit resource reallocation (Schwachtje et al. 2006; Machado et al. 2013), suggesting that other signaling pathways may be involved. A recent study in tobacco plants shows that the levels of indole-3-acetic acid (IAA), a precursor of the hormone auxin, increase in roots after AGH and application of IAA resulted in the root sequestration in a JA-independent manner (Machado et al. 2013). A complete picture of the mechanisms of initiation and control of reallocation in response AGH is however far from complete.

17.4 Recruitment of Beneficial Soil Microbes

The environment in which roots exist, the rhizosphere, supports a diverse array of soil-borne microbes. Plant roots produce copious amounts of exudates that are mainly comprised of an enormous range of small molecular weight compounds which serve as a major source of carbon to soil microbes (Walker et al. 2003; Bais et al. 2006). As a result, the composition of the rhizosphere is strongly influenced by the plant itself, and interactions mediated by these exudates can exert a strong positive or negative effect on plant growth and health (Van Der Heijden et al. 2008;

Raaijmakers et al. 2009). Soil microbes such as pathogenic fungi, oomycetes, bacteria, and nematodes negatively impact plant growth. On the other hand, arbuscular mycorrhizal fungi (AMF), nitrogen-fixing bacteria, plant growth-promoting rhizobacteria (PGPR), and plant growth-promoting endophytic fungi (PGPF) exert a positive influence directly by promoting plant growth and indirectly through induced systemic resistance (ISR) (Bezemer and van Dam 2005; Van Loon 2007). ISR refers to the systemic induction of plant defenses in the whole plant as result of the association of roots with certain beneficial soil microbes. ISR provides the plant with broad spectrum protection against a wide range of insect herbivores and pathogens including those that occur only on aboveground plant tissue (Bent 2006; Pozo and Azcon-Aguilar 2007; Van Oosten et al. 2008; Doornbos et al. 2010).

The mechanism of induction of ISR shares several similarities with defenses that are initiated against AGH (Pieterse and Dicke 2007; Pieterse et al. 2009). The defense hormone, JA, plays a central role in ISR and also negatively impacts chewing insects. For example, the larvae of the diamondback moth (*Plutella xylostella*) feeding on cabbage plants associated with an endophytic fungus (*Acremonium alternatum* Gams) suffered from reduced growth and increased mortality (Raps and Vidal 1998). With respect to phloem-feeders, the result of ISR can be either a negative or neutral effect on herbivore performance and seems to depend on the plant, microbe, and the AGH. Phloem-feeding insects, aphids and whiteflies, mainly activate salicylic acid (SA)-mediated defenses that can negatively affect JA-mediated defenses via crosstalk (Kunkel and Brooks 2002). Although, JA-mediated defenses have also been shown to be involved in defense against phloem-feeders (Kunkel and Brooks 2002; Zhu-Salzman et al. 2005; Zarate et al. 2007). Examples of negative effects occur in the case of the phloem-feeders such as the green peach aphid and silverleaf whitefly (*Bemisia argentifolii*) which perform poorly on tomato or sweet pepper (*Capsicum annuum*) plants, respectively, whose roots have formed an association with a PGPR (*Bacillus amyloliquefaciens*) (Murphy et al. 2000; Herman et al. 2008). The association of another PGPR, *B. subtilis*, with tomato leaves also retards the development of silverleaf whitefly (Valenzuela-Soto et al. 2010). An example of neutral effect on a phloem-feeder, green peach aphid, occurs in white leaf clover (*Trifolium repens*) plants associated with rhizobia (*Rhizobium leguminosarum*) (Kempel et al. 2009). Additional biotic factors like the degree of specialization of the insect, microbe identity, plant developmental stage, and genotype have also been known to modulate ISR and as a result influence the AGH (Pineda et al. 2010). Abiotic factors like drought stress also seem to influence the effect of beneficial microorganisms. For example, in tomato plants associated with an endophytic fungus (*A. strictum*), whitefly mortality was higher only in plants that were undergoing drought stress (Vidal 1996). Altogether, these examples demonstrate that roots can act as important modulators of aboveground defenses.

Arbuscular mycorrhizal fungi (AMF) form intricate associations with roots of plant and aid the plant in the uptake of nutrients and water by effectively increasing the surface area occupied by the roots in the soil. By a mechanism that is similar to

but not the same as ISR, AMF can impact AGH (Fritz et al. 2006; Hempel et al. 2009; Pozo et al. 2010). It has been shown that resistance against a bacterial pathogen (*Xanthomonas campestris*) in *Medicago truncatula* and a fungal pathogen in tomato is induced by the association of AMF with the roots of these plants (Fritz et al. 2006; Liu et al. 2007). With respect to insect herbivores, factors such as degree of specialization and feeding guild impact the outcome of the interaction [reviewed in Jung et al. (2012)]. For example, root colonization of tomato plants with AMF was recently shown to affect the performance of cotton bollworm (*Helicoverpa armigera*) caterpillars with the JA pathway playing a crucial role in defense mediated by the mycorrhiza (Song et al. 2013). Mycorrhizal mycelia also form long interconnected networks known as common mycorrhizal networks (CMNs) that can connect multiple plants via their roots. In addition, to acting as conduits for the exchange of water and nutrients like nitrogen and phosphorous, they act as information "superhighways" between them (He et al. 2003; Selosse et al. 2006; Mikkelsen et al. 2008; Barto et al. 2012). Pathogen-infected tomato plants transmit defense signals to healthy plants via CMNs resulting in the induction of defense genes resulting in induced resistance to future attacks (Song et al. 2010). In bean (*Vicia faba*) plants, infestation by the pea aphid (*Acyrthosiphon pisum*) results in the activation of SA-mediated defenses, and the induction of these defenses also occurs in uninfested plants only when the plants are connected by CMNs (Babikova et al. 2013b). Additionally, the transmission of a defense signal to healthy plants occurs rapidly within 0–24 h resulting in the production of volatiles that make the healthy plant unattractive to aphids (Babikova et al. 2013a). The nature of the signal that is transmitted is unknown; however sufficient evidence now exists to show that plants communicate the presence of AGH with each other's CMNs.

17.5 Conclusion

Plants have developed sophisticated strategies to protect themselves against insect herbivores. The impact insect herbivores cause on world agriculture has resulted in intense research on plant defense mechanisms against insect herbivory. Roots are increasingly being recognized as important contributors to plant defenses not only against belowground insect herbivores but also against aboveground herbivores (AGH). Transcriptional profiling of roots during AGH has revealed that plants are capable of fine-tuning their response depending on which part of the plant, the root or the shoot, is under attack. Roots also serve as the sites of synthesis of numerous defensive compounds that exert anti-herbivore effects in the shoots. Roots act as sites of storage during herbivore induced resource sequestration. The interactions of roots with a wide variety of soil microbes also influence the outcome of plant-insect interactions. This chapter summarizes the current status of research on roots as important contributors to plant defense against AGH. In addition to possessing a potent defense system against BGH, roots also contribute significantly to plant defense strategies to AGH. Although there are several questions that yet remain to

be answered, a survey of current literature highlights the importance of roots in plant response to AGH. It is evident that a comprehensive understanding of plant-insect interactions can only be achieved by including the role of roots in the conservation. Far away and safe from the AGH, roots serve as sites for the synthesis of defensive compounds and as sites for the storage of valuable photoassimilates and recruit the help of beneficial soil microbes for protection.

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Part IV
Applied Engineering of Roots

Chapter 18
Advances in Transformed Root Cultures for Root Biofactory and Phytoremediation Research

Jutta Ludwig-Müller, Jianfeng Xu, Elizabeth Agostini, and Milen I. Georgiev

18.1 Hairy Roots Induction: From an Infection Disease to a Biotechnological Resource

Agrobacterium rhizogenes belongs to a group of phytopathogenic bacteria within the order Rhizobiales. The disease, called hairy root disease, is caused by the transformation of the plant cell by plasmid-derived DNA of the bacteria. This process proceeds in analogy to the transformation of plant cells by *Agrobacterium tumefaciens*, the causal agent of crown gall disease (Pitzschke and Hirt 2010). However, for both processes different genes are transferred into the host, which cause on the one hand the tumorous growth of crown gall and on the other hand the extensive root system of hairy roots. The principles of transformation, however, seem to be quite similar. Both species contain plasmids, which are coined Ti-plasmids, for tumor inducing in the case of *A. tumefaciens*, and in analogy Ri-plasmids, for root inducing in the case of *A. rhizogenes*. These contain the T-DNA, for transfer DNA, which is the part of the plasmid transferred into the host plant's nucleus. The T-DNA of *A. tumefaciens* contains the genes for the synthesis of two plant hormones auxin and cytokinin, as well as the genes for the

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synthesis of the opines. The T-DNA of *A. rhizogenes* contains the so-called *rol* genes, of which the function until today is not yet elucidated (Veena and Taylor 2007). Even though four *rol* genes (A, B, C, and D) are transferred to induce hairy roots, the major player seems to be *rolB*, because, first, loss-of-function mutation renders the bacteria avirulent and, second, when *rolB* is introduced into the host plant genome as a single gene, it is capable of hairy root induction (Altamura 2004).

While for genetic engineering the *A. tumefaciens* T-DNA is "disarmed," i.e., the tumor-inducing genes are removed (Tzfira and Citovsky 2006), the complete T-DNA of *A. rhizogenes* is used for genetic transformation to generate organ cultures, the hairy roots. While *A. tumefaciens* transformation is one method of choice to generate transgenic plants with novel traits, generation of hairy roots has been used to increase the production of highly bioactive plant secondary metabolites, potentially beneficial in medicine (Georgiev et al. 2010). Therefore, the focus within the last decades has not so much been on the biology of *A. rhizogenes*, but rather on improving transformation protocols for different plant species with the bacterium.

Biotechnological production of secondary plant metabolites has been of interest for many decades (Georgiev et al. 2007; Verpoorte et al. 2002). Many plant species with metabolites having interesting pharmaceutical properties are on a list of endangered species, and therefore novel approaches have to be found to get hands on these compounds (Gómez-Galera et al. 2007). Organ cultures have been used to produce secondary metabolites under sterile conditions, but these are more expensive in cultivation because they rely on the addition of plant hormones to the culture medium. On the contrary, transformation of plant tissues with *A. rhizogenes* generates organ cultures, which are independent of plant hormones for their growth (Georgiev et al. 2007; Vasilev et al. 2006; Zhang et al. 2009). In addition, a hairy root is a more organized structure, so biosynthetic processes, which need compartmentation in the plant cells, can be much better simulated in a complex organ. Similarly to cell cultures, hairy roots can be cultivated under sterile and controlled conditions depending on the type of cultivation method used. In both cultivation systems, cell and organ cultures, it is possible to produce higher amounts of a given compound in comparison to the mother plant (Srivastava and Srivastava 2007). However, a preselection of plant cultivars before the culture is induced might be advantageous for the production of higher levels of a secondary metabolite. In addition, during the cultivation method the biosynthesis of compounds can be induced by so-called elicitors, either abiotic or chemical factors added during the cultivation procedure (Staniszewska et al. 2003; Zhang et al. 2009). Finally, the generation of hairy root cultures with novel traits is possible by using *A. rhizogenes* with additional information on the T-DNA (Fig. 18.1). These can be genes encoding enzymes for metabolic pathways, which might be rate limiting, but also transcription factors, which could control major steps in a given pathway (Georgiev et al. 2010). Combining these transgenic approaches with elicitation could result in much increased production rates.

One major factor to consider for the genetic alteration of a given pathway is to gain as much knowledge on enzymatic steps, competing reactions and regulatory

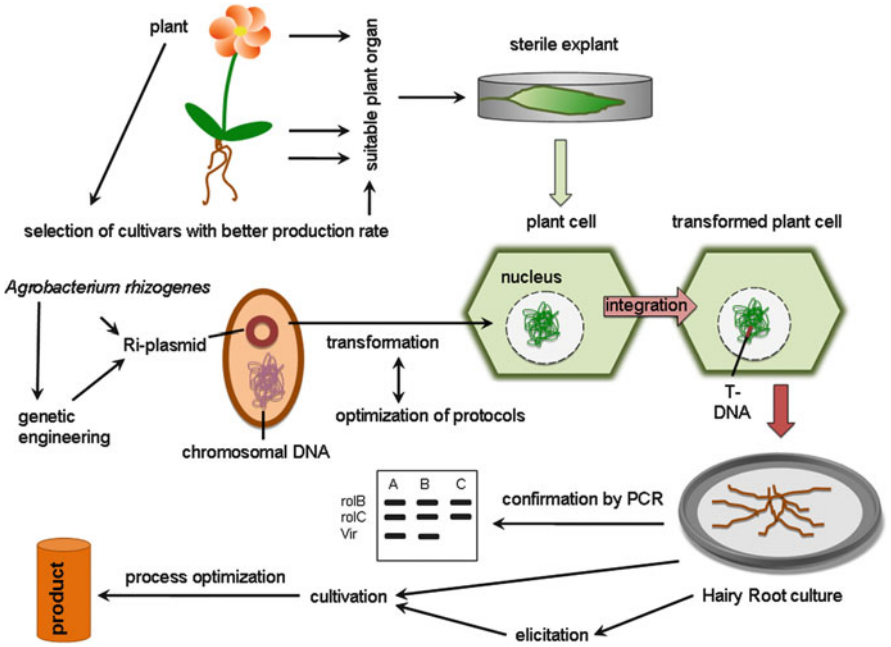

Fig. 18.1 Scheme to demonstrate an example for a common transformation protocol for the generation of hairy root cultures using either wild type or genetically modified *Agrobacterium rhizogenes* strains. In the gel lane A represents bacteria in culture, lane B a contaminated hairy root culture, and lane C a transformed, uncontaminated culture

factors of the native pathway (Georgiev et al. 2010). Alterations can include the increase of a specific bottleneck enzyme (or several), the decrease of an unwanted side pathway to elevate availability of precursors for the main pathway, and the increase in regulatory proteins, i.e., transcription factors. One has to take into account also the transport rates, if several cellular compartments are involved, the availability of precursors and cofactors and possible unwanted effect such as co-suppression of homologous genes, which would actually lead to a decrease in the amount of transcript. Finally, unwanted products could be removed from a beneficial culture by using transgenic approaches to suppress a biosynthetic pathway deliberately (Georgiev et al. 2012). The T-DNA can be transformed with the gene of choice and the plasmid is then brought back into suitable *A. rhizogenes* strains. Suitable strains in this case should be highly virulent, so that transformation events lead to the strong hairy root phenotype.

One of the main obstacles so far is still the transformation protocol. *A. rhizogenes* has a wide host range within monocots and dicots, and in theory, hairy roots can be obtained from any tissue of a plant, i.e., leaves, roots, and flower organs (Ono and Tian 2011; Porter 1991). Mostly parts of leaves are taken, which are then sterilized and incubated with the bacteria. Cutting the leaves induces wounds necessary for penetration of the bacteria. Transformation events are

increased when phenolic compounds, which are signals for the bacteria in nature, are added. For example, acetosyringone and others can enhance the transformation of plant tissues by *A. rhizogenes* as well as *A. tumefaciens* (Giri and Narasu 2000; Joubert et al. 2002). Even though hairy roots can be used themselves to produce antimicrobial compounds (Wang et al. 2012), sometimes the endogenous compounds of a given plant reduce or completely inhibit bacterial growth and thereby transformation. To reduce antimicrobial plant compounds, adsorbing compounds such as charcoal can be added, which often results in increased transformation events. Other protocols use additional sonication to increase the penetration with agrobacteria (Georgiev et al. 2011b). While growth of hairy roots from leaves can be rather easily monitored, the outgrowth of hairy roots, if a root is the origin for transformation, is much more difficult. Here, especially the need for inclusion of selection markers in the *A. rhizogenes* strain might be advantageous. Otherwise, the roots need to be screened by PCR to test for integration events of the T-DNA. This should be in general tested for all hairy root cultures. Here, routine protocols include the amplification of *rol* genes, which are transferred into the plant genome, but could also be present in contaminating bacteria, which have not been removed by antibiotics treatment during initial cultivation periods (Rahman et al. 2004). Therefore, additional PCR reactions are carried out using genes only present in the bacterial chromosome. The expected result for hairy roots would be the amplification of the *rol* genes and not the gene(s) from the chromosome, whereas a control using free bacteria should amplify all bands. This can be done by single PCR reactions, but also multiplex protocols are used for the amplification of several bands in one assay. In these tests the identification of foreign genes could also be included (Georgiev et al. 2011b).

18.2 Bioproduction of High-Value Plant-Derived Molecules by Hairy Roots

Early years of transformed root culture research were mainly focused on underlying the hairy root syndrome (e.g., the host range of plant species susceptible to infection, the mechanism of the infection and genetic transfer from bacteria to host cell). Thus, the immense biosynthetic potential of hairy root cultures was largely neglected for years. Since the 1990s, however, hairy root cultures received increasing attention as biotechnological matrices for the mass production of valuable molecules, because of their several attractive features, as high genetic and biochemical stability (compared to dedifferentiated plant in vitro systems) and relatively fast growth rates (compared to adventitious roots) in hormone-free media (Georgiev et al. 2007, 2012). To date, hairy roots are induced from over 500 plant species, including dozens of endangered and threatened medicinal plant species (Georgiev et al. 2012). The mainstreams of hairy roots application include production of value-added plant-derived metabolites and therapeutic proteins,

phytoremediation, biotransformation, and assistance in molecular breeding (Georgiev et al. 2010; Guillon et al. 2006).

Plants are used since time immemorial to feed people and in the production of beverages but also provide essential materials for clothing and shelter, for writing and coloring materials, for hunting and murdering, and even for ritualistic purposes (as hallucinogens). Moreover plants accumulate a wide spectrum of metabolites (mainly secondary ones), used for centuries as vital source of drugs for treatment of numerous diseases. Nowadays over a quarter of all prescribed current drugs are derived either directly or indirectly from plants (Georgiev 2012). This is especially the case in oncology, immunosuppression, and metabolic disorder therapeutic areas where natural products have played a central role in lead discovery (Butler 2005). The continuously increasing demands for drug leads, produced by ever-greener processes, along with significant reductions in biodiversity, are driving efforts to find alternatives to produce high-value plant-derived metabolites (Georgiev et al. 2007).

Some remarkable examples of high-value metabolites produced by hairy root cultures, including anticancer (paclitaxel, camptothecin, and justicidine B), antimalarial (artemisinin), and anti-inflammatory (harpagoside and verbascoside) substances, are emphasized in Fig. 18.2 and discussed further.

Paclitaxel (Fig. 18.2), a complex diterpenoid that is the active ingredient of Taxol®, has been isolated for the first time in the early 1970s by Wani and his coauthors from the stem bark of the pacific yew *Taxus brevifolia* (Wani et al. 1971). Paclitaxel and related taxanes are microtubule-stabilizing drugs widely used in the treatment of various kinds of cancer, inter alia breast cancer, ovarian cancer, AIDS-related Kaposi's sarcoma, and non-small cell lung cancer (Onrubia et al. 2013). The market of paclitaxel and related taxanes is still expanding, as currently annual global sales are worth over five billion US dollars (Malic et al. 2011). Commercial production of paclitaxel from natural sources is not economically feasible as *Taxus* plants grow very slowly, and their paclitaxel contents are very low (<0.02 % of the dry weight of the bark, where levels are highest). Hairy root cultures of *Taxus* x *media* var. *Hicksii* have been found to produce paclitaxel at levels of 40 μg/g dry roots. Syklowska-Baranek et al. (2009) recently found that the content of paclitaxel in transformed roots can be further enhanced through combinatorial feeding with L-phenylalanine and elicitation with methyl jasmonate, reaching 568 μg/g dry roots or about 14 times higher compared to the control non-stimulated roots.

Camptothecin (Fig. 18.2), a pentacyclic quinoline alkaloid, exhibits anticancer properties due to its inhibition of DNA topoisomerase (Yamazaki et al. 2010). Hairy root cultures of *Ophiorrhiza pumila* were found to produce up to 0.1 % camptothecin per unit dry weight (Yamazaki et al. 2010), which clearly outlines the perspectives for its bioproduction by green root factories. Justicidine B, an arylnaphthalene lignan with cytotoxic, antiviral, fungicidal, antiprotozoal, and antiplatelet properties, was produced by transformed root cultures of *Linum leonii* (Vasilev et al. 2006) in amounts (10.8 mg/g dry weight per unit dry weight) significantly exceeding those obtained from callus cultures (five times less compared to the respective hairy roots) of the same species.

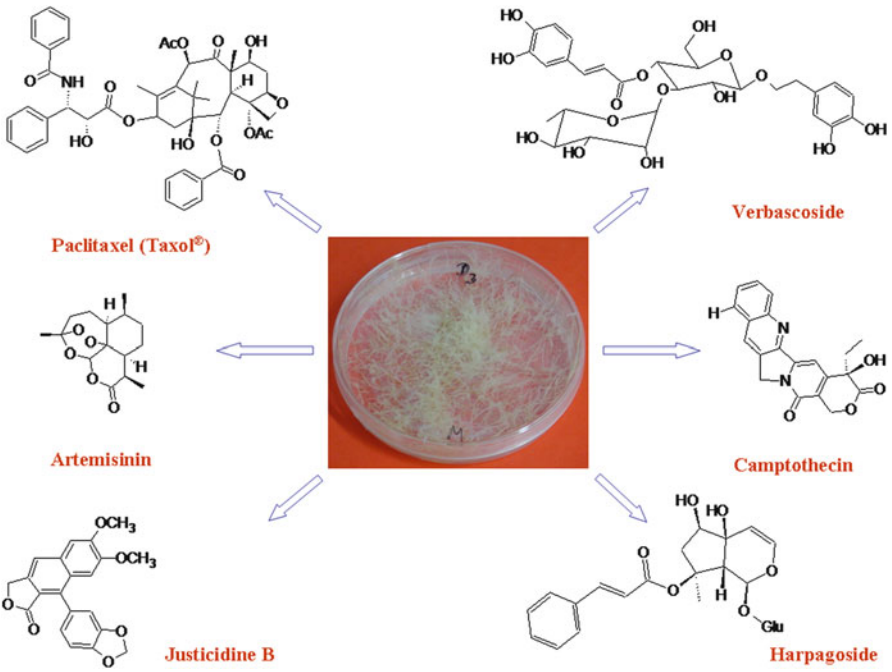

Fig. 18.2 Chemical structures of high-value plant-derived secondary metabolites produced by hairy root culture systems

Artemisinin (Fig. 18.2), a sesquiterpene lactone produced by *Artemisia annua*, is a highly active compound against the parasitic protozoa of the genus *Plasmodium*, the causative agent of malaria. Malaria is a very common and virulent tropical disease, which causes about two million deaths per year (mainly of infants and children; Potterat and Hamburger 2008). Large-scale artemisinin extraction from the natural sources is laborious and costly, because of the low yields in the intact plants (0.01–0.8 %; Potterat and Hamburger 2008). In searching of alternative sources for artemisinin supply, hairy root cultures of *A. annua* were induced in Weathers' laboratory in Worcester Polytechnic Institute (USA). Moreover, the artemisinin biosynthetic process was upscaled in several bioreactors, as the most promising appeared to be mist bioreactor configurations (Kim et al. 2001).

