Nitrogen (N) and phosphorus (P) are major nutrients required for plant growth. Both nutrients can be supplied by fertilizers or manures. However, leguminous plants have the ability to fix N from the atmosphere through a symbiosis with host-specific gram-negative soil bacteria belonging to the family Rhizobiaceae, universally known as rhizobia (Killham, 1995). Symbiotic dinitrogen (N₂) fixation by legumes can account for as much as 97% of the total plant N (Peoples and Craswell, 1992). Such a contribution reduces the need for mineral N fertilizer and, even if the yield of the grain legume is not enhanced, any increase in N₂ fixation may lead to greater protein content of seed (Fabre and Planchon, 2000), or more N being available to subsequent nonfixing crops (Karpenstein-Machan and Stuelpnagel, 2000; Goss et al., 2002; Jensen and Hauggaard-Nielsen, 2003). Symbiotic N₂ fixation is thus considered an 'environmentally friendly' means of supplying N to crops.

The vast majority of all land plants form a symbiotic relationship with arbuscular mycorrhizal fungi (AMF), taxonomically grouped in the order Glomales (Bentivenga and Morton, 1994). Photosynthetic rates of mycorrhizal plants increase in compensation for the portion of net photosynthates allocated to the roots for fungal growth and maintenance (e.g., Jakobsen and Rosendahl, 1990). In return for carbon (C), the fungus provides the host with mineral nutrients, of which P is of considerable importance (Smith and Read, 1997). Working with 

\[ \text{Glomus fasciculatum} \text{ (Thaxter) Gerdemann and Trappe emend. Walker and Koske, Lambert and Weidensaul (1991) observed that the P content of soybean \text{ [Glycine max (L.) Merr.]} shoots was approximately 60\% greater in mycorrhizal plants than in nonmycorrhizal plants, even when high rates of phosphatic fertilizer (e.g., 150 mg kg}^{-1} \text{ soil}) were supplied. Results such as these highlight the significance of effectively managing AMF for an optimum efficiency of phosphatic fertilizer utilization and the potential for preventing environmental contamination.
Mycorrhizal colonization alters the quantity and composition of rhizosphere microorganisms (e.g., Secilia and Bagyaraj, 1987). Microbial interactions in the rhizosphere are complex and our understanding of their mechanisms of regulation is far from complete. Legumes can host AMF and N\textsubscript{2}-fixing bacteria at the same time. However, the two symbioses are rarely studied together because of the obligate biotrophy of arbuscular mycorrhizal fungi (Gianinazzi-Pearson et al., 1995). Although at first glance the symbioses of plants with rhizobia and AMF seem to have little in common as the phenotypic impacts are different, new phenomena result from the interaction between the three symbiotic partners. Asai (1944) was likely the first to study this interaction, reporting that several legume species failed to nodulate in autoclaved soil unless they were mycorrhizal. It was 30 yr later before this subject received more attention. Crush (1974) observed that the presence of mycorrhizas stimulated nodulation and growth of several legumes. Many others followed, reporting additional effects produced by the interaction on the plant: enhanced symbiotic N\textsubscript{2} fixation, greater number and dry weight of nodules, larger N content (e.g., Daft and El-Giahmi, 1974; Mosse et al., 1976; Carling et al., 1978; Smith et al., 1979; Kawai and Yamamoto, 1986; Barea et al., 1987; Luis and Lim, 1988; Vejsadová et al., 1992; Barea et al., 1992; Vejsadová et al., 1993). Since both nodules and arbuscular mycorrhizas are maintained by energy provided by the plant, it was initially discussed whether or not the three-way relationship was competitive or mutualistic. Mutualism was consistent with nodule number or size being increased consistently in the presence of AMF. Cluett and Boucher (1983) analyzed 20 data sets from published literature on the relationship, and concluded that mycorrhizal colonization generally has a positive effect on nodulation. Much less is known about the impact of rhizobial colonization on AMF activity. Bagyaraj et al. (1979) and Kucey and Paul (1982) found no significant change in AMF colonization after inoculation with rhizobia. In contrast, Pacovsky et al. (1986) found \textit{Bradyrhizobium japonicum} (Kirchner) Jordan effects on the development of AMF and, more recently, Xie et al. (1995) reported results that involved signalling between the symbionts. The two groups of microorganisms do not seem to compete for colonization sites (Barea and Azcon-Aguilar, 1983; Tobar et al., 1996), and only under conditions of stress, the presence of one microsymbiont was observed to significantly inhibit the development of the other (Bethlenfalvay et al., 1985). Thus, the idea of a tripartite symbiosis started to expand (e.g., El-Hassanin and Lynd, 1985), and many similarities have been discovered in the signal transduction pathways that lead to the successful establishment of both rhizobial and mycorrhizal partnerships with soybean and other legumes (e.g., Hirsch and Kaplanik, 1998; Albrecht et al., 1999; Guinel and Geil, 2002; Stracke et al., 2002; Vierheilig and Piché, 2002).