Hairy root cultures were also found to be attractive producers of anti-inflammatory substances. Harpagoside (Fig. 18.2) is an iridoid glycoside (cinnamic ester) with remarkable biological properties, such as antiarthritis, anti-inflammatory, and analgesic effects. It is the major constituent of the iridoid pool in devil's claw plants (*Harpagophytum procumbens*, Pedaliaceae). By transforming shoot tip explants of devil's claw with *A. rhizogenes*, Grabkowska et al. (2010) successfully induced hairy roots capable of growing under submerged conditions and producing ca. 0.32 mg harpagoside/g dry root mass. Verbascoside (Fig. 18.2) is a water-soluble naturally occurring phenylethanoid glycoside, isolated from several

medicinal plants. Several pharmacological studies have shown verbascoside to have a wide spectrum of biological activities (in vitro and in vivo) including antimicrobial, immunomodulatory, anti-inflammatory, and cholinesterase inhibitory properties (Georgiev et al. 2011a; Gyurkovska et al. 2011). Hairy root culture of *Verbascum xanthophoeniceum* was found to produce significant amounts of bioactive verbascoside (over six times more than in mother plant leaves) during their submerged shake-flask cultivation (Georgiev et al. 2011b).

Although no commercial processes based on hairy root culture have been yet established, there have been many proof-of-concept studies (e.g., the production of anticancer, anti-inflammatory, and antimalarial compounds), which allow hairy root-based processes to be upscaled while keeping their immense biosynthetic potential.

18.3 Hairy Root Culture: Green Factories for Biopharmaceuticals

Biopharmaceuticals are the next generation of high-value therapeutic proteins that offer great importance in the treatment of various diseases like cancers, heart attacks, strokes, diabetes, anemia, and hemophilia (Walsh 2005). Commercial production of biopharmaceutical proteins has traditionally relied on bacterial fermentation and mammalian cell cultures. However, the inherent disadvantages of these expression platforms in terms of cost, scalability, safety, and authenticity of proteins produced have prompted research into alternatives. Plants are emerging as one of the most powerful alternative bioproduction platform because of their economic and safety advantages over the traditional systems and the presence of posttranslational modification machinery, such as glycosylation that enables the production of complex human proteins (Hood et al. 2012; Ma et al. 2003; Ono and Tian 2011; Xu et al. 2012). Hairy roots that can be grown in vitro and in bioreactors, just as plant suspension cells, represent an attractive contained plant production system preferred to plants grown in open field. The contained in vitro culture systems combine the merits of whole-plant system with microbial and mammalian cell culture benefits (Georgiev et al. 2012; Xu et al. 2011). They show intrinsic benefits like fast-growing, batch-to-batch consistency, production in compliance with good manufacturing practice (GMP), less concerns over regulatory compliance and product safety, and simpler procedures for downstream processing and protein purification, especially when proteins are secreted into the culture medium (rhizosecretion; Hellwig et al. 2004; Huang and McDonald 2009). Yet as a more organized tissue, hairy root presents additional benefits over suspension cells, including genotype and phenotype stability and being able to grow on plant hormone-free media (Guillon et al. 2006).

The hairy root culture-based process for biopharmaceutical production is shown in Fig. 18.3. Tobacco (*Nicotiana tabacum*) is by far the most largely used plant species for the production of recombinant biopharmaceutical (Daniell et al. 2009). For high-level protein expression and secretion in hairy root system, a strong constitutive promoter, e.g., *cauliflower mosaic virus 35S* (*CaMV35S*), and a signal peptide to direct nascent proteins through the default ER-Golgi secretory pathway are strategically designed. Further enhancement of the transgene expression can be achieved by using a double-enhanced *CaMV35S* promoter (Medina-Bolivar et al. 2003; Woods et al. 2008) or a chimeric super-promoter [*(Aocs)3AmasPmas*] (Nopo et al. 2012; Rukavtsova et al. 2007) as well as introduction of a translational enhancer (5′-untranslated leader sequence), such as that derived from tobacco etch virus (TEV) (Liu et al. 2008; Nopo et al. 2012) or from alfalfa mosaic virus (AMV) (Anuar et al. 2011; Gallie et al. 1987). Except for constitutive expression, controlled and temporal gene expression in the hairy root system by inducible promoters, such as *Arabidopsis small heat shock protein 18.2* promoter (Lee et al. 2007) and *glucocorticoid-inducible* promoter (Peebles et al. 2007), has been achieved. Since a fully functional murine IgG1 monoclonal antibody was produced by tobacco hairy roots in 1997 (Wongsamuth and Doran 1997), nearly 20 therapeutic proteins, including antibodies, vaccines, cytokines, therapeutic enzymes, etc., have been successfully expressed in hairy root systems, which were summarized in a recent review (Georgiev et al. 2012). Protein yields as high as 64 mg/L of a murine IgG1 produced by tobacco hairy root were achieved (Martinez et al. 2005). In addition, the rhizosecretion feature of hairy roots has also been exploited. For example, up to 43 % of the murine IgG1 was found to be secreted into tobacco hairy root culture medium when polyvinylpyrrolidone (PVP) and gelatin were supplemented (Wongsamuth and Doran 1997).

The scaling-up of culture systems is of critical importance for achieving commercial level of biopharmaceutical production. However, the special morphological characteristics of hairy roots including nonhomogenous growth and highly branched phenotypes present major challenges to culture scale-up in bioreactors (Ono and Tian 2011). Compared with the production of phytochemicals by hairy roots, protein offers additional challenges due to the susceptibility of products to proteolytic degradation and their increased sensitivity to shear stresses (Hood et al. 2012). To achieve high production yields, a culture system with low shear stresses and higher oxygen transfer efficiency (thus low levels of protease generation) is especially needed. So far, two types of bioreactors have been successfully used for scaling up hairy root cultures for biopharmaceutical production: The first, based on airlift concept, is a liquid-phase bioreactor where compressed air released from the base of the culture vessel provides aeration and agitation as the air moves up through the root bed (Caspeta et al. 2005). The second type, the mist reactor, is a gas-phase reactor where hairy roots are exposed to humidified air or a gas mixture and nutrients delivered as droplets by spray nozzles or ultrasonic transducers (Eibl and Eibl 2008). Compared to liquid-phase bioreactors, the mist bioreactor offers advantages in that it reduces the volume of culture medium, enhances oxygen transfer efficiency, and totally eliminates hydrodynamic stress imparted on root

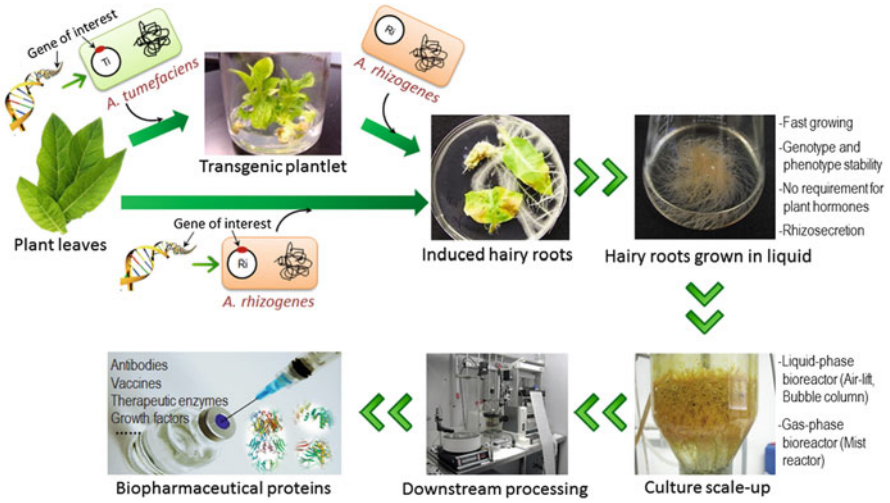

Fig. 18.3 Schematic illustration of the hairy root culture process for producing biopharmaceuticals. Transgenic hairy roots expressing certain therapeutic protein can be established either by infecting stably transformed plants (expressing the target protein) with *Agrobacterium rhizogenes* (*upper pathway*) or by directly infecting wild-type plants with *A. rhizogenes* harboring binary vectors containing gene of interest (*lower pathway*) (Pham et al. 2012). Scale-up production and downstream purification methodologies adapted from other cell-based bioproduction systems can be used in delivering biopharmaceuticals to the market. *Ti* Ti-plasmid, *Ri* Ri-plasmid

biomass (Liu et al. 2009; Towler et al. 2006; Weathers and Giles 1988). A recent study scaling up the tobacco hairy root cultures for production of murine interlukin-12 (mIL-12) showed that a mist reactor made from disposable plastic bag supported better protein production than an airlift reactor (Liu et al. 2009). This is actually the first demonstration of successful production of a pharmaceutical protein in a mist bioreactor. Future research should focus on establishing effective and economical bioreactor culture systems for commercial-scale production.

18.4 Applications of Hairy Roots for Phytoremediation Research: Current Knowledge and Challenges

Hairy roots have been frequently applied in phytoremediation research as model plant systems, for screening the potentialities of different plant species to tolerate, accumulate, remove, and/or degrade environmental pollutants. This is in part due to several advantages of hairy roots and the fact that roots have evolved specific mechanisms to deal with pollutants, because they are the first organs to have contact with them. Therefore, they are the sites where the first reactions against the contaminant take place. Among the main advantages, they are independent of site and weather conditions, and they have a short sub-cultivation period (2 or 3 weeks)

which substantially reduces the time required to carry out studies. The experimental variables are easily controlled, thus providing a more reliable and reproducible experimental system over time, for phytoremediation purposes (Doran 2009; Harms et al. 2003). They also have a prolific root growth, and thus, large amounts of biomass are generated in a controlling setting (Erlenmeyer's or bioreactors). Moreover, hairy roots grow in an environment that is totally free of microbes and can be used to distinguish the responses and capabilities of plant cells from rhizospheric microorganisms, without the interference of soil matrix.

The results derived from these in vitro cultures can be used to predict the responses of plants to environmental contaminants because if a compound is metabolized by in vitro hairy roots, this is a clear indication that the plant has the genetic capacity of biotransforming this compound (Doran 2009). In fact, hairy roots closely reflect the biochemical pathways typical of the parent plant, and although the natural translocation from roots to shoots is obviously blocked, this characteristic frequently constitutes an additional advantage of this plant system. They can also improve the design and reduce the cost of subsequent conventional whole-plant experiments. Furthermore, hairy roots have the ability to produce large amounts of exudates which may contribute not only to remove harmful pollutants but also to sequester them.

As it is well known, the metabolism of xenobiotic organic pollutants is a very complex process and a great variation was observed in the fate of pollutants and their degradation products among different plant species. In this context, hairy roots could provide valuable information regarding plant metabolic pathways. For instance, they have been used to explore metabolism of explosives, phenolics, polychlorinated biphenyls (PCBs), and textile dyes as well as to analyze the effect of these pollutants on the activity of plant pollutant-converting enzymes [glutathione S-transferase (GST); peroxidases (Px); cytochrome P450, laccases, lignin peroxidases, tyrosinases among others] (Agostini et al. 2011; Ghodake et al. 2009; Govindwar and Kagalkar 2010; Patil et al. 2009; van Aken et al. 2010). The chemical mechanism of degradation, the involvement of reactive oxygen species (ROS) in the removal process (Gujarathi et al. 2005), the nature, and the compartmentalization of some final products of the removal reaction (Talano et al. 2010) and several biochemical and physiological processes such as the antioxidative stress responses (Ibáñez et al. 2011), lipid peroxidation, as well as the signaling mechanisms involved in the responses to environmental pollutants (Sosa Alderete et al. 2011, 2012a) were also studied using hairy roots (Table 18.1). All these studies not only help in understanding the pollutant uptake, their metabolism, and removal but also open the possibility of designing new genetic and biochemical approaches to perform a deep study of transformation pathways.

On the other hand, hairy roots could be used as sources of enzymes (Px and laccases) which demonstrated to be powerful catalysts for the removal of harmful pollutants, and they provide valuable information about them, such as variations in substrate preference and catalytic efficiencies toward contaminants (Coniglio et al. 2008; González et al. 2008; Sosa Alderete et al. 2012b; Telke et al. 2011).

Table 18.1 Phytoremediation of organic environmental pollutants using hairy root cultures from different plant species

Plant species	Summary of remediation strategies and/or main aspects studied	References
Brassica juncea, Beta vulgaris, Raphanus sativus, Azadirachta indica	Screening of some plant species to find those with the highest potential for phenol removal	Singh et al. (2006)
Solanum lycopersicon, Brassica napus	Removal of phenol and/or 2,4-DCP with high efficiency. Establishment of optimal conditions (pH, temperature, concentration of co-substrate, time of treatment) for the removal process. Tolerance studies	Agostini et al. (2003), González et al. (2006, 2008), Coniglio et al. (2008)
Nicotiana tabacum	Studies related to physiological responses of plant tissues against phenol (oxidative stress, signal transduction pathways)	Ibáñez et al. (2011), Sosa Alderete et al. (2011, 2012a)
Brassica napus	Removal of 2,4-DCP on a large-scale in bioreactors. Hairy roots could be reused in several consecutive cycles, with high efficiency. The compartmentalization of some of the final products of the 2,4-DCP removal was shown	Angelini et al. (2011), Talano et al. (2010)
Armoracia rusticana	Plant metabolism of various explosives (TNT, DNT, ADNTs, DANTs) was studied as well as the effect of these pollutants on enzymes such as GST and Px	Nepovim et al. (2004)
Solanum nigrum	Detoxification products of PCB metabolism were identified. Newly discovered plant metabolites of PCBs were described: hydroxy-PCBs, methoxy-PCBs, and hydroxy-methoxy-PCBs	Rezek et al. (2007, 2012)
Tagetes patula L.	High concentrations of the textile dye Reactive Red 198 were removed. Hairy roots could be reused for five consecutive decolorization cycles. A possible pathway for the biodegradation was proposed and some metabolites were identified	Patil et al. (2009)
Brassica juncea L.	An intracellular laccase was purified, characterized, and applied for the removal of textile dyes. The dye decolorization rate was significantly enhanced in the presence of various redox mediators	Telke et al. (2011)

(continued)

Table 18.1 (continued)

Plant species	Summary of remediation strategies and/or main aspects studied	References
Armoracia rusticana L.	Hairy roots were able to take up and detoxify *N*-acetyl-4-aminophenol (paracetamol). A paracetamol-glucoside was described as the dominant metabolite, which would be a precursor of insoluble bound residues associated to the cell wall	Huber et al. (2009)

2,4-DCP 2,4-dichlorophenol, *TNT* 2,4,6-trinitrotoluene, *DNT* 2,4-dinitrotoluene, *ADNTs* aminodinitrotoluenes, *DANTs* diaminonitrotoluenes, *PCBs* polychlorinated biphenyls, *GST* glutathione S-transferases, *Px* peroxidases

Toxicity of final degradation products is another important feature to be addressed, because frequently unknown and final transformation products are difficult to identify. Transformation into nontoxic products is preferable for phytoremediation, and hairy roots can help researchers in such studies (González et al. 2012a; Paisio et al. 2010). As could be seen, there are several examples in the literature which illustrate the potentialities of hairy roots to understand the complex biochemical and molecular mechanisms involved in phytoremediation of organic pollutants. Some selected examples are summarized in Table 18.1.

Hairy roots are also suitable for studying the mechanisms of metal uptake, as well as to investigate the physiology and biochemistry of metal accumulation and tolerance in a wide range of plant species (Doran 2011). Although the mechanisms involved are still only partially understood, several sequestration and detoxification strategies are known to occur in plant cells; among them complexation with phytochelatins (PCs) synthesized from glutathione has been identified as an important mechanism for detoxifying metals. In this way, hairy roots seem to be adequate systems for studying PC induction, as was first demonstrated by Maitani et al. (1996) in *Rubia tinctorum* hairy roots. Furthermore, some analogous new families of PC-related peptides as well as PCs were detected using *Armoracia rusticana* hairy roots exposed to Cd (Kubota et al. 2000). These and other works (Sanità di Toppi et al. 1999; Wu et al. 2001) highlight the valuable contribution of hairy roots to improve understanding of the complex mechanisms triggered by inorganic pollutants. Hairy roots are also a convenient laboratory tool for investigating hyperaccumulation mechanisms of heavy metals. Moreover, they contribute to understanding the role of organic acids in heavy metal detoxification as well as their localization in roots (Boominathan and Doran 2003). In addition, hairy roots of *Alyssum bertolonii* were used for generating a metal-enriched product from the harvested plant biomass, which might be useful for phytomining operations (Boominathan et al. 2004).

Hairy roots have a large surface area in comparison with nontransformed roots, due to their highly branched nature. Therefore, they can also be used for rhizofiltration purposes, i.e., to absorb, concentrate, and/or precipitate hazardous compounds, particularly heavy metals or radionuclides, from aqueous solutions

Table 18.2 Use of hairy root cultures from different plant species to study several aspects of inorganic compound phytoremediation

Plant species	Summary of remediation strategies and/or main aspects studied	References
Rubia tinctorum	Induction of PCs containing Cd and Cu by adding Cd to the culture medium	Maitani et al. (1996)
Daucus carota	Short-term response to Cd mainly in terms of production of PCs and stress proteins, synthesis of stress ethylene, and involvement of lipid peroxidation	Sanità di Toppi et al. (1999)
Armoracia rusticana	A new group of SH-containing peptides as well as PCs were induced after exposure to Cd	Kubota et al. (2000)
Adenophora lobophylla; A. potaninii	A comparative study on Cd detoxification was performed. PCs have been isolated and characterized from hairy roots. Different strategies for Cd detoxification in both species were suggested	Wu et al. (2001)
Thlaspi caerulescens; Alyssum bertolonii	Uptake and storage of Cd and Ni, respectively. Role of organic acids in Cd detoxification; localization of heavy metals in hyperaccumulator and non-hyperaccumulator roots and the distribution of metals. Investigation of the energy source for transmembrane transport of Cd and Ni	Boominathan and Doran (2003)
Brassica juncea	Rhizofiltration of Cd and Pb with high efficiency within a short period of exposure. The influence of high concentrations of these heavy metals on antioxidative enzymes was studied	Eapen et al. (2007)
B. juncea and Chenopodium amaranticolor	Both hairy roots removed U within a short period of incubation. *B. juncea* hairy roots proved to be a better candidate for U removal, as they could take up two to four times more U from the solutions, at concentrations up to 5,000 μM	Eapen et al. (2003)
Solanum nigrum	High efficiency for Zn uptake and accumulation	Subroto et al. (2007)
Daucus carota	A test to determine the threshold toxicity of U in optimal conditions of both exposure and observations was proposed	Straczek et al. (2009)
A. rusticana	High accumulation of U in the presence of phosphate, which had a stimulating effect on growth of hairy roots and accumulation of the pollutant	Soudek et al. (2011)

PCs phytochelatins

(Eapen et al. 2007). For instance, *Brassica juncea*, *Chenopodium amaranticolor*, *Daucus carota*, and *A. rusticana* hairy roots were applied for the removal of high U concentrations (Eapen et al. 2003; Soudek et al. 2011; Straczek et al. 2009). These authors also demonstrated that hairy roots would be appropriate to study toxicity and distribution of U in plant roots in optimal conditions and to examine further physiological processes triggered by radionuclides. A summary of the main applications of hairy roots for inorganic phytoremediation is provided in Table 18.2.

Until now, limited studies were carried out using transgenic hairy roots in order to improve pollutant removal. Nevertheless, encouraging results were obtained. For instance, Banerjee et al. (2002) demonstrated that *Atropa belladonna* hairy roots expressing a rabbit P4502E1 enzyme were able to metabolize trichloroethylene,

whereas transgenic tomato and tobacco hairy roots were used to remove phenol with higher efficiency than non-transgenic roots (Sosa Alderete et al. 2009, 2012b; Wevar Oller et al. 2005).

A very recent and emerging field of research is the application of hairy roots to investigate microbe-assisted phytoremediation of pollutants, which offers important advantages and challenges. As was demonstrated, the association between hairy roots and microorganisms, such as arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria, could positively contribute to the improvement of the phytoremediation process (González et al. 2012b; Ibáñez et al. 2011). Studying these complex relationships at this appropriate scale would probably be the key to answering several aspects that are unknown about rhizoremediation.

In conclusion, the combinatorial use of different strategies and adequate approaches at the laboratory scale would be very helpful to increase tolerance and removal of pollutants and thus to improve the efficiency of phytoremediation in the field.

18.5 Perspectives

Today, hairy root cultures can be induced from any plant species, including rare and threatened plant species (e.g., some medicinal plants), which have a clear ecological benefit and contribute to the biodiversity preservation. The immense biotechnological potential of hairy root cultures to serve as green factories for mass production of high-value plant-derived metabolites and recombinant therapeutic proteins is clearly recognized and acknowledged. In addition, transformed root cultures show promising potential for other ecologically valuable applications, e.g., their utilization for phytoremediation purposes. Although no commercial processes based on hairy root culture have been yet established, there have been many proof-of-concept studies, which allow hairy root-based (bio)processes to be upscaled while keeping their biosynthetic potential. Finally, recent developments in so-called "omics" approaches help us to understand the roots' biochemical machinery and give us a powerful tool for deliberate adjustment of desired metabolites' biosynthetic pathways as well as to improve pollutant removal. Of course, several fundamental and technological challenges still remain to be addressed. It can be concluded that hairy root culture technology is entering an exciting period and will undoubtfully have a bright future for diverse purposes.

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Chapter 19
Root Proteomics

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19.1 Introduction

Plant roots are dynamic systems that provide nutrients, water, as well as physical support to plants, and are critical for plant growth and survival. Upon seed germination, primary root meristem growth is initiated under the control of several biological and biochemical reactions. Growth of the root meristem is promoted by an increased rate of cell differentiation through hormonal control rather than by cell division (Moubayidin et al. 2010). The primary root and associated secondary structures, including lateral roots, form the architecture of the root system, which is affected by intrinsic developmental programs and external biotic and abiotic stimuli. The development of lateral roots involves several factors, such as the plant species, soil composition, water and mineral nutrient availability, and hormonal changes (Lynch 1995; Malamy 2005; Malamy and Benfey 1997). These factors alone or in combination can influence the growth and proliferation of roots.

Nutrient distribution is one of the major environmental signals affecting lateral root development, as evidenced by the proliferation of lateral roots in nutrient-rich zones (Zhang and Forde 1999). Water is another determining factor influencing the growth and development of the root system. In soils with low water content, root growth is stimulated as an adaptive mechanism to facilitate water uptake from deeper soil layers. In contrast, the growth and development of roots are negatively impacted in soils with excess water. The regulation of growth and organogenesis in roots in response to abiotic and biotic environmental stimuli is basically controlled

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by endogenous genetic programs. Changes in the expression of genes and accumulation of proteins under normal and water stress conditions are the fundamental factors controlling root architecture. Specifically, external stimuli trigger the regulation of genes and proteins that control cell division and differentiation mainly through the actions of phytohormones.

The role of hormones in the spatial configuration and growth (Hodge et al. 2009) of the root system, particularly the number and length of lateral organs, has been well documented and reviewed. The signaling molecule auxin plays a major role in root growth by controlling root formation, apical dominance, and lateral root proliferation. The application of exogenous auxin increases the initiation and development of lateral roots (Blakely et al. 1988), and the accumulation of auxin in *Arabidopsis* root pericycle cells triggers lateral root initiation by respecifying these cells into lateral root founder cells (Dubrovsky et al. 2008). Cell signaling and transport strongly influence root growth and development. Benková et al. (2003) showed that root formation in *Arabidopsis* involves dynamic gradients of auxin, which maximally accumulates in the tips of growing primordia. Regulation of local auxin gradients by the auxin transport protein PIN represents a common module for the formation of plant organs. Thus, the regulation of root growth is closely associated with the production, transport, and response to plant hormones.

The interactions of plants with soil microorganisms can be beneficial, neutral, or detrimental to the plant host. Plant–microbe interactions greatly influence the entire plant, particularly the root system. Numerous studies have examined the complex mechanisms underlying the interactions of plant roots with soilborne microorganisms (Mathesius 2009) and have revealed that these interactions are mainly controlled and regulated by changes in protein accumulation. However, root exudates also contain proteins that influence the interactions between plants and soil organisms (De-la-Peña et al. 2010). Therefore, proteomic studies of plant roots are expected to provide new insights into the mechanisms underlying and controlling these interactions.

A wide range of proteins are involved in the complex regulatory networks controlling plant growth. Among the various plant organs, the targeting of roots for plant proteome studies has several advantages. First, roots contain relatively low levels of the ribulose bisphosphate carboxylase/oxygenase large subunit, which accounts for approximately 50 % of total proteins in green tissues (leaf and stem) (Hashimoto and Komatsu 2007) and therefore limits the detection of proteins, particularly those in low abundance, in these tissues. This is particularly critical in comparative proteomics, in which the detection of slight changes of protein is desired. Second, the abundance of certain functional groups of proteins is tissue specific. A comparison of the leaf, stem, and root proteomes of rice during growth showed that green tissues had highly similar profiles that differed from those of the root. In particular, photosynthesis- and energy production-related proteins were most abundant in the leaf and stem, whereas those involved in cellular defense were higher in roots (Nozu et al. 2006). In addition, Mooney et al. (2006) showed that 13 % of root proteins are involved in disease resistance compared to only 7 % of leaf proteins. Third, in the case of root-specific studies, such as the proteomic

analysis of plant–microbe interactions, the use of roots is an absolute requirement. Root proteome analyses of plant–pathogen interactions provide information about the total proteins accumulated in roots in response to biotic stresses (Mehta et al. 2008). Fourth, the root can be the main target of study in plants that have roots with high nutritional value, such as sugar beet, cassava, and ginseng. Ma et al. (2013) explained the association of metabolic proteins with the growth strategies of ginseng by root proteome analysis. Fifth, root proteome analysis is a prerequisite for the study of proteins secreted from roots, especially in response to nutrient starvation, which helps the plant survive under unfavorable conditions. For instance, plants actively modify the rhizosphere by the secretion of enzymes such as RNases and purple acid phosphatases, which scavenge and promote the uptake of phosphate from organophosphates (Alexova and Millar 2013).

The functions of plant roots in environmental sensing and communication, gravitropism, uptake of nutrients and water, growth, and development are predominantly controlled at the protein. To determine and assign specific functions to proteins involved in these processes, proteins need to be efficiently extracted, detected, and identified. In this chapter, techniques for protein extraction from roots are described, and root proteins related to hormonal regulation, gravitropism, and abiotic stress responses are reviewed.

19.2 Methodology of Root Proteomics

19.2.1 Extraction of Proteins from Root

The extraction and preparation of protein samples is one of the most challenging steps in any proteomic study. In plant proteomics, the type of plant species, tissue, and organ and the nature of the target proteins affect the techniques that can be used for protein extraction. Furthermore, the presence of vacuoles, rigid cell walls, and membrane plastids makes the extraction process more difficult (Komatsu 2008; Lee and Cooper 2006). An ideal extraction method would reproducibly capture and solubilize the full complement of proteins from a given sample while minimizing post-extraction artifact formation, proteolytic degradation, and nonproteinaceous contamination (Cho et al. 2006; Rose et al. 2004). In protein extraction and solubilization procedures, the combinations and concentrations of detergents, reducing agents, and chaotropic agents that are present in the buffer also have marked effects on the quality of the extracted proteins. In addition, the presence of secondary metabolites and lipids, as well as large amounts of carbohydrates, not only hampers high-quality protein extraction but also impedes high-resolution protein separation in two-dimensional polyacrylamide gel electrophoresis (2-DE), resulting in streaking and reduced resolution of protein spots (Komatsu and Ahsan 2009).

The inherent properties of root tissues have several limitations that can interfere with protein extraction and subsequent 2-DE separation. For example, it is difficult to obtain clean root material from roots grown in soil (Neilson et al. 2010), and root tissue often contains low amounts of proteins (Mehta et al. 2008) and is highly vacuolated, and contaminants such as phenolics and flavonoids are often present (Sumner et al. 2007). In addition, different parts of a root often contain markedly differing amounts of protein. It is known that less vacuolated tissues, such as root tips and nodules, have up to ten times more proteins than other root sections (Sumner et al. 2007). Mehta et al. (2008) reviewed the sample preparation and protein extraction techniques used for studying plant–microorganism interactions. A list of differentially accumulated proteins in roots during plant–microorganism interactions has been reported.

Two main types of extraction buffers are typically used for protein extraction: trichloroacetic acid/acetone-based and phenol-based buffers. The selection of a suitable extraction buffer is influenced by several determining factors, including the type of tissue, aim of the protein extraction, and existence of interfering compounds. A literature search of the extraction procedures used for plant proteins confirmed that various unique techniques are used for protein extraction. Ahsan and Komatsu (2009) performed a comprehensive proteomic study on nine organs from soybean plants in various developmental stages using three different protein extraction and solubilization methods. These analyses revealed that the use of an alkaline phosphatase buffer followed by trichloroacetic acid/acetone precipitation caused horizontal streaking in 2-DE, whereas Mg/NP-40 buffer followed by extraction with alkaline phenol and methanol/ammonium acetate produced high-quality proteome maps with well-separated and high-intensity spots (Ahsan and Komatsu 2009). Thus, phenol extraction might be more suitable for tissues such as leaves with a higher proportion of interfering compounds, whereas both the phenol and trichloroacetic acid/acetone methods might be useful for other tissues.

19.2.2 Root Proteome Analysis Using Gel-Based and Gel-Free Techniques

Once extracted, proteins need to be separated, detected, and identified. Current methods for protein identification and quantitation fall roughly into two groups, gel-based and gel-free techniques, which may be further divided into subgroups that have been extensively described by Nanjo et al. (2011). Gel-based proteomic methods are based on the separation of proteins by 2-DE, which first resolves proteins according to their isoelectric point and then by molecular mass. In comparative proteomic analyses, the intensities of proteins spots on gel images are quantified to detect differentially accumulated proteins. In gel-free proteomics, protein solutions are treated with a protease, such as trypsin, and the resulting

peptides are directly separated and quantified by reverse-phase liquid chromatography coupled to an MS/MS system.

Despite the wide use of gel-based proteomic techniques, this approach does not represent a truly global method because of limitations in identifying specific classes of proteins, such as low-abundance and hydrophobic proteins, excessively large and small proteins, and basic proteins (Zhang et al. 2007). The gel-free proteomics is frequently used for the identification of a wide range of proteins, including highly basic or acidic, and low-abundance or hydrophobic proteins. In addition, Blackler et al. (2008) and Hahne et al. (2008) demonstrated the efficiency of gel-free techniques for identifying integral membrane proteins. Nanjo et al. (2011) compared the efficiency of gel-based and gel-free proteomic approaches for protein identification and found that although gel-based methods provide a visual representation of the proteome, including intact protein maps, such an approach is not suitable for the identification of low-abundance proteins, those with extreme molecular weights, isoelectric points, or hydrophobicity. Therefore, the sample type and study target should determine whether gel-based and gel-free techniques are use alone single or in combination for proteome analysis.

The efficiency of gel-based and gel-free proteomics was examined using samples extracted from soybean root. A total of 161 proteins were identified by gel-based proteomics, whereas 341 proteins were identified using gel-free proteomics (Fig. 19.1). A comparative proteomic analysis of root and hypocotyl proteins using gel-based and gel-free techniques was also performed to examine soybean seedling responses to flooding stress. Using the gel-based approach, a total of 17 spots were found to be differentially regulated by flooding, whereas the gel-free approach detected 81 proteins that were significantly affected by flooding (Nanjo et al. 2010). In another study, proteins were extracted from the root tips of soybean seedlings exposed to flooding treatment and analyzed using a gel-free technique for quantification and phosphoproteomic analyses (Nanjo et al. 2012). The accumulations of a total of 109 proteins were significantly increased by flooding stress, and 40 unique proteins were identified in the phosphoproteomic analysis. The abundance and phosphorylation status of seed lipoxygenase, pyruvate kinase, heat-shock protein 70, and alpha-tubulin were all affected by flooding (Nanjo et al. 2012). These studies demonstrate that gel-free proteomic approaches can identify a significantly higher number of proteins compared to gel-based proteomics and are a good option to decipher the complex regulatory systems of plant roots.

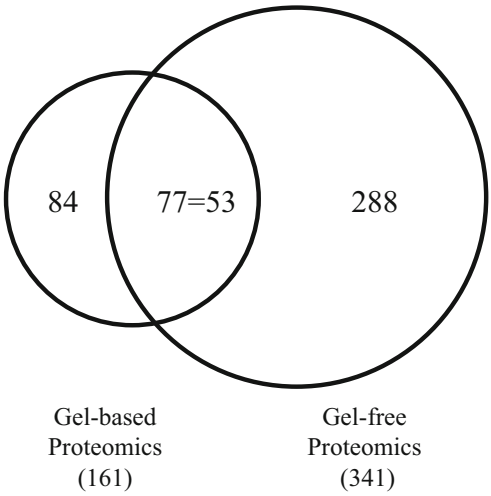

Fig. 19.1 Comparison of gel-based and gel-free proteomic techniques for the analysis of root proteins. Root proteins were extracted from 2-day-old soybeans and then identified using gel-based and gel-free proteomic techniques. The *numbers* indicate the unique proteins identified by both techniques

19.3 Proteomics of Gravitropism and Phytohormones in Roots

19.3.1 Proteomics of Root Phytohormones

Environmental signals can modulate plant responses by inducing changes in the concentration of phytohormones. Puig et al. (2012) reviewed the regulation of shoot and root development mediated by cell signaling pathways and described that a systematic signaling pathway involving a β-carotene-derived hormone expressed in roots regulates shoot development. The role of phytohormones in plant growth and survival is more critical under conditions of nutrient starvation or abiotic stress. For instance, hormonal signals, rather than water availability, control plant growth at high salinity (Munns 2011). In addition, exposure to externally applied or internally generated hormones increase protein accumulation in plants. A review by Komatsu and Tanaka (2004) found that gibberellic acid (GA) application induced changes in the rice proteome and that several hormones with synergistic or antagonistic effects are involved in the growth regulation of roots. Therefore, a wide range of proteins are likely involved in the control or modulation of plant growth and stress responses.

Auxin, cytokinin, ethylene, abscisic acid (ABA), and GA are the main hormones studied in plant root systems. Auxin is the predominant hormone involved in the maintenance of root meristem and regulation of root architecture (Dello Ioio et al. 2008). Auxin influx carriers such as auxin transporter protein (AUX1), p-glycoprotein auxin efflux carrier (PGP), and other members of the AUX1-like (LAX) family of putative transmembrane transporters facilitate auxin influx within transporting cells. In contrast, members of several families of transmembrane proteins, including the PIN family of auxin efflux facilitators and ABC transporters, are

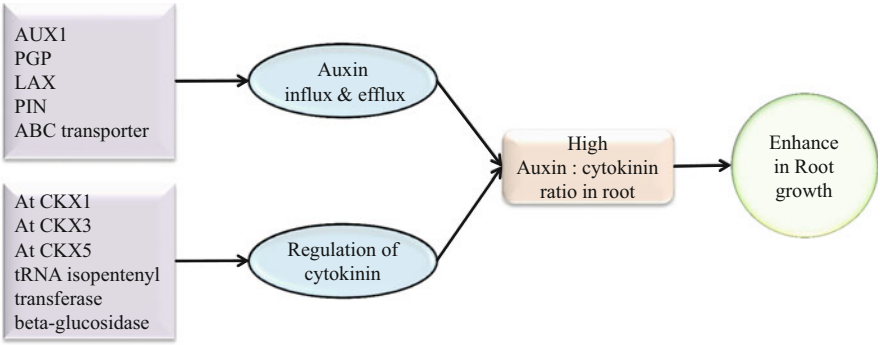

Fig. 19.2 The function and effects of auxin- and cytokinin-regulating proteins (*left*) on root growth. The growth and development of roots are highly influenced by the accumulation and relative ratios of two main phytohormones, auxin and cytokinin

involved in auxin efflux (Band et al. 2012; Bennett et al. 1996; Leyser 2006). These influx and efflux transporters facilitate polar transport and asymmetric distribution of auxin and regulate root morphogenesis (Fig. 19.2).

Cytokinin plays key roles in the regulation of root development and functions as a positive regulator of shoot apical meristem development and as a negative regulator of root apical meristem development (De-la-Peña et al. 2010). Several genes and proteins have been identified that control cytokinin concentrations in plants. In *Arabidopsis*, constitutive expression of AtCKX1, AtCKX3, and AtCKX5 significantly reduces levels of endogenous cytokinins by 30–60 % of that of wild type in both root and shoot meristems (Werner et al. 2003). Cytokinin and auxin interaction controls the balance between cell division and differentiation in the root meristem, as well as root nodule formation (Mathesius 2008). Pernisová et al. (2011) reviewed the interactions of auxin and cytokinin in plant development and showed that changes in auxin levels can affect the concentration of cytokinin and the mechanisms by which proteins modulate hormone accumulation.

Cytokinin may have a close relationship with several nutrients. For example, cytokinin and nitrogen play the same role in the balancing of energy and amino acid metabolism and are involved in the accumulation of several proteins, including adenosine kinase, adenylate kinase, 26S proteasome regulatory particle non-ATPase subunit 10, and NBS-LRR proteins (Ding et al. 2012). Phosphorus deficiency suppresses cytokinin synthesis in plants by decreasing the accumulation of tRNA isopentenyl transferase and beta-glucosidase, which are involved in cytokinin synthesis in the root (Li et al. 2007). Komatsu and Tanaka (2005) reviewed the mechanism of root formation in rice by the treatment of cultured suspension of cells with auxin and zinc and found that the accumulations of seven proteins were increased. Therefore, these results suggest that the interactions between cytokinin and nutrients increase the metabolic activity of plant cells.

Several other hormones are involved in root development. Ethylene promotes adventitious and lateral root formation and is produced in response to certain

abiotic stresses, such as flooding. ABA also stimulates elongation of the main root and proliferation of lateral roots in response to drought (De Smet et al. 2006). Recently, ABA was reported to play a significant role in response to drought (Mirzaei et al. 2012), cold (Neilson et al. 2013), and salinity (Pérez-Alfocea et al. 2011). GA is a regulator of cell elongation in roots and may play a role in defense responses in rice root (Tanaka et al. 2004). In rice roots, exogenous GA application increases aldolase expression, which in turn enhances the metabolic rate of glycolysis and cell growth in seedling roots through the physical interaction and activation of V-ATPase by aldolase (Komatsu and Konishi 2005). Taken together, these findings demonstrate that phytohormones form complex network of signaling pathways in plant roots and are actively involved in various functions in roots, including the development of primary and lateral root in response to stresses.

19.3.2 Proteomics of Gravitropism in Root

Gravitropism in roots is driven by differential growth in the elongation zone that is under hormonal control. The distribution of auxin determines the growth rate and pattern of root cells, which in turn is regulated by gravity-sensing cells in the root tip. When roots are oriented horizontally, auxin accumulation along the lower side of the elongation zone inhibits cell growth in that region, whereas the growth of cells located in the upper regions is stimulated, a process that leads to the downward bending of the root (Luschnig et al. 1998). Not only primary roots, but also lateral roots, respond to gravistimulation. Kiss et al. (2002) evaluated the effects of gravity and light on lateral root growth in *Arabidopsis* and showed that in addition to positive gravitropism, lateral roots also exhibit negative phototropism depending on light quality, with blue light inducing negative phototropism and red light inducing positive phototropism. Therefore, both environmental and internal factors determine gravitropism in plant roots.

The transport of auxin involves the coordinated action of several proteins. Luschnig et al. (1998) explained how a root-specific protein, EIR1, regulates auxin transport in the process of root gravitropism in *Arabidopsis*. EIR1 not only is involved in auxin homeostasis in root cells but also has a role in response to endogenous ethylene. PIN3, which is typically symmetrically localized along the plasma membrane of statocytes of the root cap columella, is another important protein involved in gravitropism. This protein relocalizes to lower membranes via vesicle trafficking along actin microfilaments within 2 min of gravistimulation (Friml et al. 2002). The auxin influx carrier AUX1 is also involved in root gravitropism. Using an in situ hybridization technique, Bennett et al. (1996) showed that AUX1 expression is associated with root apical tissues, which control the root gravitropic response.

In addition to the aforementioned hormonal-related proteins involved in gravitropism, several groups of plant proteins are accumulated upon gravistimulation. Azri et al. (2009) performed 2-DE on proteins isolated from the apical

and basal regions of poplar stems to analyze the regulatory mechanisms involved in gravitropic stimulation and identified 60 proteins that were significantly accumulated after inclination. A review on signal transduction in primary roots showed that multiple pathways may modulate gravity signal transduction within root tips (Perrin et al. 2005). Recently, a quantitative proteomic approach was used to identify key proteins that modulate the early events of gravitropism (Schenck et al. 2013). *Arabidopsis* plants were exposed to constant gravistimulation, and proteins were then identified using an iTRAQ proteomic technique, which detected a total of 82 proteins that were significantly accumulated as early as 2 and 4 min after gravistimulation (Schenck et al. 2013). Hence, although the proteins related to auxin transport play a main role in root gravity-sensing cells, a wide range of proteins with various functions may be involved in root gravitropism.

19.4 Root Proteomics Under Stress Conditions

19.4.1 Root Proteomics Under Flooding

The root is the first plant organ to sense flooding stress. To understand how plants respond and adapt to flooding, studies on the root system and the surrounding environment are important. Flooding initially triggers cellular signal transduction pathways in the root system, leading to molecular- and metabolic-level changes in the plant. Flooding causes a decrease in aquaporins (Vandeleur et al. 2005), which leads to decreased hydraulic conductivity and decreased root permeability (Clarkson et al. 2000).

Plants respond to flooding stress by shifting to alternative pathways of energy generation. Proteomic analysis of soybean roots exposed to flooding stress at an early growth stage revealed that proteins related to glycolysis, such as UDP-glucose pyrophosphorylase and fructose-bisphosphate aldolase, and/or disease/defense-related proteins, such as reactive oxygen species scavengers, chaperones, hemoglobin, and acid phosphatase, are among the most highly affected proteins (Hashiguchi et al. 2009; Komatsu et al. 2009). The response mechanisms of soybean at later growth stages were reported in 3-week-old soybean roots exposed to flooding for 3 and 7 days (Alam et al. 2010a, b). Enzymes involved in glycolysis and fermentation pathways were the major changed proteins, suggesting that the response of soybean seedlings to flooding stress at later growth stages is similar to that at an early growth stage.

Proteomic analysis was also performed to investigate the response mechanism of wheat roots to flooding stress (Kong et al. 2010). The decreased accumulation of proteins related to the glycolysis pathway supported the speculation that carbohydrate metabolism and reduced energy consumption are the primary responses to cope with flooding stress. In addition, the increased accumulation of proteins related to disease/defense was observed, whereas cell wall structure/modification-

related proteins, including methionine synthase, were decreased, suggesting that plants restrict cell growth to conserve energy in the unfavorable environmental conditions imposed by flooding. Proteomics was also used to elucidate the response mechanisms of wheat roots to flooding stress at different soil depths (Haque et al. 2011). Proteins showing increased accumulation were related to energy and redox status, defense responses, and cell wall turnover, suggesting that these proteins have possible roles in alternative respiration and cell degeneration pathways that modulate metabolic adjustment in wheat plants under hypoxia stress induced by flooding.

Tomato undergoes several changes to cope with the severe conditions experienced under submerged conditions. In particular, secondary metabolite biosynthesis, programmed cell death, and accumulation of diseases/defense-related proteins are increased in flooded tomato roots and lead to plant survival through the management of energy consumption in cellular processes (Ahsan et al. 2007). Metabolic adjustment is the key adaptive response in tomato roots exposed to waterlogged conditions, as evidenced by the increase in alcohol dehydrogenase and enolase and decreased levels of pyruvate dehydrogenase (Ahsan et al. 2007). Screening of proteins involved in conferring flooding tolerance to plants using proteomic technique can provide strategies for developing flood-tolerant plants.

19.4.2 Root Proteomics Under Drought Stress

Root development is strongly influenced by adverse growing conditions. However, root growth is typically less affected by drought stress than shoot growth (Franco et al. 2011). Thus, the shoot/root ratio is commonly decreased under drought stress, resulting either from an increase in root growth or from a comparatively larger decrease in shoot growth. Drought stress leads to a greater percentage of fine roots, which are capable of penetrating smaller soil pores and presumably optimize the exploratory capabilities of the root system and, as such, likely improve the survival of plants under conditions of drought stress. The root color of plants changes from white to brown in response to drought and is associated with suberization of the exodermis, which may reflect a metacutization process, and is correlated with the capacity to grow under drought conditions (Franco et al. 2011).

Proteomic analysis of the roots of soybean (Alam et al. 2010a, b; Mohammadi et al. 2012a, b; Toorchi et al. 2009), rice (Mirzaei et al. 2012), and *Brassica napus* (Damerval et al. 1988; Mohammadi et al. 2012a, b) under drought stress condition has identified a wide range of proteins that have led to a better understanding of the mechanism of drought stress tolerance in plants. For example, drought stress causes an increase in the pH of xylem sap, which promotes the loading of ABA into the root xylem and its transport to the shoot (Hartung et al. 2002). Because roots are the first plant tissue to encounter drought stress, it is likely that roots are able to sense and respond to this stress condition. Mirzaei et al. (2012) investigated how rice root systems in heterogeneous soils adapt to drought by comparing root tissues exposed

to one of four conditions. Label-free proteomic analyses of samples collected from these roots identified 1,487 nonredundant proteins. Drought caused marked changes in protein accumulation, most notably in partially droughted roots, in which the levels of 38 % of proteins were altered compared to adjacent, watered roots. In response to drought, pathogenesis-related proteins were generally increased, whereas heat-shock proteins were not detected in the roots of fully watered plants. The accumulations of proteins involved in transport and oxidation–reduction reactions were also increased and decreased, respectively, in response to drought signals. This comparison revealed that nine tubulins were strongly reduced in droughted roots, whereas the levels of six chitinases were increased, even in watered plants located adjacent to droughted roots. This label-free proteomic analysis of water stress in split-root systems of rice has provided novel molecular insights into the heterogeneous translation patterns that occur in wet and dry soil zones.

19.4.3 Root Proteomics Under Salt Stress

The root is also the first plant organ to encounter salt stress. Proteomic analyses of the roots of soybean (Sobhanian et al. 2010), rice (Liu et al. 2012), wheat (Guo et al. 2012), maize (Zörb et al. 2010), barley (Sugimoto and Takeda 2009), and potato (Aghaei et al. 2008) under salt stress have been reported. These studies have revealed that a number of salt stress-responsive genes and proteins are more strongly induced in roots than in other plant organs (Yan et al. 2005).

Sobhanian et al. (2010) examined changes in the proteome of soybean roots exposed to high salinity and found that the accumulation of several metabolism-related proteins were predominantly decreased under salt stress. Among the decreased proteins, a dienelactone hydrolase, which hydrolyzes the conversion of dienelactone to maleylacetate, which are both intermediates for the aerobic degradation of haloaromatic compounds (Schlomann et al. 1990; Blasco et al. 1995), was identified, suggesting that these secondary metabolites are not effectively degraded under salt stress. Sugimoto and Takeda (2009) performed proteomic analyses of specific proteins in the root of salt-tolerant barley and identified six stress-/defense-related proteins that do not scavenge reactive oxygen species directly, as had been reported in other plants. These findings indicate that in the course of evolution, a common salt tolerance mechanism may have developed in plants.

19.5 Conclusion

Plant roots form a complex network of interactions with the surrounding rhizosphere upon seed germination. Plants respond to environmental signals through changes in the concentration of phytohormones, such as auxin and cytokinin, whose

relative ratios influence primary and secondary root growth, downward bending, and several other processes. In this chapter, the role of auxin in the control of root formation, lateral root proliferation, and gravitropism was discussed. Several proteins are involved in auxin transport and include auxin influx carrier proteins, such as AUX1, PGP, and LAX. These proteins, along with transmembrane proteins from the PIN family and ABC transporters, facilitate the influx and efflux of auxin within transporting cells. Plant roots have several specific features that require adapted extraction techniques for protein identification using both gel-based and gel-free proteomic analyses. Proteomic analyses of roots exposed to abiotic stresses are expected to provide new insights into the understanding of plant cell physiology. Abiotic stress and nutrient starvation are the main factors affecting the function of hormones, which can be extensively studied using proteomic approaches.

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Chapter 20
Adventitious Root Development in Ornamental Plants: Insights from Carnation Stem Cuttings

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20.1 Ornamental Plant Propagation

The economic value of ornamental plants has increased significantly worldwide and is increasing annually by 8–10 % (Jain and Ochatt 2010). Micropropagation is the most commonly used method for the clonal propagation of many plant species. Current ornamental breeding depends on rapid multiplication of elite clones, production of healthy and disease-free plants, and faster introduction of novel cultivars. It is well known that several factors can affect in vitro micropropagation (George and Debergh 2008). Hence, to boost production economics in ornamentals, the existing protocols for plant micropropagation should be improved (Pati et al. 2006). In orchids, for example, clonal propagation has been traditionally achieved through the separation of root-bearing adventitious plantlets (*keiki*) from the mother stem. However, this is a slow and unsuitable method for mass propagation. An efficient protocol for in vitro propagation of the epiphytic orchid *Dendrobium longicornu* from nodal explants that relies on the appropriate combination of hormones, mainly auxins and cytokinins (CKs), has been recently published (Dohling et al. 2012). Apical buds, stems, and leaves of *Kalanchoe blossfeldiana* were used as explants for efficient in vitro regeneration of entire plantlets (Kordi et al. 2013). Interestingly, in some Kalanchoë species, such as *Bryophyllum daigremontianum* or *B. marnierianum*, plantlets develop spontaneously on leaves (Kulka 2006), which suggests a complex and species-specific regulation of micropropagation. Moreover, most of the commercially grown

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cultivars of gerbera are vegetatively propagated through shoot tissue culture (Aswath et al. 2003). In all the examples so far shown, regenerated plantlets initially lack functional roots, and they need to undergo a rooting and acclimatization process which, in some cases, might limit its commercial scale-up.

20.2 Adventitious Root Formation

Adventitious roots (ARs) are distinct from lateral roots in that they form from any tissue that is not a root, such as leaves and stems, naturally or in response to altered environments (Geiss et al. 2010). AR formation is a complex process regulated by both environmental and endogenous factors, among which the plant hormone auxin plays a central role (Geiss et al. 2010; Pop et al. 2011). AR formation from stem cuttings is usually divided into several stages according to physiological and metabolic markers (de Klerk et al. 1999). In the early dedifferentiation phase, specific cells along the cambium become competent to respond to the rooting signal. During the induction phase, these cells undergo cell reprogramming and become committed as root initials. Subsequent cell proliferation will lead to the formation of small clusters of cells in which a new root meristem begins to organize (initiation phase). Next, in the expression phase, the growth of the root primordia and the establishment of their vascular connection with the stem will take place, and the new functional root system will emerge. Hence, it is not unforeseen that the same genetic pathways and hormonal signals required for the specification of root tissues in the primary and lateral roots might be also involved in AR formation.

20.2.1 Genetic Determinants

Various molecular and genetic approaches have been used to study AR development in Arabidopsis and other model plants (Geiss et al. 2010). In rice, the disruption of the auxin-inducible *CROWN ROOTLESS1*/*ADVENTITIOUS ROOTLESS1* (*CRL1*/*ARL1*) gene, which encodes a member of the plant-specific LOB protein family, prevents the initiation of primordia in the crown root system (Inukai et al. 2005; Liu et al. 2005). The promoter of the *CRL1*/*ARL1* gene contains specific *cis*-regulatory elements that interact with a rice transcription factor from the auxin response factor (ARF) family (Inukai et al. 2005). In the *Arabidopsis thaliana* model plant, the balance between ARF17, a negative regulator of adventitious rooting, and ARF6 and ARF8, positive regulators of AR formation, plays a critical role in AR formation (Gutierrez et al. 2009; Sorin et al. 2005). Additionally, the proteomic analysis of mutants affected in adventitious rooting led to the identification of 11 proteins, among them three auxin-inducible GH3-like proteins (Sorin et al. 2006). It has been recently demonstrated that these GH3-like proteins are required, downstream of the ARF6 and ARF8 pathway, for the fine-tuning of AR

initiation, through modulating jasmonic acid homeostasis (Gutierrez et al. 2012). Another gene required for crown root formation in rice is *CROWN ROOTLESS5* (Kitomi et al. 2011), which encodes the homologue of the *AINTEGUMENTA* (*ANT*) gene encoding an APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factor required for organ growth regulation in Arabidopsis (Mizukami and Fischer 2000). Recent work in poplar has identified a key role for PtAIL1, an AP2/ERF transcription factor homologous to ANT, in the formation of AR primordia in this species (Rigal et al. 2012). These results suggest that the early stages of AR formation need to be tightly regulated at the genetic level and that this regulation requires specific factors that ultimately modulate the hormonal signals triggering AR formation.

20.2.2 Phytohormone Regulation

Auxin is a well-known trigger for AR formation in stem cuttings of several species (de Klerk et al. 1999). High auxin levels in the basal region of the cuttings are required for the competent cells in the cambium to resume proliferation and to start the root-specific developmental program (Ahkami et al. 2013; Garrido et al. 2002). Despite both active basipetal auxin transport from leaves and local auxin biosynthesis might account for the increased auxin levels observed in the stem base, the observation that chemical inhibition of auxin efflux completely abolishes adventitious rooting indicates a major function of the transport of auxin in this response (Acosta et al. 2009; Garrido et al. 2002; Guerrero et al. 1999). Consistent with a positive role for auxin in AR formation, Arabidopsis mutants overproducing auxin spontaneously develop ARs on the hypocotyl (Boerjan et al. 1995; Zhao 2008). The auxin signal has been recently shown to act locally by binding to the AUXIN-BINDING PROTEIN1 (ABP1) receptor (Robert et al. 2010), although the main transductional output of the auxin signal is channeled to the degradation of the Aux/IAA corepressors through their auxin-dependent binding to the SCF TIR1/AFB (AUXIN SIGNALING F-BOX) E3 ligase complex (Dharmasiri et al. 2005; Kepinski and Leyser 2005). As a consequence of the latter, AUXIN-RESPONSIVE FACTORS (ARFs) are released and transcriptionally regulate their target genes. In lateral roots, for example, the Aux/IAA-ARF module BODENLOS (IAA12) and MONOPTEROS (ARF5) is downstream of the auxin signal for the specification of the new root primordia (Lau et al. 2011). Whether a similar module involving this ARF or other ARFs is responsible for AR formation in stem cuttings needs to be established.

It has been observed that once auxin induces cells to enter the AR formation program, the auxin levels in the carnation stem cutting base decay up to the steady state (Agulló-Antón et al. 2014), which is consistent with the inhibitory action of exogenously applied auxin observed in later stages of adventitious rooting, during primordia outgrowth (de Klerk et al. 1999). Auxin can be actively degraded via oxidative decarboxylation by peroxidases (Caboni et al. 1997) or inactivated by

their conjugation to sugars and amino acids (Nordstrom et al. 1991). Ultimately, vascularization of the new root primordia will channel the auxin stream to their apices. Hence, tight regulation of the auxin homeostasis in the site of AR formation is a critical factor ensuring the dedifferentiation and redifferentiation of competent cells leading to the new root primordia.

In addition to auxin, CKs are important regulators of AR development (de Klerk et al. 2001; Konieczny et al. 2009; Werner et al. 2003). The treatment of stem cuttings with exogenously applied CK resulted in a strong suppression of AR formation (de Klerk et al. 2001). Consistent with a negative role for CKs in AR formation, mutants defective in CK biosynthesis or perception displayed increased production of ARs, whereas enhanced CK biosynthesis has the opposite effects (Rasmussen et al. 2012; Riefler et al. 2006; Werner et al. 2003). CK biosynthesis occurs in various cell types in both aerial organs and roots (Miyawaki et al. 2004). Reciprocal grafting experiments showed that some CKs, such as *trans*-zeatin, were preferentially transported from the root to the shoot (Matsumoto-Kitano et al. 2008). Such root-to-shoot transport is thought to signal the nitrogen and nutrient status of the soil and thus coordinate soil nutrient availability with above-ground plant growth (Hirose et al. 2008). Transcriptome analysis during early stages of AR formation in poplar cuttings has identified the type B cytokinin response regulator PtRR13 as a negative regulator of AR in this species (Ramirez-Carvajal et al. 2009). Their results are consistent with a model in which some CKs, synthesized in the roots and transported throughout the stem, act through PtRR13 to repress AR development in intact plants; hence, removing of CK supply from the roots downregulates CK signaling in the stem leading to the inactivation of PtRR13 (Ramirez-Carvajal et al. 2009). Auxin and CK have been known to play a crucial role in many aspects of plant development, often acting antagonistically (Skoog and Miller 1957). Besides cross talk between auxin and CK signaling components (El-Showk et al. 2013; Moubayidin et al. 2009), CK also interferes with the auxin distribution by modulating the activity of auxin transport facilitators (Bishopp et al. 2011; Pernisova et al. 2009). Although the molecular events involved in the auxin–CK cross talk have been clarified in different processes (El-Showk et al. 2013; Moubayidin et al. 2009), our knowledge on the key players in AR formation in ornamental species such as carnation is still unknown. Hence, novel approaches there will be necessary to identify the molecular components of the auxin–CK interaction network in AR formation in this and other non-model species.

Strigolactones (SLs) are a novel class of plant hormones that were originally discovered for their promotion of mycorrhizal association (Akiyama et al. 2005) and parasitic weed seed germination (Matusova et al. 2005). The adventitious rooting phenotype of mutants with defects in SL biosynthesis or SL response highlights a negative role for these hormones in adventitious rooting, independently of the action of CKs (Rasmussen et al. 2012). The authors proposed a model where SL signaling regulates AR initiation in stem tissues directly by inhibiting formative divisions and indirectly through the regulation of basipetal auxin transport that is

required for the local auxin buildup triggering AR initiation (Rasmussen et al. 2012). Consistent with the latter, SL signaling has been found to induce clathrin-mediated PIN1 depletion from the plasma membrane in stem tissues (Shinohara et al. 2013). Roots and stems are the main site of SL biosynthesis (Alder et al. 2012), and SLs are transported acropetally to the shoot, where they play a role in axillary bud activity, in contrasting concert with auxin (Prusinkiewicz et al. 2009; Shinohara et al. 2013). In stem cuttings, SL levels are supposed to be low; hence, PIN1-mediated auxin transport is not restricted through the stem. Later, when the new roots are functional, SLs move upward through the xylem and actively downregulate auxin transport. The experimental validation of this model in stem cuttings will give some light on the cross talk between auxin and SLs observed during AR formation.

Both the mechanical wounding and the water imbalance caused by the excision of the stem cuttings from the mother plant might alter the endogenous levels of the hormones regulating stress responses, particularly jasmonic acid (JA), abscisic acid (ABA), and salicylic acid (SA) (Schilmiller and Howe 2005). JA levels were found to be upregulated in the stem base of *Petunia hybrida* cuttings during the first hours after their excision from the mother plant, which was dependent on local JA biosynthesis (Ahkami et al. 2009). Additionally, exogenously applied JA accelerated adventitious rooting in apple stem slices (de Klerk et al. 1999), and it was suggested that JA enhanced the sensitivity of founder cells to respond to the auxin signal (Calamar and De Klerk 2002). Recent data in Arabidopsis hypocotyls, however, has revealed a different scenario for JA in this process; JA-deficient and JA-signaling mutants produced more ARs from the hypocotyl, suggesting that JA plays a negative role in AR formation (Gutierrez et al. 2012). In response to the auxin signal and downstream of the ARF6-, ARF8-, and ARF17-regulatory pathway (Gutierrez et al. 2009), three *GH3* genes encoding broader acyl-adenylate-/thioester-forming enzymes are expressed, whose activities will lead to an increase in JA conjugation and, as a consequence, to a reduction in free JA levels (Gutierrez et al. 2012). According to this scenario, elevated JA levels in the base of the stem cuttings after the excision will prevent premature AR formation; later, when auxin levels peak in this region because of active basipetal transport, subsequent signaling events will lead to the inactivation of JA and to the auxin-dependent establishment of founder cells.

It is largely known that wounding stimulates ethylene biosynthesis in different tissues (Blakesley 1994; Cheong et al. 2002; O'Donnell et al. 1996). In tomato, ethylene has been shown to positively enhance AR formation from excised hypocotyls (Negi et al. 2010). Although the exact mechanism how ethylene promotes AR formation is not known, a plausible hypothesis is that a delay in auxin transport through the hypocotyl in response to ethylene might increase auxin concentration in this tissue above the level required to trigger AR formation. However, a direct effect of ethylene signaling in AR formation, independently of auxin, could not be discarded with the current data.

ABA is a known inhibitor of lateral root development, and it has been shown to promote the quiescence of the root primordia at the postemergence stage by

signaling in specific tissues, such as the endodermis (Duan et al. 2013). In deep-water rice, ARs are produced in response to hypoxia caused by flooding conditions (Mergemann and Sauter 2000). In this species, emergence and growth of ARs are both controlled by ethylene signaling which is enhanced by GA and inhibited by ABA (Steffens et al. 2006). Additional investigations involving ABA-deficient and ABA-signaling mutants in model species, such as Arabidopsis and rice, will be required to shed some light on the complex role of ABA in AR formation.

Despite the importance of AR formation in plants, most of the mechanisms behind the hormonal cross talk observed have not been elucidated yet. Recently, the combination of biochemical and genetic approaches started to shed some light on the molecular players involved in such cross talk.

20.2.3 Nutrient Signaling

The nutrient status of the stem cutting is very important as it has a profound effect on their subsequent growth and development. With regard to AR formation, results with *Pelargonium* cuttings indicate that adventitious rooting can be limited by the initial amount of nitrogen reserves (Druege et al. 2004). However, cutting survival and root regeneration become predominantly determined by the initial sugar availability when high-light adaptation or low light conditions impair net carbon assimilation. In this context, AR formation relies on an adequate supply of carbohydrates to the region of root regeneration, where they provide energy and carbon skeletons required for root initiation and root development (Correa et al. 2005; Druege et al. 2004).

Interrelationships between auxin and carbohydrate metabolism during adventitious rooting have been investigated by the application of exogenous auxins and monitoring of carbohydrate levels, carbon translocation, and activities of key enzymes involved in sugar metabolism in the rooting zone (Agulló-Antón et al. 2011; Ahkami et al. 2009; Husen and Pal 2007). The rapid increase observed in carbohydrate levels at the stem base of carnation cuttings after exogenous auxin application suggests that auxin stimulates the establishment of a new carbohydrate sink, which directly contributes to the formation of the new root primordia (Agulló-Antón et al. 2011). Hence, low allocation of carbohydrates to the rooting zone may limit AR formation. In a recent work in *P. hybrida* cuttings, Druege and coworkers have elegantly shown that the endogenous accumulation of free auxin in the rooting zone contributes to the establishment of a new carbohydrate sink in this region via stimulation of cell wall and vacuolar invertases (Ahkami et al. 2013). Later, the decrease of auxin levels allows for subsequent root development, which in turn stimulates the entry of glucose into glycolysis and the pentose phosphate pathway to produce energy and carbon skeletons (Ahkami et al. 2013). Additionally, new evidences suggest that auxin homeostasis and auxin response can in turn be modulated by sugar signaling during root development (Mishra et al. 2009): based on whole-genome expression profiling and mutant analyses, glucose

signaling has been shown to affect the expression of some *Aux/IAA* genes as well as other genes involved in auxin biosynthesis (*YUCCA*) or auxin transport (*PIN2*) in Arabidopsis roots (Mishra et al. 2009).

Before being planted for rooting, excised cuttings are commonly stored in darkness and at low temperatures, which allows producers to regulate market supply (Garrido et al. 1996). Rooting of cuttings of storage-sensitive plant species like *Pelargonium* seems to be negatively affected by dark treatment (Druege et al. 2004). A positive effect on adventitious rooting occurred, however, when carnation, chrysanthemum, and petunia cuttings were stored in darkness at low temperatures before rooting (Druege et al. 2000; Garrido et al. 1996; Klopotek et al. 2010). Shortly after exposing cold dark-treated cuttings to the rooting conditions, soluble sugars and starch increased significantly to higher levels in the leaf and basal stem compared to their nontreated counterparts. The generally accelerated and more intense AR formation observed in stored cuttings than in non-stored cuttings suggests that certain changes in the endogenous auxin pool, the rooting induction factor, occur during the storage (Agulló-Antón et al. 2011). A challenge in the future is to unravel the underlying complex regulatory processes at the molecular level responsible for the sugar and auxin interaction observed during AR formation.

20.3 Carnation as a Model to Study AR Formation

Carnation is, after rose, the most important species on the worldwide market of cut flowers, with a yearly sales volume of almost 200 million plants (Sheela 2008). There is a strong requirement for the clonal propagation of commercial carnation cultivars from stem cuttings obtained from mother plants of elite cultivars. On the one hand, most of the cultivars are of hybrid nature as they arose by intra- but also by interspecific hybridizations with other *Dianthus* species (Sheela 2008). On the other hand, an efficient propagation of uniform starting material is a strong requirement for mass production of cut flowers. Our current knowledge about adventitious rooting in carnation has gain insight from physiological studies showing that root induction is affected by complex interactions between sucrose and hormone levels (Agulló-Antón et al. 2011). Polar auxin transport has been shown to contribute significantly to this process (Garrido et al. 2002; Guerrero et al. 1999). In addition, a large variation in AR formation in this species is found to be genotype dependent (Garrido et al. 2002, 2003). Hence, AR formation is a genetically and environmentally controlled process, indispensable for the commercial propagation of elite carnation cultivars (Garrido et al. 2002, 2003). For some of the new cultivars developed by breeders, poor AR formation limits its commercial scale-up, because of a significant increase in the production costs caused by rooting losses (E.A. Cano, personal communication).

We are interested to determine the genetic basis of the differences found between carnation cultivars during adventitious rooting. Good-rooting cultivars

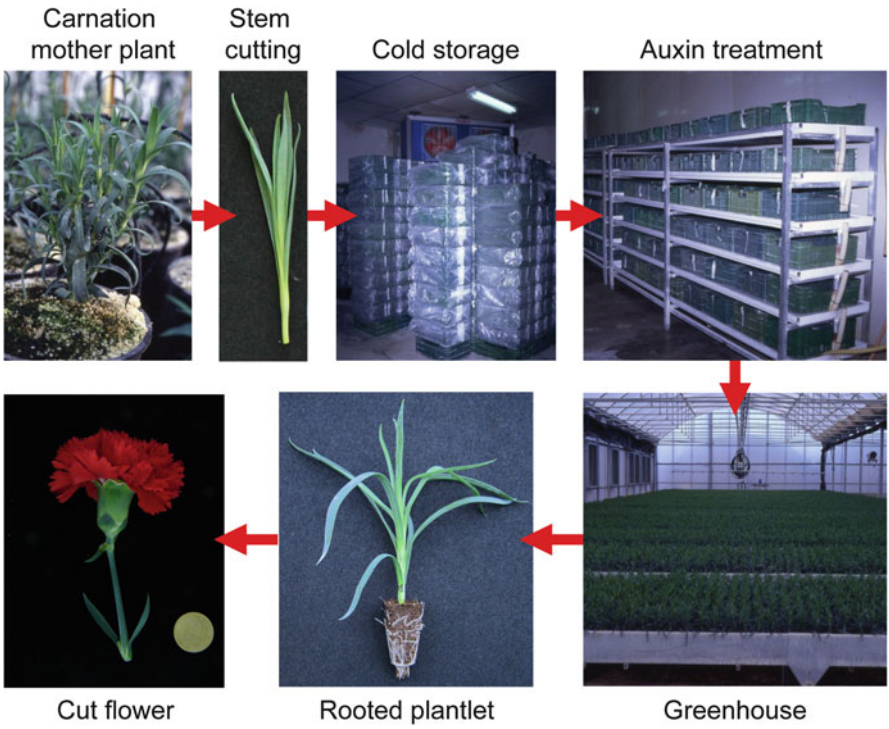

Fig. 20.1 Cut flower production scheme from carnation stem cuttings. Pictures were taken at the production facilities of Barberet & Blanc, S.A. (Puerto Lumbreras, Spain; http://www.barberet.com)

and bad-rooting cultivars have been selected for a detailed characterization of AR formation. For the commercial propagation of a given cultivar, terminal stem cuttings with 4–6 pairs of leaves are obtained from several mother plants of clonal origin. To match market demand, fresh cuttings are cold stored in darkness, where they must remain deprived of the root during storage (Garrido et al. 1996, 1998). Next, stem cuttings will be treated overnight with auxin before being planted to soil substrate in the greenhouse. Rooted stems will then be transferred to soil at different locations for flower production (Fig. 20.1).

Several endogenous and environmental factors might contribute to the differences in adventitious rooting observed between carnation cultivars (Garrido et al. 1996, 1998, 2003), such as the age of the mother plants, the storage period of the cuttings, the developmental stage of the cuttings, or their sensitivity to the auxin treatment, among others. To reduce the contribution of some of these factors to the differences found in AR formation, phenotyping could be performed directly on fresh stem cuttings, without the aid of an external hormone source. Also, rooting stem cuttings on defined culture media in a rhizotron will minimize the effects of some environmental conditions affecting AR formation, such as soil heterogeneity,

air temperature, and solar irradiation. Next, we will discuss the approaches we are using in our lab to describe the characteristics of the stem cuttings before rooting and their dynamic root architecture during AR formation.

20.3.1 Stem Cutting Phenotyping

Several reports indicate that the physiological characteristics of the stem cuttings are determinant for their adventitious rooting success which, in turn, is strongly influenced by the storage conditions of the cuttings (Agulló-Antón et al. 2011; Garrido et al. 1996, 1998) (see above). To understand the differences found in the rooting ability shown by different carnation cultivars, one has to characterize the stem cuttings in great detail. In our experimental approach, we randomly collect several stem cuttings from different mother plants to quantify some of their morphometric characteristics, such as their weight, length, and width of the stem and number and area of leaves, as well as to obtain additional physiological information from the cuttings, such as photosynthesis rate and water turgor pressure, among others. Additionally, since hormonal cross talk at the stem base is responsible for triggering AR formation from this region, quantification of the levels of hormones involved in wound response and AR formation, particularly JA, IAA, and CKs but also ABA, SA, and SLs, is routinely performed in our laboratory (Albacete et al. 2008). It might be possible that histological differences in the structure of the stem cuttings would also contribute to the differences in the rooting performance between cultivars. Last, but not least, carbohydrate partitioning between the leaves and the stem base also influences the adventitious rooting response, which makes necessary the quantification of the differences found in sugar levels and activities of their metabolic enzymes among cultivars. In Table 20.1, results for some ecophysiological measurements in two carnation cultivars that differ in their AR performance are shown. Phenotyping data from the stem cutting is also been taken at different stages during the rooting process. Finally, statistical associations will help to unravel the complex interaction between stem characteristics, root architectural traits, and AR performance.

20.3.2 Phenotyping the Architecture of ARs

Root systems are complex and dynamic three-dimensional (3D) structures that remain hidden in the soil, which make their study using nondestructive imaging methods difficult (Fang et al. 2009; Perret et al. 2007). In the last years, several programs have been developed for the 2D study of simple dicot root systems, such as those of Arabidopsis, growing on transparent or semitransparent media (Armengaud et al. 2009; French et al. 2009; Zeng et al. 2008). However, none of them allow the morphometric characterization of complex root systems, such as

Table 20.1 Some ecophysiological traits analyzed in carnation cultivars differing in AR performance

	Stem cutting weight (g)	Stem cutting length (cm)	DS (g/dm^2)	SLA (dm^2/g)	LDMC (g/g)
Bad-rooting cultivar	0.4 ± 0.1	8.1 ± 1.0	1.0 ± 0.1	4.70 ± 0.20	0.10 ± 0.01
Good-rooting cultivar	3.8 ± 0.8	17.0 ± 1.0	2.6 ± 0.1	1.54 ± 0.04	0.11 ± 0.01

Data are the mean of 10 cuttings ± standard error

Degree of succulence (DS) is calculated as the water content of the stem cutting at saturation (g) divided by the leaf area (dm^2). Specific leaf area (SLA) is defined as the ratio of leaf area (dm^2) to leaf dry weight (g). Leaf dry matter content (LDMC) is calculated as the leaf dry weight (g) divided by leaf water-saturated weight (g)

rice or maize, or the intricate root architecture found in adventitious rooting of carnation stems (V. Birlanga and J.M. Pérez-Pérez, unpublished). Recently, a novel imaging and analysis platform for automated phenotyping of complex root systems has become available (Iyer-Pascuzzi et al. 2010). It allows the imaging of root growth in 3D for at least 2 weeks and overcomes most of the limitations of previous methods (Armengaud et al. 2009; French et al. 2009; Zeng et al. 2008). The accompanying analysis software can analyze a single image up to many thousands of images and, for each image, extract the root network, estimate its traits, and report quantitative trait estimates (Galkovskyi et al. 2012). Despite this imaging analysis platform relies on artificial growth media, current data showed high correlation between root architecture parameters and their associated quantitative trait loci (QTL) for laboratory-grown plants and for field-grown plants in several species (Cui et al. 2008; Fang et al. 2009). In a recent study using this 3D imaging and phenotyping system, the genetic basis of root architecture in a rice mapping population has been unveiled (Topp et al. 2013). Several clusters of linked QTL contributing to root system architecture (RSA) traits have been identified, which will allow further identification of the genes controlling RSA in this species (Topp et al. 2013).

To study AR formation in carnation, the entire root system arising at the base of the cutting was imaged at regular time intervals from a number of fresh stem cuttings of several cultivars differing in their rooting losses (Fig. 20.2). To allow nondestructive quantification of the root system, the stem cuttings were grown on transparent media during 1 month. Images were processed using available software (Galkovskyi et al. 2012), and several features were chosen to characterize the architecture of AR: average root width, maximum number of roots, network length, network perimeter, and network surface area, among others. In Fig. 20.2b, images of a representative stem cutting from a good-rooting (top) and a bad-rooting (bottom) cultivars are shown. Most of the observed differences between these two cultivars are caused by a delay in root initiation and a reduced number of root primordia in the bad-rooting cultivar, whereas the elongation rate of the newly formed roots was higher in the good-rooting cultivar. Additional phenotyping of

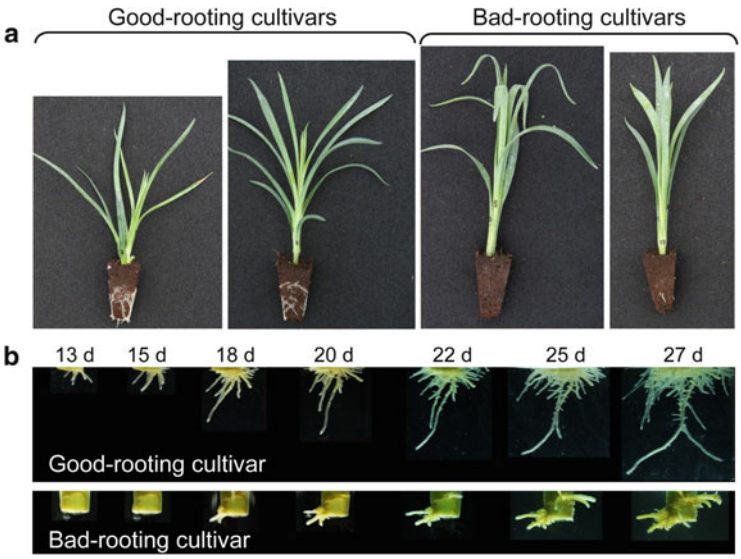

Fig. 20.2 Phenotyping AR formation in carnation stem cuttings. (**a**) Pictures from rooted plantlets of good-rooting and bad-rooting cultivars are shown. (**b**) Representative images from a time-course analysis of AR formation in two carnation cultivars with strong differences in their rooting performance. Images in (**a**) were kindly provided by Emilio A. Cano (Barberet & Blanc, S.A.)

AR formation in soil substrate needs to be performed to confirm the observations found in our in vitro system for adventitious rooting from carnation stem cuttings.

20.4 Toward the Identification of Genes Involved in AR Formation

The genetic loci determining the variation found in AR formation among carnation cultivars can be identified by correlating phenotypic values with genetic polymorphisms on a large mapping population derived from two parents differing in their rooting performance. High-resolution linkage mapping, however, only allows the localization of large-effect QTL to wide genomic intervals due to the low number of recombination events arisen during building the population (Holland 2007). Moreover, phenotyping and maintaining a large mapping population are costly and labor-intensive. Recently, the first simple sequence repeat (SSR)-based genetic linkage map for carnation has been published (Yagi et al. 2012); the map included 178 SSR loci into 16 linkage groups that cover 843.6 cM and has allowed the localization of the main QTL responsible for resistance to bacterial wilt in the studied population (Yagi et al. 2012).

Recent developments in DNA sequencing technologies make the direct identification of molecular polymorphisms among landraces and cultivars feasible, which can then be used for the discovery of trait-linked markers through genome-wide association (GWA) studies (Davey et al. 2011; Han and Huang 2013; Varshney et al. 2009). Successful application of GWA may depend on the genetic diversity of the germplasm collection, the quality of available phenotyping data, and a sufficient number of polymorphisms with genome-wide coverage. Once the molecular bases of the traits contributing to the desired phenotype have been identified, technologies of marker-assisted selection (MAS) can be used to introduce these alleles into current cultivars (Roy et al. 2011). Since the choice of germplasm is critical to the success of association analysis (Zhu et al. 2008), a preliminary study on the current carnation germplasm collection to determine population structure and ancestral kinship is essential.

Following a candidate gene association mapping approach, single nucleotide polymorphisms (SNPs) in selected candidate genes whose homologues have been shown to participate in the regulation of adventitious rooting in other species will be identified (Zhu et al. 2008). The most straightforward method of identifying SNPs relies on re-sequencing the transcriptome of several genetically distinct individuals of a larger association population. Usually, a small fraction of the identified SNPs will capture most of the haplotype structure of the population, which facilitates subsequent statistical analyses. Additionally, a GWA mapping approach using a broad germplasm collection could be envisioned; thus, genetic variation in the whole genome will be systematically surveyed to find statistical association of the identified SNPs with the traits measured during adventitious rooting (Fig. 20.3).

Collection of high-quality and robust phenotypic data is essential for association mapping studies (Rafalski 2010). Hence, phenotyping the population is the most laborious and technically challenging part of this process, as replicate trials are necessary across multiple environments over a number of seasons (Furbank and Tester 2011).

As an alternative strategy to identify the genes involved in AR formation in carnation cuttings, the gene expression profiles in the stem base of cultivars with contrasting effects on AR formation could be analyzed. An earlier study has used *Pinus contorta* to investigate the temporal distribution of specifically regulated transcripts during AR formation induced by exogenous auxin application (Brinker et al. 2004). Using whole-genome microarrays, the analysis of gene expression in poplar cuttings was determined during the first days after excision (Ramirez-Carvajal et al. 2009). The authors reported that extensive transcriptome remodeling and gene clustering allowed them to identify patterns of gene expression that reflect contrasting roles of ethylene, auxin, and cytokinin in this process (Ramirez-Carvajal et al. 2009). In another study (Abu-Abied et al. 2012), *Eucalyptus grandis* has been used as a model system to determine the differences in gene expression between juvenile and mature cuttings, as the loss of rooting capability following the transition from the juvenile to the mature phase is a well-known phenomenon in this species. Recently, the characterization of the transcriptome profiling during the early development of the maize brace root system, which has an adventitious origin

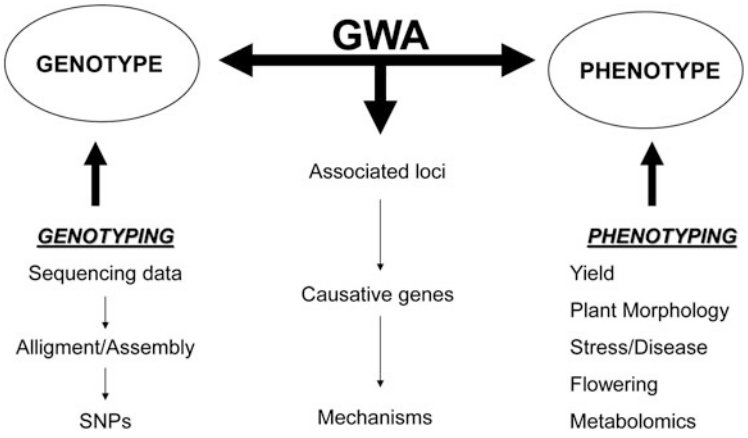

Fig. 20.3 Data integration scheme for GWA of AR formation in carnation stem cuttings. Adapted from Han and Huang (2013)

on the stem nodes, has been published (Li et al. 2011). Interestingly, the authors compared the differentially expressed transcripts involved in the maize brace root and primary root development and found little overlap in transcript identity; their discrepancies between the two root expression profiles imply that different regulation mechanisms are involved in the development of primary root and adventitious roots in maize.

20.5 Perspectives

Recent improvements in next-generation sequencing (NGS) technologies have resulted in the reduction of costs and time efforts (Morozova and Marra 2008; Werner 2010), rendering the technology now amenable for genetically less studied crops like carnation (Tanase et al. 2012). These recent technological advances will make GWA mapping a valuable strategy for the identification of trait-linked markers required for establishing a MAS approach to select for adventitious rooting performance in current carnation germplasm. The identification of the genes involved in AR formation in this species will contribute to our basic understanding of the molecular events leading to this complex developmental response and to the eventual manipulation of AR formation in other recalcitrant, ornamental species.

20.6 Conclusion

Adventitious rooting is an important factor ensuring vegetative propagation of a number of plant species. Carnation is, after rose, the most important species on the worldwide market of cut flowers. Our current knowledge about adventitious rooting in carnation has gain insight from physiological studies showing that root induction in the cutting is affected by complex interactions between sucrose and hormone levels, particularly auxin. However, the genetic determinants of the differences found in rooting performance between carnation cultivars are still unknown. We are developing new approaches to characterize in detail stem cutting morphology and adventitious root (AR) architecture in carnation cuttings. Recent developments in sequencing technologies allow genome-wide genetic variation discovery among landraces and cultivars, which can then be used for the discovery of trait-linked markers through genome-wide association (GWA) studies. The identification of the genes involved in AR formation in this species will help establishing a marker-assisted selection (MAS) approach to select for improved adventitious rooting performance in current carnation breeding programs.

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Chapter 21
Roots of Medicinal Importance

Aparajita Das, Vipin M. Dan, George Varughese, and Ajit Varma

21.1 Introduction

Success of plant growth and its adaptability to the environment depend on the shoot as well as root growth of the plant. It is because root and shoot growths are so interdependent that one cannot succeed without the other. The right root architecture in a given environment condition affects the volume of soil available as a source of water and mineral nutrients for the plant. The extents of root systems help plants to survive periods of water and nutrient deficit, as well as compete effectively for resources for better survivability. Thus, it is important to understand the development and architecture of roots that hold promising potential for the utilization and management of root characteristics for enhancement in food plant yield and optimize agricultural land use. Root systems act by both recognizing and reacting to environmental cues and thus help plants to overcome different challenges posed by soil environmental conditions. Root system architecture varies between species and also within species, subject to genotype and environment also (Smith and Smet 2012). Root systems also provide an optimal system for studying developmental plasticity, a typical feature of plant growth and development. Phenotypic plasticity is the ability of an organism to change its phenotype in response to changes in the environment. Examples of phenotypic plasticity in plants include

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the allocation of more resources to the roots in soils that contain low concentrations of nutrients and the alteration of leaf size and thickness.

Plants have long been used as major ingredients for traditional medicines worldwide. Medicinal plants are being used for centuries as primary healthcare need for the welfare of mankind. Although modern medicines may be available, but traditional herbal medicine are still being used for historical, cultural, and ecological reasons, specially due to continued availability, better compatibility and high acceptance due to their less side effects. These benefits have led to a worldwide search for new pharmacologically essential substances derived from natural products like plants. The beneficial effects of the medicinal plants in health care can be well judged from the WHO estimate that around 80 % of the world population uses them in some form or the other. It is significant to note that homeopathy and modern medicine have their ancestry in medicinal plants. The bioactive compounds derived from medicinal plants form the ingredients of analgesics, antibiotics, heart drugs, laxatives, anticancer agents, ulcer treatments, contraceptives, diuretics, etc. (Kunwar et al. 2010; Ahmed et al. 2012). Plant-based natural products or bioactive compounds can be obtained from any plant parts like barks, leaves, flowers, fruits, roots, seeds, etc. Roots, one of the most vital organs of plants for its survival, not only provide plants the anchorage and help plants to absorb nutrition for its growth and development but also store bioactive compounds which can be used a medicinal importance. Bioactive compounds from medicinal plants have tremendous utility in modern days for the treatment of many diseases for the welfare of the human population worldwide. Over three-quarters of the human population depend mainly on plants and its extracts for health care. More than 30 % of the entire plant species, at one time or another, are used for medicinal purposes. Medicinal plants can also be classified according to the part used, habit, habitat, curative value, etc., besides the usual botanical classification (Joy et al. 1998). There are different methods of classifying medicinal plants; one of the methods can be based on plant parts used for extraction of bioactive compounds from medicinal plants for treatment of diseases; medicinal plants can be classified as:

1. Whole plant: *Boerhaavia diffusa*, *Phyllanthus niruri*, and *Mimosa pudica*
2. Root: *Asparagus racemosus*, *Coleus forskohlii*, and *Withania somnifera*
3. Stem: *Tinospora cordifolia* and *Acorus calamus*
4. Bark: *Saraca asoca*, *Terminalia arjuna*, and *Cinchona officinalis*
5. Leaf: *Bacopa monnieri, Aloe vera*, and *Ocimum sanctum*
6. Flower: *Punica granatum*, *Biophytum sensitivum*, and *Crocus sativus*
7. Fruit: *Aegle marmelos*, *Emblica officinalis*, and *Solanum nigrum*
8. Seed: *Trigonella foenum-graecum*, and *Abrus precatorius*

There are large numbers of medicinal plants available worldwide where roots have the bioactive compounds of medicinal importance. The present chapter discusses an overview of some selected medicinal plants where the roots are the source of bioactive compounds used for the well-being of the humankind.

21.2 Roots: The Organ of Anchorage and Its Types

Radicle is the first organ that comes out when the seed germinates. Radicle grows into the root system of the plant, which grows downward in the soil. Roots are nongreen due to the absence of chlorophyll in them and are not divided into nodes and internodes. It is also characterized by the absence of leaves and buds. Roots are generally positively geotropic (grow toward gravity), positively hydrotropic (grow toward water), and negatively phototropic (grow away from light). There are two main types of root systems:

1. Tap root system
2. Fibrous root systems

1. Tap root system
   Radicle may elongate to form the primary or the tap root. It gives off lateral branches (secondary and tertiary roots). The primary or main root grows vertically downward and is longer than its branches. The tap root along with its lateral branches constitutes the plant's root system. This kind of root is found generally in dicots like carrot, mustard, gram, radish, beans, and pea. The principal advantages of root systems are that they penetrate deeper and anchor plants. It helps to obtain water for the plants from other levels (http://www.nios.ac.in/srsec314newE/PDFBIO.EL6.pdf dated 27 June 2012).
2. Fibrous root system
   A number of thread-like roots arising from the base of the stem are called fibrous roots. The radicle is short-lived, and numerous roots of more or less equal size arise and form fibrous roots. This kind of root system is generally found in monocots plants like grasses, wheat, rice, maize, and barley (http://www.bcb.uwc.ac.za/ecotree/root/roottypes.htm dated 27 June 2012).

   Major functions of roots

   - Provide anchorage and mechanical support to the plant
   - Help in the absorption and conduction of water and dissolved mineral nutrients for the plant
   - Synthesize various essential compounds such as growth regulators in the plant
   - Store food in root crops such as sugar beet, carrots, and cassava in the plant
   - Act as major interface between the plant and various biotic as well as abiotic factors in the soil environment
   - Prevent soil erosion as roots of the plants help to bind to the soil particles and avert them from being blown away by wind or water

   Types of roots

   1. Tap root: It is the main root that develops from the radicle and bears many branches and remains underground. It is usually found in dicots.

2. Adventitious root: It is the root that develops from any part of the plant except radicle. These roots may be aerial or underground. They may grow from stem base or nodes or tree branch.

21.2.1 Specialized Variations of Roots

21.2.1.1 Modifications of Tap Roots

As the radicle keeps growing downward and smaller lateral roots form along with it, it forms tap root system. The shape of the tap root may vary, and many tap roots are modified into storage organs which are tabulated in Table 21.1. Tap root and its modifications are illustrated in Fig. 21.1. Modifications of adventitious roots are tabulated in Table 21.2. (http://www.botgard.ucla.edu/html/botanytextbooks/generalbotany/typesofroots/ dated 27 June 2012 and http://www.nios.ac.in/srsec314newE/PDFBIO.EL6.pdf dated 27 June 2012)

21.3 Roots: Source of Bioactive Compounds

A large number of diseases including complicated metabolic disorders have seriously disturbed the human health and quality of human life in today's world. Bioactive compounds from countless medicinal plants have long been an admirable source of pharmaceutical agents for treatment of diverse innumerable diseases. Ayurveda and other traditional medicinal system used for the treatment of various diseases describe a number of plants that are used as herbal drugs due to their less side effects and low cost. Roots of numerous medicinal plants have been used for the management of diseases as they are a great source of biological constituents also. The following paragraphs discuss about the roots of medicinal plants including modified roots for the treatment of some selected diseases (Balunas and Kinghorn 2005; Itokawa et al. 2008; Lee 2010).

21.3.1 Diabetes Mellitus

Diabetes mellitus is a complex metabolic disorder of carbohydrates, a global health crisis that has fatally affected the quality of human's life in today's world irrespective of the socioeconomic background and geographic location of the population. Type I diabetes (insulin dependent) is caused by insulin insufficiency because of lack of functional beta cells. Patients suffering from this are therefore totally dependent on exogenous source of insulin, while patients suffering from Type II diabetes (insulin independent) are unable to respond to insulin and can be

Table 21.1 Modifications of tap root

S. No.	Type	Character	Function	Example
1.	Conical	Base is broad and tapers gradually and steadily toward the apex	Storage	Carrot
2.	Fusiform	Swollen in middle, tapering toward both ends, i.e., top and bottom	Storage	Radish
3.	Napiform	Spherical at base, tapering sharply like a tail toward the tip, has top-like appearance	Storage	Turnip
4.	Tuberous	Thick and fleshy with no definite shape	Storage	Four o'clock plant

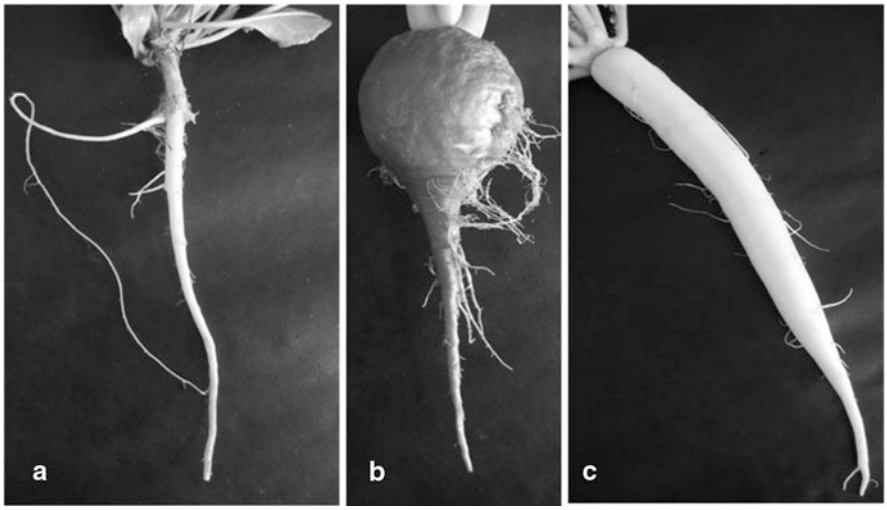

Fig. 21.1 Tap root and its modification: (**a**) Tap root, (**b**) napiform root, (**c**) fusiform root

treated with dietary changes, exercise, and medication. Type II diabetes is the more common form of diabetes, constituting 90 % of the diabetic population. Symptoms for both diabetic conditions may include (1) high levels of sugar in the blood, (2) unusual thirst, (3) frequent urination, (4) extreme hunger and loss of weight, (5) blurred vision, (6) nausea and vomiting, (7) extreme weakness and tiredness, and (8) irritability, mood changes, etc. The global prevalence of diabetes is estimated to increase, from 4 % in 1995 to 5.4 % by the year 2025. It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025. As the people suffering from diabetes mellitus have been increasing noticeably these days, thus, this demands special awareness toward its management. Besides the use of conventional methods, plants which have gargantuan medicinal importance are being explored for their use against many diseases including diabetes. Drugs obtained from

Table 21.2 Modifications of adventitious roots (http://www.botgard.ucla.edu/html/botanytextbooks/generalbotany/typesofroots/ dated 27 June 2012 and http://www.nios.ac.in/srsec314newE/PDFBIO.EL6.pdf dated 27 June 2012)

S. No.	Type	Character	Function	Example
1.	Tuberous	Swollen roots developing from nodes of prostrate stem	Storage	Sweet potato
2.	Fasciculated	Swollen roots developing in a cluster from the stem	Storage	Dahlia
3.	Nodulose	Only apices of roots become swollen like single beads	Storage	Mango ginger
4.	Moniliform	Roots alternately swollen and constricted, presenting a beaded or moniliform appearance	Storage	Grasses, sedges
5.	Annulated	Look as if formed by a number of discs placed one above the other	Storage	Ipecac
6.	Assimilatory roots	Roots which when exposed to sun develop chlorophyll, turn green (aerial root), and manufacture food	Photosynthesis	*Tinospora*
7.	Epiphytic roots	Aerial roots of epiphytes are greenish and covered with spongy tissue (velamen) with which they absorb atmospheric moisture	Absorbing atmospheric moisture	Orchids (*Vanda*)
8.	Pneumatophores or respiratory roots	Some roots grow vertically up (negatively geotropic) into air. Exposed root tip possesses minute pores through which roots respire and appear like conical spikes coming out of water	Gaseous exchange	Mangroves (marshy plants) *Rhizophora*
9	Sucking roots or haustoria	Parasitic plants give out sucking roots or haustoria which penetrate living host plant and suck food	Sucking nutrition from hosts	*Cuscuta*
11.	Prop roots	Roots develop from tree branches, hang downward, and ultimately penetrate the ground, thus support heavy branches	Strong support	Banyan
12.	Stilt roots	Extra roots developing from nodes near the base of stem grow obliquely and penetrate the soil giving strong anchorage	Strong support	Sugarcane, Screw pine
13.	Climbing roots	Weak climbers twine around and clasp the support with the help of climbing roots arising from their nodes	Strong support	Betel

(continued)

Table 21.2 (continued)

S. No.	Type	Character	Function	Example
14.	Clinging roots	Special clinging roots arise, enter the crevices of support, and fix the epiphyte	Strong support	Epiphytes orchids
15.	Floating roots	Spongy, floating roots filled with air arise from nodes of some aquatic plants and help in floating and respiration	Buoyancy and respiration	*Jussiaea*

medicinal plants are quite acceptable as these drugs are known to cause less adverse effects on human health and low cost (Patel et al. 2012; Khan et al. 2012; Modak et al. 2007).

Plants of medicinal importance have been explored scientifically and systematically and claimed to be helpful for the treatment of diabetes by various scientific research groups globally. Medicinal plants having the active compounds like glycosides, alkaloids, terpenoids, flavonoids, etc., are the potential hyperglycemic agents. Medicinal plants having hyperglycemic agents can be used as potential antidiabetic agents as these are less toxic and free from side effects. Worldwide large numbers of medicinal plants are used for the treatment of diabetics. Some of the important medicinal plants whose roots are used for the treatment of diabetics are tabulated in Table 21.3.

Medicinal plants can provide better alternative for the management of diabetics as the number of people suffering from diabetes mellitus has been increasing noticeably over the past few decades.

21.3.2 Cardiovascular Disease

Cardiovascular diseases account for large number of deaths and disabilities worldwide. Finding solutions to lessen the mortality of cardiovascular disease remains a significant health goal. The continuous increase in incidences of cardiovascular disease is a manifestation of persistent poor diet and lifestyle choices, which also lead to diabetes and obesity. A large number of plants, nearly 2,000 plants, are listed in the traditional (herbal/alternative) systems of medicine, and some of these medicinal plants are providing comprehensive relief to the people suffering from cardiovascular diseases, specially “hyperlipidemia” and “ischemic heart disease” (Arya and Gupta 2011). Some of the cardioprotective medicinal plants whose root extracts are used are discussed below.

Table 21.3 Roots of medicinal plants with antidiabetic and related beneficial properties

S. No.	Botanical name	Common name	Family	Phytochemical present	Antidiabetic effect and other important effect	References
1.	*Astragalus membranaceus*	Yellow leader	Leguminosae	Polysaccharides, saponins, alkaloids	Hypoglycemic, decrease in insulin resistance	Khan et al. (2012)
2.	*Aegle marmelos*	Bael	Rutaceae	Coumarins, alkaloids, scoparone, marmesin	Hypoglycemic, antidiarrheal	Joy et al. (1998)
3.	*Berberis aristata*	Barberries	Berberidaceae	Flavonoids, terpenoids, alkaloids, phenols, sterols	Antihyperglycemic, antioxidant	Khan et al. (2012)
4.	*Beta vulgaris*	Beetroot or garden beet	Chenopodiaceae	Alkaloids, flavonoids, terpenoids, saponins, betaine, neobetain	Hypoglycemic, antioxidant	Pal et al. (2010), Donga et al. (2011), Khan et al. (2012)
5.	*Casearia esculenta*	Saptarangi	Salicaceae		Antihyperglycemic, antioxidant, antihyperlipidemic	Khan et al. (2012)
6.	*Ceiba pentandra*	Silk cotton tree	Bombacaceae	Epicatechin and flavonoids	Improves hyperglycemia effect	Dzeufiet et al. (2006), (2007)
7.	*Costus speciosus*	Crepe ginger, kebu	Costaceae/ Zingiberaceae	Diosgenin, dioscin, gracillin, quinones, etc.	Antihyperglycemic, antihyperlipidemic and antioxidative effects	Khan et al. (2012)
8.	*Dioscorea polygonoides*	Jamaican bitter yam	Dioscoreaceae	Diosgenin, saponins	Hypoglycemic, hypolipidemic	Khan et al. (2012)
9.	*Ficus benghalensis*	Banyan	Moraceae	Flavonoids	Hypoglycemic, antioxidant	Donga et al. (2011), Khan et al. (2012)
10.	*Glycyrrhiza glabra*	Mulhatti, sweet wood	Fabaceae/tap root	Glycyrrhizin, saponins, flavonoids	Hypoglycemic, antioxidant	Saxena (2005), Jatav et al. (2011)
11.	*Helicteres isora*	East Indian screw tree	Sterculiaceae	Cucurbitacin B and isocucurbitacin B	Anti hyperglycemic activity	Venkatesh et al. (2007)

12.	*Hypoxis hemerocallidea*	African star grass, African potato	Hypoxidaceae	β-sitosterol, stigmasterol	Antidiabetic and anti-infective properties, stimulate insulin release	Nair and Kanfer (2008), Khan et al. (2012)
13.	*Lantana aculeata*	Spanish flag	Verbenaceae	Oleanolic acid, alkaloids	Hypoglycemic	Kumar et al. (2010)
14.	*Orchis anatolica*	Dilicikik	Orchidaceae		Hypoglycemic	Khouri and Daradka (2012)
15.	*Panax pseudoginseng*	Ginseng	Araliaceae	Ginsenosides, polysaccharides, peptides	Hypoglycemic	Lee et al. (2008)
16.	*Polygonatum odoratum*	Angular Solomon's seal	Ruscaceae	Dipeptide, glucoside	Insulin sensitizer	Khan et al. (2012)
17.	*Raphanus sativus*	Radish	Cruciferae	Glucosinolates, isothiocyanates	Hypoglycemic	Hanlon and Barnes (2011), Shukla et al. (2011)
18.	*Rhodiola sachalinensis*	Roseroot, golden root, hong jing tian	Crassulaceae	Polysaccharide, saponins, flavonoids, salidroside	Hypoglycemic, hypolipidemic, increase in serum insulin level	Gao et al. (2009), Khan et al. (2012)
19.	*Tinospora cordifolia*	Giloe	Menispermaceae	Cordifol, tinosporidine, tinosporide, perberilin, etc.	Antihyperglycemic	Joy et al. (1998)
20.	*Withania somnifera*	Ashwagandha, Indian ginseng	Solanaceae	Alkaloids, withanine, somniferine, somniferinine, somnine	Hypoglycemic	Khan et al. (2012)

21.3.2.1 *Desmodium gangeticum*

Desmodium gangeticum is an herb belonging to the family Fabaceae. The herb is widely distributed in tropical and subtropical habitats worldwide. It is extensively used in Ayurveda for the treatment of various diseases like typhoid fever, urinary discharges, piles, and inflammations. It is also used in the treatment of ischemic heart disease in Indian system of medicine. The root of the plant is one of the components of Ayurvedic preparations frequently used for the management of different types of heart diseases. Three pterocarpenoids, gangetin, gangetinin, and desmodin, are the major phytochemical constituents of the roots (Shabi and Upadhyay 2012).

21.3.2.2 *Raphanus sativus*

Botanically, the common radish which grows in our vegetable garden is known as *Raphanus sativus*. It belongs to family Brassicaceae. Although radish is native to Asia, nowadays, it is grown and consumed as a garden vegetable throughout the world. The plant has a long, round, bitter, and pungent root. The root also varies in shape, size, and color. The roots also have medicinal importance. The main bioactive compounds present in the roots are raphanin, glucosinolates, vitamin C, and volatile oil. Radish is considered to be an antiseptic, antirheumatic, appetite stimulant, diuretic, diaphoretic, and rubefacient. Radish is an excellent source of vitamin C and a powerful immune booster. It is used for the treatment of gastrointestinal and cardiovascular disorders. It is also used in inflammations, hiccough, leprosy, and cholera too (Arya and Gupta 2011; Zaman 2004).

21.3.2.3 *Inula racemosa*

The genus *Inula* consists of 100 species that belong to the family Asteraceae. This perennial herb is distributed all over East Asia, Europe, and Africa. In India, it is also known as Pushkarmula and grows in the hilly regions of northwestern Himalayas. The root extracts of *I. racemosa* have been prescribed as medicine in China for various human diseases, such as abdominal pain, acute enteritis, and bacillary dysentery, to relieve the depression of the liver qi, alleviate pain especially between the neck and the shoulders, and to prevent abortion. In India, the plant root powder is reported to have shown potential effect on cardiovascular system and also used as Ayurvedic medicine for angina and dyspnea. In addition, root extract along with guggul (*Commiphora mukul*) is also employed for curing myocardial ischemia. The various biological properties of this plant root are mainly through a variety of bioactive phytochemicals such as alantolactone, isoalantolactone, dihydroalantolactone, dihydroisoalantolactone, sitosterol, daucosterol, inunolide, aplotaxene, phenylacetonitrile, and isoinunal (Arumugam et al. 2012; Rawat and Everson 2011).

Fig. 21.2 *In vitro* shoots of *Picrorrhiza kurroa*

21.3.2.4 *Picrorhiza kurroa*

Picrorhiza kurroa, which belongs to family Scrophulariaceae, is a medicinal plant of high-altitude areas (Fig. 21.2). This medicinal plant is known to cure cardiac ailments also, besides having many more curative properties (Arya and Gupta 2011). *P. kurroa*, popularly known as "Kutki" or "Kurro" in Hindi, is a mild hairy perennial herb growing wild in the Alpine-Himalayan region from Kashmir to Sikkim at an altitude of 2,700–5,000 m. In Indian Ayurvedic medicine,

Picrorhiza kurroa has been used to heal cardiac ailments (Rajaprabhu et al. 2007). In traditional medicine, it has also been used to treat hepatitis, abdominal pain, stomach disorders, anemia, and jaundice and to promote bile secretion. Picroside I, picroside II, and kutkoside are the naturally occurring free radical-scavenging principles present in the roots and rhizomes of *P. kurroa*. They are credited with antiallergic, antianaphylactic, antidiabetic, antitumor, and other beneficial properties (Rajaprabhu et al. 2007). Studies demonstrated that the ethanol extract of *Picrorhiza kurroa* rhizomes and roots significantly prevented the isoproterenol-induced myocardial infarction and maintained the rats at near normal status with respect to lipid metabolism in serum (Senthil Kumar et al. 2001; Rajaprabhu et al. 2007).

21.3.2.5 *Salvia miltiorrhiza*

The medicinal plant *Salvia miltiorrhiza* (Lamiaceae) is also commonly known as "Danshen." The plant is native to China and Japan. Danshen is also known as red sage root. It contains several distinctive bioactive compounds (tanshinoates and salvianolic acids) with cardioprotective and antioxidant properties and hence has been widely used in Chinese medicine for numerous cardiovascular disorders. *Salvia* dilates arteries and blood vessels, thus increasing blood flow. This effect is very significant as strokes and heart attacks are caused by blood platelets that aggregate and form clots. A heart attack occurs when one of the coronary arteries becomes totally blocked, generally by a blood clot. Salvianolic acid A (Sal A), the water-soluble constituent from the root of the *Salvia miltiorrhiza* plant, has antioxidant, antiproliferative, and antiplatelet properties. Studies have shown that Sal A prevents I/R-induced myocardial damage by reducing necrosis and apoptosis in isolated rat hearts and cardiomyocytes (Cao et al. 2009; Pan et al. 2011).

21.3.2.6 *Coleus forskohlii*

Coleus forskohlii Briq., a medicinal plant, is a member of the mint family, Lamiaceae. It is indigenous to India and is recorded in Ayurvedic *Materia Medica* under the Sanskrit name "Makandi" and "Mayani." *C. forskohlii* being aromatic, its roots, flowering shoots, and leaves are also aromatic (Fig. 21.3). Roots are the source of an active principle forskolin, which is a diterpenoid and used as a drug for the treatment of cardiovascular diseases. Besides, roots also contain the phytochemicals coleosol and colenone. Leaves contain the diterpenoids methylene quinine, barbatusin, and cyclobutatusin. Barbatusin has inhibitory action against lung carcinoma and lymphatic leukemia. In traditional Ayurvedic systems of medicine, *C. forskohlii* has been used for treating heart diseases, abdominal colic, respiratory disorder, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy, and angina (Kavitha et al. 2010; Jagtap et al. 2011; Das et al. 2012).

Fig. 21.3 *In vitro* shoots of *Coleus forskohlii*

Cardiovascular disease is a quite bothersome disease in today's world affecting large number of population due to lifestyle misbalance. Hopefully new pharma compounds can be isolated from various medicinal plants for the treatment of cardiovascular diseases.

21.4 Hepatoprotective

The liver regulates various physiological functions and also controls many vital functions such as secretion, storage, and metabolism. It plays the main role in the detoxification of toxic compounds and also helps in the production of useful substances (Subramoniam and Pushpangadan 1999). It plays an important role in homeostasis of the body and is involved in major biochemical pathways. It is involved in the removal of various compounds from the portal circulation, and this role of the organ makes it susceptible to many harmful substances, thus resulting in liver dysfunction (Bodakhe and Ram 2007). Liver diseases are caused by chemicals such as acetaminophen or paracetamol in higher doses, overconsumption of alcohol, microbial infections, and autoimmune diseases. Research studies have made many advances, but the search for an effective drug for liver diseases still remains unanswered. Compounds that effectively protect or stimulate liver function or help in reviving damaged liver cells are still lacking at

the clinical level for human use. Medicinal plants and their combinations with strong traditional knowledge base for treatment of liver diseases have a long history in human civilization. Hepatoprotective plants have many compounds that offer protective action to the liver; these chemicals include phenols, coumarins, monoterpenes, glycosides, alkaloids, and xanthenes (Bhawna and Kumar 2009).

21.4.1 Vitis vinifera *L. (Vitaceae)*

V. vinifera is well known as grape vine; it grows in the Mediterranean region, Central Europe, and southwestern Asia, from Morocco and Spain, north to southern Germany, and east to northern Iran. Traditional use of the plant parts of *Vitis vinifera* for medicinal purposes is well documented (Sharma et al. 2012). The ethanolic root extract of the plant has been shown to possess liver-protective action against carbon tetrachloride-induced liver damage in rats. The extract was able to reverse the hepatic damage caused by CCl_4 in rat model (Sharma et al. 2012). This mechanism of action in liver protection may be correlated to the presence of membrane-stabilizing agents in the root extract, which averts the enzyme leakage in tissues in response to the toxic damage caused by CCl_4. *V. vinifera* has a high concentration of polyphenols; the phenolic content and composition depend on factors such as the cultivar and environmental conditions. The phenolic compounds most notably well distributed in grapes are anthocyanins, catechins, and procyanidins, which can be related to the hepatoprotective activity offered by the plant (Xia et al. 2010).

21.4.2 Scrophularia buergeriana *(Scrophulariaceae)*

This group has its habitats in Oriental countries; the root of the plant is used for treatment of fever, swelling, neuritis, and laryngitis. The genus *Scrophularia* has been scientifically investigated, and a number of natural compounds have been studied such as terpenoids, flavonoids, phenylpropanoids, and iridoid glycosides; some of these compounds are effective in treating liver diseases and can be developed into possible drug candidate in the future. Lee et al. (2002) demonstrated that phenylpropanoids extracted from the roots of *S. buergeriana* were able to protect primary rat hepatocyte cultures from the toxicity induced by carbon tetrachloride. Eleven phenylpropanoids were isolated from the root of the plant; among these, three compounds, namely, 4-*O*-E-*p*-methoxycinnamoyl-α-L-rhamnopyranoside ester, isoferulic acid, and *p*-methoxycinnamic acid, were the most effective in hepatoprotective action. The presence of α,β-unsaturated carboxyl moiety in phenylpropanoid chemical structure imparts the major role in hepatoprotective activity.

21.4.3 Asparagus racemosus

It belongs to the family Liliaceae and is used across the globe as a vegetable in culinary preparations. The plant habitat is throughout Asia, Australia, and Africa. Research investigations conducted on *A. racemosus* have disclosed a wide range of biological activities such as antimutagenic, antitumor, antifungal, diuretic, and antiulcer and immunostimulatory effects (Om et al. 2011). Traditional knowledge base strongly supports the hepatoprotective activity of *A. racemosus*. The research study by Om et al. (2011) revealed decrease in serum enzymes, alanine aminotransferase and aspartate aminotransferase activities, on treatment with the root extracts, thus indicating repair of damaged hepatic tissue caused by paracetamol. In toxic conditions, as the liver damage occurs, the levels of bilirubin increase, and the root extracts of *A. racemosus* were able to restore bilirubin to its normal level, thus indicating hepatoprotective activity. Different types of flavonoids and saponins have been reported to actively participate in hepatoprotective activity (Fuentes et al. 2008). Phytochemical screening has disclosed the presence of flavonoids in the ethanolic extract of *Asparagus racemosus*. *A. racemosus* roots have the presence of four saponins, for example, shatavarin I–IV, the glycosides of sarsasapogenin (Negi et al. 2010). So the hepatoprotective effect of Asparagus racemosus can be correlated to both its flavonoid and saponin content.

21.4.4 Daucus carota *L. (Apiaceae)*

D. carota is native to Europe and has various medicinal properties; it is used as antibacterial, stimulant, antiseptic, carminative, and diuretic and has hepatoprotective properties. Bishayee et al. (1995) investigated the liver-protecting effects of the extracts of tuber roots of *D. carota* L. on CCl_4-induced liver toxicity in animal models. The extract was able to reduce the elevated levels of serum enzymes and also decreased the high content of bilirubin and urea that resulted due to liver toxicity. Phytochemical screening has revealed a variety of compounds in this plant such as the daucane-type sesquiterpenes (carotol, daucol, and furocoumarins), flavonoids, polyacetylenes, fatty oil, and beta-carotene (Ahmed et al. 2005).

21.4.5 Actinidia valvata *Dunn*

A. valvata belongs to genus Actinidia, is a shrub widely distributed, and is native to eastern China. The plant has documented traditional use in Chinese medicine and folk herb. Research study by Qu et al. (2012) revealed that in CCl_4-induced acute liver damage models, the levels of alanine aminotransferase, aspartate

aminotransferase, and ALP in serum were significantly decreased by treatment with the root extract of *A. valvata*. The study also revealed that the extract showed effective antioxidant properties. Total saponin present in *A. valvata* root is considered to be the compounds that constitute the pharmacologically active ingredients against inflammation and tumor (Yi et al. 2009). The saponins present in root can be correlated to the hepatoprotective action. Two polyoxygenated triterpenoids were extracted from *A. valvata* root; the triterpenoids, namely, (2β,3α,6α)-2,3,6,20,23,30-hexahydroxyurs-12-en-28-oic acid and (2β,3α)-2,3,20,23,24,30-hexahydroxyurs-12-en-28-oic acid *O*-β-D-glucopyranosyl ester, were elucidated by means of extensive spectroscopic studies (Xin et al. 2008).

The present trend in food habits and lifestyle are major factors that lead to liver problems. The traditional knowledge wealth available globally should be harnessed and scientifically validated to prove their claims in hepatoprotective action. The answer to a cure for liver diseases lies within the herbal combinations that have been passed on and successfully used for generations, which we call traditional medicine.

21.5 Cancer

Cancer is a prominent disease which leads to large percent of mortality worldwide. As per the data published in 2005 by World Health Organization, from a total of 58 million deaths worldwide, cancer covers 7.6 million (13 %) of all the deaths. The near future will see an increase in cancer-related deaths due to a variety of reasons like steadily aging population in both developed and developing countries, existing smoking prevalence, growing preference for unhealthy lifestyles, evolution of multidrug-resistant cancer cells, and scarcity in discovery of effective novel anticancer drugs.

Cancer research has been heavily funded by various organizations globally in pursuit of a cure for this dreadful disease. Cancer chemoprevention mainly employs chemical compounds, natural products, or natural compound derivatives that can revert or inhibit malignant cell transformation, prevent invasion and metastasis, and provide an economical and rational approach for cancer control. The use of herbal medicine or dietary agents is being increasingly utilized as an effective way for the management of many cancer treatments (Zarei and Javarappa 2012). Antitumor research has witnessed the introduction of many clinically successful plant-derived compounds such as taxol, vinblastine, vincristine, and camptothecin. Hence, there is a great potential for the development of anticancer drugs from the essentially untapped reservoir of the plant kingdom (Zarei and Javarappa 2012). Since the 1940s, more than 175 anticancer drugs were developed and clinically used for treatment for various cancers. In this the major percentage of anticancer drugs was inspired from plant-based phytochemicals (Newman and Cragg 2007; Rosangkima et al. 2010).

Some phytochemicals extracted from plant root are in clinical use, such as epipodophyllotoxin which is an isomer of podophyllotoxin, which was isolated as

an effective anticancer agent from the roots of Podophyllum species, *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich (Stahelin 1973). *Podophyllum peltatum* is native to eastern parts of North America, covering from Ontario to Florida and eastern Texas, while *Podophyllum emodi* is native to the Himalayan region in Asia. Etoposide and teniposide are well-known clinical agents which are semisynthetic drugs made by chemically modifying the basic structure of epipodophyllotoxin and are used in treatment of lymphomas and bronchial and testicular cancers (Cragg and Newman 2005; Harvey 1999). VP-163 is a well-known semisynthetic podophyllotoxin that has a key role in the chemotherapeutic approach to a variety of hematopoietic and solid tumors (Ross 1985). These drugs which are chemically modified from podophyllotoxin are representative of a class of agents whose mechanism of action is mediated by their ability to stabilize complexes involving topoisomerase II and DNA. Topoisomerase II-active drugs, including VP-16, act by making a stable enzyme–DNA complex, thus resulting in the prevention of the rapid turnover of the protein-cross-linked DNA strand breaks, and thus, it interferes with processes that require alteration in DNA topology, such as DNA replication, repair, and transcription (Chen et al. 1984).

Cancer chemotherapy has been facing many road blocks at the clinical level due to the rise of multidrug-resistant tumor cells during the period of treatment. Scientific studies have revealed that the mechanism behind developing the multidrug-resistant phenotype in tumor cells is the increased expression of a membrane glycoprotein, P-glycoprotein (Gottesman and Pastan 1993). Pervilleine A is another anticancer agent isolated from the roots of *Erythroxylum pervillei* Baill. (Erythroxylaceae) that is effective against P-glycoprotein (Silva et al. 2001). The plant inhabits tropical regions on the globe such as South America, Africa, and Madagascar. The phytochemistry of *Erythroxylum* has revealed the presence of tropane alkaloids and flavonoids. The first tropane alkaloid isolated from *E. pervillei* was pervilleine A; it was effective against oral epidermoid cancer cell line (KB-V1) in the presence of the anticancer agent vinblastine (Mi et al. 2001). Research studies related to pervilleine A is in preclinical stage. Pervilleines B and C are two other tropane alkaloid aromatic esters obtained from the chloroform extract of the roots of *Erythroxylum pervillei* as the result of bioactivity-guided fractionation, which were found to restore the vinblastine (VLB) sensitivity of cultured multidrug-resistant cancer cells (Mi et al. 2002).

21.5.1 Hemidesmus indicus *L*

H. indicus belongs to the family Apocynaceae and is commonly referred to as Indian sarsaparilla, Anantamool, or Nannari. It is native to India and is also found in other south tropical Asian countries. The plant is a well known source of medicine in traditional practice owing to its antioxidant and anti-inflammatory properties. The root extract of *H. indicus* was tested on hepatoma HepG2 cell lines and was found to be effective in inhibiting growth of cancer cell lines (Hu and Kavanagh 2003).

In another study *H. indicus* methanolic root extract showed effective anticarcinogenic and cytotoxic potential. The root extract showed significant in vitro cytotoxic activity against Ehrlich ascites tumor (EAT) cell line, and anticarcinogenic activity of the extract was determined by using EAT cell line-induced ascites tumor model in mice (Zarei and Javarappa 2012). Phytochemical study of the root extract of *H. indicus* conducted by Rajan et al. (2011) revealed the presence of components like alkaloids, terpenoids, flavonoids, phenolic compounds, and tannins. *Glochidion zeylanicum* (Euphorbiaceae) commonly named as Neeru mamidi in Telugu is commonly grown in slopes and altitudes of various places in Andhra Pradesh, India. In traditional system of medicine, it plays an important role in curing many diseases like cancer, stomachic, and diabetes. Methanolic extract of *Glochidion zeylanicum* root has shown potential cytotoxic activity on HepG2, HT29, and PC3 cancer cell lines. The root extract was able to decrease cell viability and increase growth inhibition in a concentration-dependent manner and also alter the cell morphology (Sharma et al. 2011). *Juglans regia* L. (Juglandaceae) commonly known as walnut is an important species of deciduous trees found principally in temperate areas across the world. In India it is found in Kashmir, Himachal Pradesh, and Uttarakhand states. It is largely consumed as part of the diet, and different parts of plant are used as local folk medicine. Phytochemical screening of the root bark of *Juglans regia* disclosed presence of naphthoquinones like juglone and bisjuglone (Pardhasaradhi and Babu 1978). Bisjuglone had been reported for its antitumor property in mouse skin carcinogenesis (Kapadia et al. 1997). In a study conducted by Hasan et al. (2011), organic extracts from the root bark of *Juglans regia* L. were treated on MDA-MB-231 human breast cancer cells. The treated breast cancer cells demonstrated that Bcl-2 and Mdm-2 expression was significantly inhibited, while Bax, Tp53, caspase-3, caspase-8, and TNF-α expression was markedly increased in all extract treatments. The experiment places the plant root as a source for potential anticancer drug candidate for future.

Cancer is a disease that still requires a lot of scientific investigation at the molecular level, and the search for a cure requires exploration and close scientific analysis of all possible potential drug candidates. The global plant diversity has in store many untouched species and many with strong traditional base, which requires immediate scientific attention to bring into light a hope for the ailing cancer patients.

21.6 Ulcer

Peptic ulcer is a disease that includes gastric and duodenal ulcer; this is the most common gastrointestinal disorder. The pathophysiology of this disease is caused by a difference in proportion of activity between two factors—the first being the offensive factors, which include pepsin, acid, and the bacterium *Helicobacter pylori*, and the second being the defensive factors, which encompass mucin, nitric

oxide, bicarbonate, and growth factors. The treatment of peptic ulcer employs two methods. The first involves reduction of gastric acid production, and the second method deals with ensuring the protection of gastric mucosa (Hoogerwerf and Pasricha 2001).

The pathogenesis of peptic ulcer was effectively researched upon in the past leading to its understanding, thus helping in evolution of newer and effective drug therapies. The treatment methods like proton pump inhibitors, histamine receptor blockers, prostaglandin analog, and compounds directed on mucosal barriers initially showed promise and were efficiently used in the clinic (Manonmani et al. 1995). The promises and scientific expectations were short-lived as later clinical evaluations indicated the development of tolerance, evidences of relapses, and side effects that questioned the efficacy and long-term usage of these new-age drugs (Dharmani and Palit 2006). Medicinal plants have always offered remedy for many human ailments including peptic ulcer. Herbal drugs that can treat peptic ulcer gained acceptance globally with many research studies focusing on uncovering many traditional medicinal plants that have documented antiulcer potential. In the present scenario major concentration is focused on research of indigenous drugs with lesser side effects and aimed at better management of peptic ulcer disease.

21.6.1 Desmodium gangeticum *DC (Leguminosae)*

D. gangeticum is a plant of traditional importance in India; it is known as "Shaparni" in Hindi language. The plant is a small shrub that is well adapted to the climatic conditions of tropical region. The root of this plant is used in combination with other plants for treatment of many diseases. The ethanolic root extract of the plant was used to evaluate the antiulcerogenic potential in four animal ulcer models, ulcer induced by cold restraint (CRU), alcohol (AL), aspirin (ASP), and pyloric ligation (PL) (Dharmani and Palit 2006). The effectiveness of the extract in CRU model was correlated to antioxidant property; research studies in the past have revealed free radical-scavenging activity of *D. gangeticum*. The extract was also able to provide protection to gastric mucosa by reducing gastric acid secretion in pyloric ligation animal models. AL and ASP animal models showed increase in mucin production on treatment with root extract of *D. gangeticum*, thus indicating cytoprotective ability (Dharmani and Palit 2006). Pterocarpenoids are the major chemical components in the roots of *D. gangeticum*, and among pterocarpenoids, gangetin, gangetinin, and desmodin share the major proportion (Purushothaman et al. 1975). Varaprasad et al. (2009) isolated a new compound named gangetial, another pterocarpan from the chloroform extract of the roots of *D. gangeticum*.

21.6.2 Hedranthera barteri *[(Hook F.) Pichon] (HB) (Apocynaceae)*

H. barteri is a common shrub that harbors in damp conditions of closed forest across different regions in the world. It is commonly found in countries like South Nigeria, Ghana, North/West Cameroon, and Congo-Brazzaville. The plant has many medicinal values; it is used as a laxative for children, and the leaf exudates are used for treating tumor and inflammation. A study by Onasanwo et al. (2010) revealed that the root extract of HB was effective against the four induced gastric ulcer models in rat and duodenal ulcer model in guinea pig. The gastric ulcer models in rat were cold restraint stress (CRU) induced, alcohol (AL) induced, aspirin induced (ASP), and pyloric ligation induced (PL), while histamine-induced (HST) duodenal ulcer model was employed in guinea pig. The root extract of the plant showed significant antisecretory mechanism for ulcer control in PL, CRU, and HST animal models, while the mode of ulcer control was cytoprotective mechanism in ASP and AL animal models. The study also disclosed that the dichloromethane fraction of HB root also exhibited proton pump inhibition activity and free radical-scavenging activity that can be beneficial in ulcer healing. The phytochemical analysis of the root extract showed the presence of saponins, alkaloids, flavonoids, and other secondary metabolites especially amataine and vobstusine, which can be correlated to the antiulcer activity.

The root bark of *Aralia elata* Seem. (Araliaceae) has significant traditional importance as a medicine for the treatment of gastric ulcer in Oriental countries. Research investigations have disclosed several bioactive compounds from this plant such as hypoglycemic compounds, inhibitors of ethanol absorption, and saponins with cytoprotective ability (Lee et al. 2005). The root extract of the plant has shown significant antiulcer action through antisecretory action and also showed inhibition of gastric lesion formation in animal models. Research study by Lee et al. (2005) revealed that the compound Araloside A that is present in the root bark is the reason behind the antiulcer potential of *A. elata*, and the compound employed its antiulcer action through the inhibition of gastric acid output.

Ulcer is a serious gastrointestinal disorder that requires a well-managed targeted therapeutic strategy. The failure of many promising drugs at the clinical stage is forcing scientists to uncover the potential of herbal drugs for treating ulcer. Herbal drugs that are showing promise in the preclinical studies need further research investigations to determine the safety, toxicity, and efficacy in humans. The vast traditional knowledge available globally in treatment of ulcer shows a promise for the future in developing herbal drugs that meet the expectation of ulcer management and treatment.

21.7 Conclusion

Medicinal plants have a long history in treatment of human ailments. The scientific work on different plants in the past ages has gained confidence among researchers worldwide that the global plant diversity holds the cure for many unresolved human ailments. The extensive traditional knowledge that is treasured in different parts of the globe has contributed immensely in exploring many medicinal plants, using modern biology tools. A large part of the biologically active plant-derived compounds used commercially have come into light through follow-up research to verify the authenticity of traditional knowledge. Different parts of the plant or plant as a whole has served as the source of bioactive compounds for purpose of investigation, as well as for commercial drug production. Roots are the ground anchoring part of the plant that harbors various bioactive molecules which display curative possibilities for many human ailments like cancer, diabetes, ulcer, and liver diseases. Scientific investigations in the past have helped in uncovering many phytochemicals from plant roots, some of which are now in clinical use and many others are in various stages of preclinical and clinical studies. The vast diversity of plants on the planet gives a promising journey ahead in quest for knowledge and cure for many diseases.

Research in progress in drug discovery or bioactive compounds from medicinal plants involves an all-around approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and significant leads against a range of pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain. Even though drug discovery from plants of medicinal importance continues to provide an important source of new drug leads, numerous challenges are encountered that include the procurement of original plant materials, the selection and execution of appropriate high-throughput screening bioassays, and the scale-up of bioactive compounds.

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Chapter 22
Root and Root Endophytes from the Eyes of an Electron Microscopist

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22.1 Introduction

Electron microscopes are a kind of microscopes that use a beam of highly energetic electrons to examine sample on a very fine scale and produce a magnified image. The chief advantage of the electron microscope is its increase in resolving power over the light microscope and can obtain much higher magnifications. Some electron microscopes can magnify sample up to two million times, while the best light microscopes are limited to magnifications of 2,000 times. Because of their higher resolutions and magnifications, electron microscopes can be employed in many scientific areas where light microscopes have limited utility, including biology, microorganism, cryobiology, protein localization, cellular tomography, cryo-electron microscopy, toxicology, viral load monitoring, semiconductors,

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circuit editing, defect analysis, failure analysis, electron tomography, particle analysis, pharmaceutical qualitative control, 3D tissue imaging, virology, vitrification, electron beam-induced deposition, material qualification, nanoprototyping, nanometrology, device testing and characterization, high-resolution imaging, 2D and 3D micro-characterization, macro sample to nanometer metrology, particle detection and characterization, direct beam-writing fabrication, dynamic material experiments, forensics, mining (mineral liberation analysis), and many chemical and petrochemical applications.

22.2 Scanning Electron Microscope: History and Development, Companies Manufacturing the Instrument

A scanning electron microscope (SEM) is a kind of electron microscope (EM) that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography and composition. The electron beam is generally scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nm. Specimens can be observed in high vacuum, in low vacuum, and (in environmental SEM) in wet conditions.

The most common mode of detection is by secondary electrons emitted by atoms excited by the electron beam. The number of secondary electrons is a function of the angle between the surface and the beam. On a flat surface, the plume of secondary electrons is mostly contained by the sample, but on a tilted surface, the plume is partially exposed and more electrons are emitted. By scanning the sample and detecting the secondary electrons, an image displaying the tilt of the surface is created (Fig. 22.1a).

An account of the early history of SEM has been presented by McMullan (McMullan 1988, 2006), although Max Knoll produced a photo with a 50-mm object-field-width showing channeling contrast by the use of an electron beam scanner (Knoll 1935). It was Manfred von Ardenne who in 1937 invented (von Ardenne 1937) a true microscope with high magnification by scanning a very small raster with a demagnified and finely focused electron beam. Ardenne applied the scanning principle not only to achieve magnification but also to purposefully eliminate the chromatic aberration otherwise inherent in the electron microscope. He further discussed the various detection modes and possibilities and theory of SEM (von Ardenne 1938a), together with the construction of the first high-magnification SEM (von Ardenne 1938b). Further work was reported by Zworykin's group (Zworykin et al. 1942), followed by the Cambridge groups in the 1950s and early 1960s (McMullan 1953; Oatley et al. 1965; Smith and Oatley 1955; Wells 1957) headed by Charles Oatley, all of which finally led to the

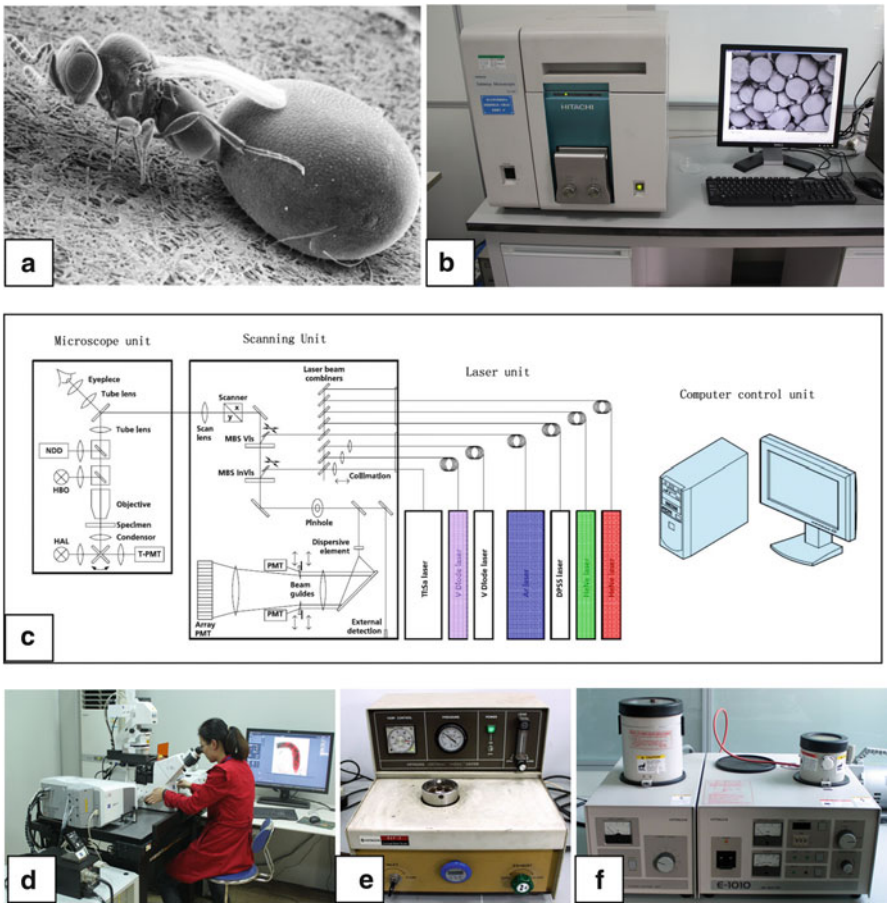

Fig. 22.1 (**a**) Image of *Telenomus theophilae* parasite the host egg with an SEM (Courtesy: Prof. Qikang Gao, China), (**b**) TM 1000 scanning electron microscope. See pear shaped chlamydospores of *Piriformospora indica* on the screen, (**c**) Scheme of system components and optical path of confocal microscopy (source of scheme is the Zeiss laser scanning confocal microscope Quick Guide), (**d**) LSM 710 confocal microscope. See the mycorrhiza colonization of root hair on the screen (Courtesy: Bing Peng, China), (**e**) HCP-2 Critical point dryer, (**f**) E-1010 ion sputter

marketing of the first commercial instrument by the Cambridge Scientific Instrument Company as the "Stereoscan" in 1965.

Companies manufacturing electron microscope
Hitachi High-Technologies Corporation: http://www.hitachi-hitec.com/global/em/index.html
JEOL Ltd: http://www.jeol.co.jp/en/products/

22.2.1 Description of SEM Instrument and Principles

TM 1000 SEM (Fig. 22.1b) was made by Hitachi High-Technologies Corporation; the system is ready for immediate use without special engineering or installation procedures. The SEM is ready to use in only 3 min, but the traditional electron microscopes need condition setting prior use in about 20 min. The "Auto-start" function allows the user to adjust focus and brightness automatically with single put on a button. The image field of view can easily be found reducing the observation time. Depending on view condition such as fungus, the charge-up may occur. The charge-up can cause the image disturbances which makes it difficult to conduct accurate image observation. The TM 1000 has a charge-up reduction mode, the image will be reduced, and observation becomes sharper by setting the view mode. The magnification of TM 1000 is from 20 times to 10,000 times; it allows for stereoscopically morphological view with high resolution and a greater depth of focus which are not available with an optical microscope (Hitachi Tabletop Microscope TM 1000). The principle is else same with the traditional SEM; the secondary electron detectors are standard equipment in all SEMs.

22.2.2 Description of Confocal Microscope and Principle

In a confocal microscope (see scheme in Fig. 22.1c), the illumination and detection light paths share a common focal plane, which is achieved by two pinholes that are equidistant to the specimen. Commonly, krypton/argon and helium/neon mixed gas lasers are used that give you a range of different distinct wavelengths. This light is sent through a pinhole and reflected by a beam splitter to the objective and specimen. The beam splitter is a dichroic filter that acts as a mirror for the excitation wavelengths and is transparent to all other wavelengths. Therefore, the emitted light from the specimen (which has a wavelength spectrum above the excitation wavelength) can go through the beam splitter to the detection pinhole and the detector (actually the beam splitter now has been replaced by an acousto-optical device). As a consequence of the pinhole arrangement, light arriving at the detector comes predominantly from a narrow focal plane, which improves the *z*-resolution significantly compared to conventional microscopy. At the high end, it is possible to achieve axial resolution in the submicron range. In the following, we will try to go through the process of preparing and scanning a fluorescent specimen, explaining a little more about the technical features of the confocal microscope to the extent one needs to know them in order to set the scanning parameters in a sensible way. Figure 22.1d shows the experiment of the mycorrhiza colonization of root hair using the confocal microscope. More recently, Rath et al. (2013) showed that the combination of physical microtomy and optical sectioning using confocal laser scanning microscopy can throw more insights into investigating plant-fungal associations.

22.3 Preparation of Samples and Operation for SEM

22.3.1 Normal Sample Preparation Method

22.3.1.1 Fixation

The fungal specimen is soft bodied and requires chemical fixation to preserve and stabilize its structure. The fungal disc was cut from a culture plate fully grown with mycelia and put into 0.1 M sodium cacodylate (pH 7.4) buffer containing 2.5 % glutaraldehyde for 30 min at room temperature. This is followed by postfixation with 1 % osmium tetraoxide.

22.3.1.2 Dehydration

The fixed tissue is then dehydrated by the HCP-2 critical point drying apparatus (Fig. 22.1e). The carbon dioxide is finally removed while in a supercritical state, so that no gas-liquid interface is present within the sample during drying.

22.3.1.3 Conductive Treatment

After the critical point drying, the fungus is usually mounted on a specimen stub using an electrically conductive double-sided adhesive tape and sputter-coated with gold/palladium alloy in E-1010 ion sputter (Fig. 22.1f).

22.3.2 Cryo-scanning Electron Microscopy Method

The scanning electron microscopy must have these equipment components: slushing station, vacuum transfer device, preparation chamber, SEM chamber components, keypad, electronics box and pumping system, nitrogen and argon, and infrared surveillance equipment.

22.3.2.1 SEM Preparation

(a) Turn on the freezer accessories power and vacuum pump will start working; evacuate the preparation chamber to prepare. Turn on the SEM power and the SEM's vacuum will start working.
(b) After preparation chamber vacuum is ready, add liquid nitrogen to preparation chamber (Fig. 22.2a) until the chamber temperature is below −150 °C.

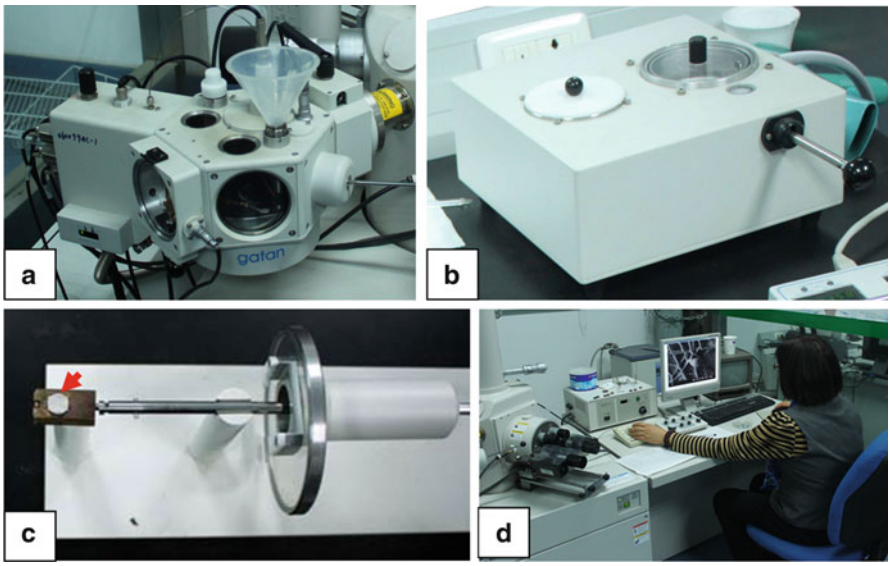

Fig. 22.2 (**a**) Gatan preparation chamber, (**b**) slushing station, (**c**) the sample stage (the *arrow* show the position of sample), (**d**) view of S-3000 N SEM with Alto 2100

(c) Add the liquid nitrogen into the slushing station (Fig. 22.2b) until the liquid nitrogen changes into nitrogen mud.
(d) Make the temperature of SEM sample chamber below −150 °C.

22.3.2.2 Sample Preparation (Refer to the Alto 2100 Operator's Handbook)

(a) Cut 5 × 5 mm of fresh sample adhered to the sample stage (Fig. 22.2c).
(b) The stage with the vacuum transfer device was quickly inserted into the liquid nitrogen, frozen completely.
(c) After vacuuming, the sample is pulled into the vacuum transfer chamber and transferred to the ready room.
(d) Sample fracture
If required, the sample is ready to be fractured in the prep chamber. To fracture the sample, remove the knife from its parking block and break the sample using the blade. Remove sample debris from the sample holder by disengaging the holder from the cold block using the transfer rod and (carefully) tapping the end onto adjacent cold surface held at base temperature, e.g., the surface of the integral Dewar. This action will remove lose fragments of specimen preventing there transfer into the SEM and ensures efficient cold trapping of potential contaminants away from the cleaved surface.

To keep the knife cold and hence optimize fracturing conditions, the knife parking block is maintained at approximately the same temperature as the

sample cold block. When in use, try to avoid dragging the knife over the sample surface, as this will cause the sample's surface to become smudged and disfigured.

Replace the knife in its parking block to keep it cold. If the knife becomes warm, any fractured samples may show signs of knife artifact.

(e) Loading a sample onto the SEM cold stage

Once the ball valve is opened, the sample may be introduced into the SEM chamber by pushing the transfer rod through the port into the SEM. The SEM lamp will come on automatically as the ball valve is opened. Provided that the SEM stage is in exchange position, it will be possible to transfer the sample holder onto the SEM cold stage. Push the sample holder as far as it will go onto the SEM cold stage: there is a stop to ensure that the holder does not go on too far. Release the rod from the sample holder's bayonet fitting by pushing it inward and turning anticlockwise until the rod hits a stop. Pull the rod back and the sample holder will remain in place on the SEM stage. Retract the rod into the prep chamber so that it is clear of the ball valve which must then be closed before the beam can be run up.

(f) Sublimation in the SEM chamber

Set the temperature of the SEM cold stage to a suitable sublimation temperature. This will depend on whether one wants to remove the bulk surface water (−85 °C to −80 °C) or else wants to sublime internal water after fracturing (−100 °C to −90 °C).

(g) Sample removal prior to coating

Open the ball valve (provided the ball valve LED is green) and advance the transfer rod to engage the sample holder's T-bar into the transfer rod's slots. Once the rod and the holder are engaged, push the rod in further until it stops; turn it clockwise until it stops. The sample is now ready to be withdrawn: pull the rod backward to disengage the sample holder from the SEM cold stage. If the sample holder remains on the cold stage, try the procedure again.

Retract the rod with the holder through the SEM port and once clear of the ball valve, the valve may be closed to isolate the vacuums of the prep chamber and SEM. Relocate the sample holder via the front of the cold block of prep chamber and push the rod forward until the sample is positioned under the sputter head. Marks for optimum position are provided on the top of the cold block.

(h) Sample coating

Make sure that the sputter system has been conditioned.

Set the sputter timer to the desired time by holding down the TIMER button while using the RAISE and LOWER buttons. Sputtering time will depend on the thickness of the coating required.

(i) Imaging and sample removal from the system

With the sample inside the SEM chamber and the ball valve closed, the beam may be run up. Bring the sample to a suitable working distance. Ideally, a CCD camera may be used to safely bring the sample to a suitable working distance without the risk of collision with the pole piece. If there is no CCD/chamber

scope, then first focus on the sample at its exchange position (long Z), change the focus to the new value and bring the sample into focus using the Z control. This will help to avoid collision with the pole piece yet still allow short working distances to be used. Figure 22.2d shows the fungal hyphae and chlamydospores.

22.4 Preparation of Samples and Operation for Confocal Microscope

22.4.1 Qualitative of Root Colonization

In 1982, Ames et al. detected autofluorescing spots in roots colonized by arbuscular mycorrhizal fungi and identified these spots as arbuscules. Ames et al. (1982) and Jabaji-Hare et al. (1984) proposed the quantification of the autofluorescing "arbuscules" as a nondestructive method for measuring root colonization by arbuscular mycorrhizal fungi. Gange et al. (1999) compared the level of arbuscular colonization in a range of plants after staining with Chlorazol Black E, acid fuchsin and trypan blue with the level of the autofluorescing "arbuscules." They concluded that in most plants the number of autofluorescing "arbuscules" seemed much higher than the number of stained arbuscules. On this basis, the quantification of autofluorescing "arbuscules" was suggested as a valid method for the quantitative determination of root colonization by arbuscular mycorrhizal fungi. But the structures detected by epifluorescence microscopy are not the same as the structures detected after staining with one of the common staining techniques. Thus, epifluorescence microscopy to study arbuscular mycorrhizal fungal structures in roots is of limited use.

Several reasons may exist for the fact that autofluorescence detection has not been more widely used as a method for visualizing AM fungal structures (Dreyer and Morte 2009). One reason may be that the title of the article by Ames et al. (1982) was misleading as the authors described the autofluorescence as induced by ultraviolet light (Dreyer and Morte 2009). If some researchers followed these indications and excited their root samples with UV light, they would have surely not achieved any results, as the AM fungal structures do not autofluoresce under UV light excitation but under blue light excitation (Dreyer et al. 2006). Another reason may be that the literature contains some contradictions concerning the reasons for autofluorescence (Dreyer and Morte 2009). Some studies attribute autofluorescence to the dead state of the arbuscules (Vierheilig et al. 1999, 2001), while others show that all AM fungal structures autofluoresce regardless of their metabolic state (Dreyer et al. 2006; Dreyer and Morte 2009).

In contrast to previous reports, Dreyer et al. (2006) clearly demonstrate that all fungal structures, both intra- and extraradical, autofluoresced under blue light excitation, regardless of their state (dead or alive). Some arbuscules isolated from

roots and mature spores showed further autofluorescence under green light excitation (Dreyer et al. 2006; Dreyer and Morte 2009). The source of the autofluorescence was localized in the fungal cell wall (Dreyer et al. 2006; Dreyer and Morte 2009).

We know that the fluorochromes can be used to visualize fungal structures in living root. Specific fluorochromes administered to abraded leaves are transported via the phloem to the roots, where they move into the pericycle, endodermal, cortical, and epidermal cells (Oparka et al. 1994, 1995). These structures in the living roots can be visualized by confocal laser scanning microscopy. Vierheilig et al. (2001) reported that after using the fluorochromes 5(6)-carboxyfluorescein (CF) or 5(6)-carboxy-seminaphthorhodafluor to tobacco leaves, all arbuscular mycorrhizal fungal structures, starting from intraradical hyphae to arbuscules, were clearly observed. This provides us with a tool to study the in vivo dynamics of the establishment of the arbuscular mycorrhizal symbiosis in the root, starting from intraradical hyphal growth and culminating in arbuscule degeneration. Hyphae in root segments were also stained either by 0.01 % acid fuchsin-lactic acid or with the chitin-specific dyes WGA-AF 488 and WGA-TMR (Molecular Probes, Karlsruhe, Germany).

To study the interaction between *Piriformospora indica* and its host plant, Deshmukh et al. (2006) described the following method: roots colonized by *P. indica* material were fixed with the trichloroacetic acid fixation solution [0.15 % (w/v) trichloroacetic acid in 4:1 (v/v) ethanol/chloroform]. Subsequently, segments were incubated at room temperature for 10 min in 1 × PBS (pH 7.4) containing each respective dye at 10 μg/mL. During incubation, segments were vacuum infiltrated three times for 1 min at 25 mmHg. After rinsing with 1 × PBS (pH 7.4), segments were mounted on glass slides. In cases that Congo red (Merck, Darmstadt, Germany) was used for counterstaining, it was added to WGA-AF 488 staining solution at a final concentration of 10 μg/mL.

The confocal fluorescence images were recorded on a multichannel TCS SP2 confocal microscope (Leica, Bensheim, Germany). WGA-AF 488 was excited with a 488-nm laser line and detected at 505–540 nm. WGA-TMR was excited with a 543-nm laser line and detected at 560–630 nm. All segments that were analyzed with an Axioplan 2 microscope were either excited at 470/20 nm and detected at 505–530 nm for WGA-AF 488 or excited at 546/12 nm and detected at 590 nm for Congo red.

22.4.2 Quantification of Root Colonization

Giovannetti and Mosse (1980) used gridline intersection method and visual and slide method to quantify mycorrhizae in cleared roots. Morphometric procedures defined as measurement techniques used to determine the area or length of structures using a microscope provide much more accurate measurements of fungal structures in roots and have been used to measure the life span of active mycorrhizal

associations (Toth et al. 1990). McGonigle et al. (1990) developed a method which quantifies different mycorrhizal structures separately (a form of morphometrics). This allows the proportion of roots containing arbuscules, vesicles, and hyphae to be separately determined. Recently, morphometric analysis has been extended by the use of computerized image analysis to measure and quantify mycorrhizal structures in digital images (Smith and Dickson 1991).

22.5 Case Study

22.5.1 Description of the Symbiont

Piriformospora indica, the novel endophytic root-colonizing fungus of the xerophytic plants of Thar Desert, India, was isolated by Verma et al. (1998). *P. indica*, a basidiomycete, resembles in many aspects the arbuscular mycorrhizal fungi (AMF) which, however, belongs to the new family Sebacinaceae and new order Sebacinales, Glomeromycota, according to the sequence analysis of ribosomal DNA (rDNA) regions (Weiß et al. 2004; Qiang et al. 2011). In contrast to AMF, *P. indica* can grow axenically. Similar to AMF, this fungus promotes plant growth, increases the resistance of colonized plants against fungal pathogens and their tolerance to abiotic stress (Harman 2011; Sun et al. 2010), and is shown to be further beneficial to plants. It also alters the secondary metabolites of many plants of economic importance and promotes overall growth and seed production of many plants. In contrast to AMF, *P. indica* colonizes *Arabidopsis thaliana*, a model plant for which a multitude of well-characterized mutants is available.

The order Sebacinales, of Hymenomycetes (Basidiomycetes), encompasses fungi with longitudinally septate basidia and imperforate parenthesomes (Verma et al. 1998, 1999, 2013a, b; Selosse et al. 2007). The fungi of order Basidiomycetes lack cystidia and structures formed during cytokinesis on some basidiomycetous hyphae, the so-called clamp connections. Like other cultivable species of the Sebacinales, *P. indica* forms moniloid hyphae, which look like pearls in a chain. Based on this phenotype and rDNA sequence analyses, this endophyte is placed in the polyphyletic genus *Rhizoctonia* (Schüßler et al. 2001; Deshmukh et al. 2006; Selosse et al. 2007).

The *P. indica* genome is assembled into 1,884 scaffolds (size: 1 kb; N50: 51.83 kb) containing 2,359 contigs with an average read coverage of 22 and a genome size of 24.97 Mb. The estimated DNA content of *P. indica* nuclei ranges from 15.3 to 21.3 Mb. To assess the genome completeness of *P. indica*, a blast search was performed with highly conserved core genes present in higher eukaryotes (Zuccaro et al. 2011). A genetic transformation system has been established using a fragment of the TEF promoter region for construction of vectors carrying the selectable marker hygromycin B phosphotransferase. It is already shown that *P. indica* can be stably transformed by random genomic integration of foreign DNA

and that it possesses a relatively small genome as compared to other members of the Basidiomycota (Zuccaro et al. 2009).

The hyphae of *P. indica* are highly interwoven, often adhere together, and appear as a simple intertwined cord. Young mycelia are white and almost hyaline but inconspicuous zones are recorded in other cultures. Hyphae are thin walled and of a diameter from 0.7 to 3.5 μm. The aseptate hyphae often show anastomosis. New branches emerge irregularly and the hyphal wall shows some external deposits at regular intervals, perhaps polysaccharides and/or some hydrophobic proteins, which stain deeply with toluidine blue. Since septation is irregular, the single compartments can contain more than one nucleus (Verma et al. 1998).

The cell walls are very thin and show multilayered structures. The septa consist of dolipores within the continuous parenthesomes, which forms the basis for the systematic position within the Hymenomycetes. The dolipores are very prominent with a multilayered crosswall and a median swelling mainly consisting of electron-transparent material. The parenthesomes are always straight and have the same diameter as the corresponding dolipore. No kind of pores could be detected, meaning thereby that they are flat discs without any perforation. The parenthesomes consist of an electron-dense outer layer and a less dense inner layer, which shows an inconspicuous dark line in the median region (Varma et al. 2012a).

Mycelium on maturity produces characteristic pear-shaped chlamydospores, which appear single or in clusters measuring 16–45 μm in length and 10–17 μm in width, and they are distinctive due to their pear-shaped structure. The average spore and hyphal wall thickness is 0.7 and 0.3 μm, respectively. Very young spores have thin, hyaline walls. At maturity, these spores have walls up to 1.5-μm thick, which appear two layered, smooth, and pale yellow. The cytoplasm of the chlamydospores is densely packed with granular material and usually contains 8–25 nuclei. Neither clamp connections nor sexual structures are observed (Varma et al. 2001).

22.6 Cultivation of Symbiont

It is shown that the fungus can grow axenically on different synthetic media. Among the tested media, the best growth is reported to be on Hill and Käfer medium (2001) which is reported from different authors (Varma et al. 1999, 2012b, 2013a; Qiang et al. 2011) (Appendix). Circular agar discs (4-mm diameter) infested with chlamydospores and actively growing hyphae of *P. indica* are placed onto Petri dishes containing solidified Hill and Käfer medium. Incubation is carried out at 25 °C in the dark for 7–10 days. Broth jars are constantly shaken at 80 rpm. After 7–10 days, the Petri plate is completely filled up with biomass. In broth jars small and large colonies appear which consist of hyphae and chlamydospores (Fig. 22.3a–c).

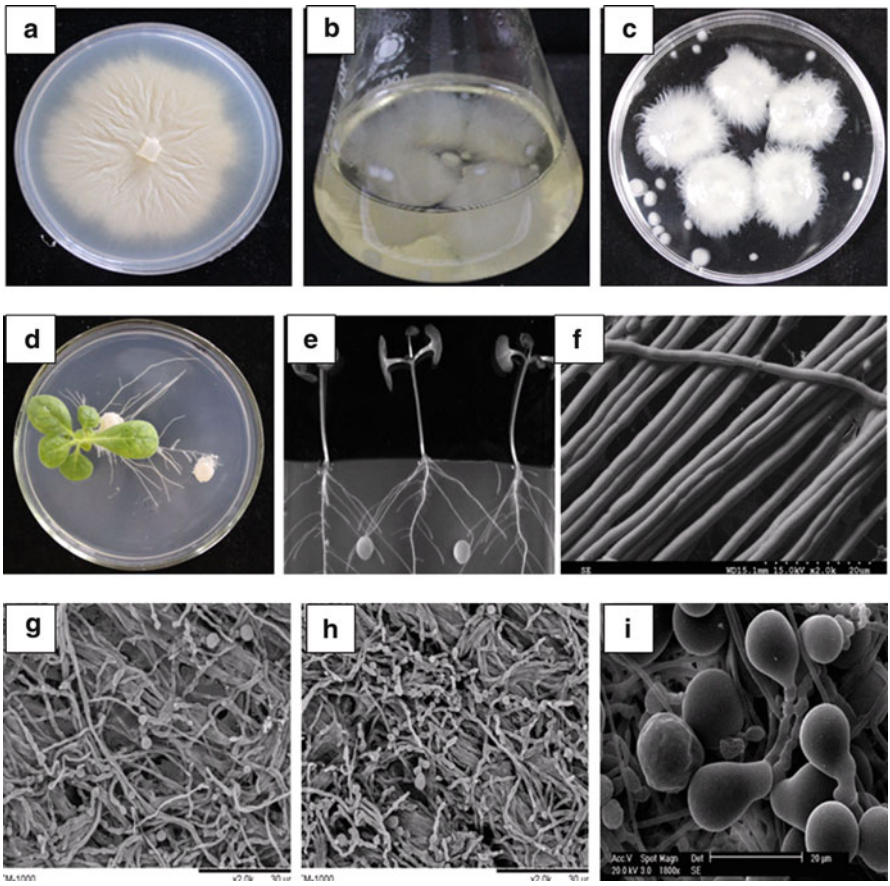

Fig. 22.3 (**a**) Circular agar disks (about 4 mm in diameter) infested with chlamydospores and actively growing hyphae of *P. indica* inoculated onto Petri dishes containing solidified (1.5 % agar agar) Hill & Kaefer medium, (**b**) growth on liquid medium, and colonies were transferred to Petri dish (**c**) for photography, note small and large colonies (Courtesy: Bing Peng, China), (**d**) Seedlings after 10 days co-cultivation with *P. indica* on MS medium, (**e**) symbiont plugs near the roots (Courtesy: Prof. Binggan Lou, China), (**f**) a view of the hyphae of *P. indica* taken by SEM (Model No TM 1000), for details of the preparation see Sect. 22.3.1.1 normally sample preparation method, (**g**) chlamydospore development after 5 days, (**h**) maturation and initiation of chlamydospores after 7 days and (**i**) clusters of mature chlamydospores after 15 days incubation, note typical pear shaped structures

22.7 Cocultivation and Interactive Studies

Cocultivation of fungus and seedlings is routinely done as described by Johnson et al. (2012). For cocultivation, a compromise MS medium was used (Murashige and Skoog 1962) which allows appropriate growth of both mycosymbiont and seedlings (Fig. 22.3d, e; Shahollari et al. 2005, 2007; Sherameti et al. 2005, 2008).

22.7.1 Surface Sterilization and Germination of Seeds

Seeds of test plants were soaked in sterile water overnight and surface sterilized with 4 % (v/v) NaOCl for 10 min. The seeds were further washed five times with sterile distilled water and rinsed with 70 % (v/v) ethanol for 30 s. This was followed by a quick treatment with 15 % (v/v) NaOCl; chemicals adhered were removed by repeated rinsing with sterile distilled water (Gamborg and Phillips 1996). Surface sterile seeds were pre-germinated either on moistened germinating paper or on water agar plates (0.8 %). The surface sterilized seeds were also germinated on half-strength MS medium supplemented with 3.0 % sucrose and solidified with 0.8 % agar. The plants were maintained at 25 ± 2 °C under 16-h photoperiod (Gamborg and Phillips 1996).

22.7.2 Root Colonization

The internal colonization of fungus in seedlings was monitored by taking periodically small root samples from seedlings cocultivated with fungus. Fine roots from treated and untreated plants were collected, rinsed well in distilled water, and cut into 1-cm pieces. Root segments were boiled with 10 % KOH to soften the root tissue. Then the tissues were neutralized with 2 % HCl for 3–4 min. Roots were stained with 0.5 % lactophenol blue solution (Conant et al. 1971). Slides were prepared from these samples, and bright field image was taken under phase contrast microscope at 40× magnification (Olympus CX41RF, model no. SN = 6K09628).

The percentage of root colonization for the inoculated plants was calculated using the formula as described.

22.7.3 *SEM of* Piriformospora indica

The young growing hyphae were straight and parallel to each other, and the surface was smooth (Fig. 22.3f).

Hyphae were undulated at maturity. No septation was seen under SEM (Fig. 22.3g). As the sporulation commenced, hyphae became more nodulated and highly branched (Fig. 22.3h). Mature chlamydospores were pear shaped (Fig. 22.3i), the junction of hypha and chlamydospores was broad.

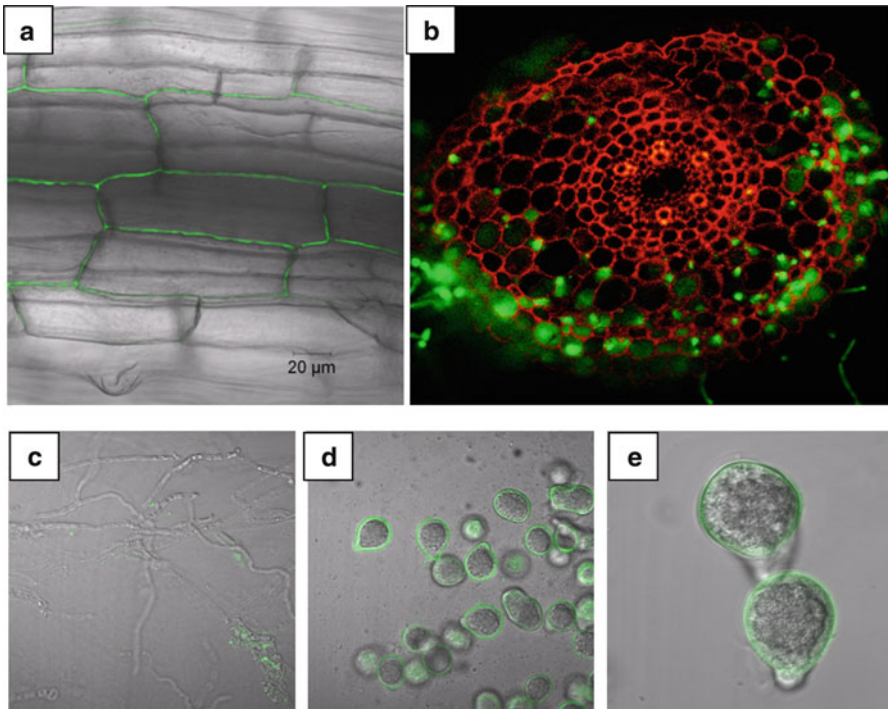

Fig. 22.4 (**a**) A view of uninoculated root viewed in Confocal microscope (Courtesy: Prof. Binggan Lou, China), (**b**) A cross section of the root as observed under confocal microscope. Note the fluorescence by fungus [Courtesy: Su et al. (2013), China], (**c**–**e**) the occurrence of fluorescence when observed under confocal microscope (Courtesy: Bing Peng, China)

22.7.4 Confocal View of Piriformospora indica

Figure 22.4a depicted root anatomy as observed under confocal microscope. Fungus colonizes the root surface and intercellularly into the cortex (Fig. 22.4b).

Mycelium showed feeble autofluorescence (Fig. 22.4c). The young spores showed fluorescence (Fig. 22.4d). At maturity, the wall was thick and emitted strong fluorescence (Fig. 22.4e). Normally qualitative and quantitative estimation of fungal root colonization takes 4–6 h following conventional staining technique. At times the cell wall does not take adequate stain, and it becomes very hard to observe the fungal hyphae and spores. Employing confocal microscopy is a matter of few minutes to observe fungal root colonization on the root surface and inter- and intracellular into the cortical regions with absolute certainty.

22.8 Conclusion and Perspective

Until few years ago, root system and fungal associations were studied by compound microscopes which are usually unable to reveal the true detailed structure and the association with root endophytes. With the development of sophisticated scanning electron microscope (SEM) and confocal microscope (CM), the structures are clearly magnified and the detailed structures were revealed. The movement of hyphae in the root system and later the production of chlamydospores can be characterized. Certain advanced software further strengthens and reveals the fine structure and precisely the mode of colonization. Confocal microscopy for the first time revealed the strong autofluorescent property of the wall of chlamydospores, whereas light fluorescence was recorded for fungal hyphae. Exact chemical nature and function(s) of autoflorescence invites future research. The ultrastructure of *P. williamsii* and strains of *Sebacina* warrant study for a comparative morphological investigation. Preliminary study revealed (data not presented) that morphologically they look alike; however, phylogenetically they are different (Varma et al. 2013a, b).

Future research on gene expression, microRNA, and the degradome sequencing to understand the plant–microbe interaction is warranted. In achieving this target, ultrastructure microscopes may play a key role.

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Appendix

Hill and Käfer medium (2001)

Composition	(g/L)
Glucose	20.0
Peptone	2.0
Yeast extract	1.0
Casein hydrolysate	1.0
Vitamin stock solution	1.0 mL
Macroelements from stock	50.0 mL
Microelements from stock	2.5 mL
Agar	0.8 % (w/v)
$CaCl_2$	0.1 M, 1.0 mL
$FeCl_3$	0.1 M, 1.0 mL
pH	5.8
Macroelements stock	(g/L)
$NaNO_3$	120.0
KCl	10.4
$MgSO_4 \cdot 7H_2O$	10.4

(continued)

Composition	(g/L)
KH_2PO_4	30.4
Minor elements stock	(g/L)
$ZnSO_4$	22.0
H_3BO_3	11.0
$MnCl_2{\cdot}4H_2O$	5.0
$CoCl_2{\cdot}6\ H_2O$	1.6
$CuSO_4{\cdot}5\ H_2O$	1.6
$(NH_4)_6Mo_7O_{24}{\cdot}7H_2O$	1.1
Na_2EDTA	50.0
Vitamins	% (w/v)
Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

The pH was adjusted to 5.8 with 1 N HCl. All stocks were stored at 4 °C except the vitamins which were stored at −20 °C

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Erratum to Chapter 8: How Ectomycorrhizae Structures Boost the Root System?

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