It has been assumed that the beneficial effect on N\textsubscript{2} fixation by AMF colonization is due to increased supply of P to the nodules by the symbiotic fungal partner and that high levels of available P can duplicate the effect of AMF inoculation (e.g., Carling et al., 1978; Asimi et al., 1980; George et al., 1995). Most studies have concentrated on indirect relationships between AMF and rhizobia but there is evidence that arbuscular mycorrhizas may directly and preferentially stimulate nodule function (Bayne and Bethlenfalvay, 1987). Ames and Bethlenfalvay (1987) suggest the existence of localized, nonsystemic, non-P-mediated influences of
AMF on root dry weight and nodule activity in cowpea \( \textit{Vigna unguiculata} \) (L.) Walp.]. This apparent evidence for the role of P has been clarified by the use of soil disturbance treatments, a simple but effective tool to study the tripartite symbiosis in real soil conditions with indigenous populations of AMF. As stated earlier, one of the difficulties in the study of the AMF symbiosis is the requirement for a host plant for these fungi to complete their life cycle. This fact limits the ability to produce a quantitatively differential colonization potential that would constitute a tool to test the contribution of the fungal symbiosis on the tripartite symbiosis. Miller et al. (1995) conducted the first series of studies with soil cores from tilled and no-tilled land and observed that soil disturbance reduced both mycorrhizal colonization and P absorption by maize \( \textit{Zea mays} \) L. and wheat \( \textit{Triticum aestivum} \) L. roots. Their study suggested that leaving the soil undisturbed prevented disruption of the extra-radical mycorrhizal hyphal network, which is likely the most effective source of inoculum for many abundant species present in agro-ecosystems such as \( \textit{Glomus} \) sp. (e.g., Oehl et al., 2003). Following this line of work, Goss and de Varennes (2002) hypothesized that more rapid and extensive colonization by AMF would result in earlier formation of nodules in soybean and that this would lead to greater N\(_2\) fixation by the soybean crop. Disturbed and undisturbed soil treatments were produced by repeatedly growing maize for 3-wk periods and sieving half of the experimental units assigned for the disturbance treatment at the end of each period to vary the rate of AMF colonization of soybean. When soybeans were planted in the disturbed or undisturbed soil, the potential for bradyrhizobial colonization was made identical in both treatments by planting pre-germinated seeds onto the same amount of peat-based inoculant. By comparing soybean plants at maturity they observed that the proportion of plant N derived from the atmosphere decreased from 32% in undisturbed soil to 12% in disturbed soil. While soil disturbance could enhance the mineralization of organic N, which can suppress N\(_2\) fixation, there was no significant difference between disturbance treatments in the amount of N removed from the soil. These data clearly established that soil disturbance can modify the tripartite symbiosis between indigenous AMF, \( \textit{B. japonicum} \) and soybean. Goss and de Varennes (2002) also emphasized that the effects of the tripartite symbiosis could not be solely a function of P uptake because, at the early stage of plant growth 10 d after emergence, the P content of the shoot was identical for both disturbance treatments whereas the number of nodules and percentage of root colonization by AMF were significantly greater in the undisturbed soil treatment. It was also taken into consideration that soil disturbance could affect the soil biota other than AMF and thus alter the activity of acid phosphatases but no difference was found between soil disturbance treatments for this trait.

Following the laboratory work of Goss and de Varennes (2002), the main hypothesis that needed to be tested was that P does not participate in the establishment of the tripartite symbiosis. Only by demonstrating this, can the alternative hypothesis (i.e., signalling processes between symbiotic partners regulate the early events leading to the tripartite symbiosis) be evaluated. Since there was no report in the literature on the functioning of the tripartite symbiosis with indigenous AMF, \( \textit{Bradyrhizobium} \) and soybean under field conditions, it was decided to establish a 3-yr field experiment which was initiated in 2000 and completed in 2002 to further investigate the transferability of previous laboratory findings to the...
field and the extent to which the early tripartite symbiosis is a function of P (Antunes, 2004). For that it was also necessary to assess the soil alkaline phosphatase activity as well as the P nutrition in all plant parts as early as 10 d after emergence, when the effects of the tripartite symbiosis on nodulation can already be detected.

The impact of the tripartite symbiosis was detectable in the field, an environment constrained by many factors. At 10 d after emergence, nodulation as well as AMF colonization were significantly greater in plants grown under undisturbed (no-till) soil than in the plants grown in soil disturbed by tillage (rotary tillage). However, the soil acid and alkaline phosphatase activity and P concentration in the shoots and roots of plants from both treatments did not differ, consistent with what had been found in the laboratory. Importantly, since the main hypothesis for a non-mediated P effect was accepted, the door was opened to test the alternative hypothesis (see Antunes, 2004).

Flavonoids are an ubiquitous group of plant secondary metabolites (Fig. 11–1). These molecules function as transcriptional signals from plants to microbial organisms. This was discovered in the last decade of the 20th century, opening a new area of study for understanding plant microbe interactions and rhizosphere ecology (Hopkins, 1995). A number of flavonoids are phytoalexins, substances which are either absent or present in extremely small concentrations (picomolar) in plants that are rapidly synthesized and released into the rhizosphere as a defense mechanism against bacterial and fungal pathogens (Recourt et al., 1992). Despite the antifungal, antimicrobial, and antioxidant properties of flavonoids that enhance

![Diagram of flavonoids](image)

**NOTE:** The numbering system for coumestan is different from that for isoflavones. The carbonyl function adjacent to the C-ring oxygen, which is equivalent to C-2 in the isoflavone system, is considered position-6 in the coumestan system (based on Bohm, 1998).
the survival of the plant (Dakora and Phillips, 1996), these phytochemicals are also recognized as early plant host signals secreted into the rhizosphere from seedlings or from seed coats during germination (Phillips, 2000). The establishment of the tripartite symbiosis likely requires coordinated gene regulation which is synchronized by mutual exchange of diffusible signal molecules that induce the transcription of genes involved in the colonization by the microbial symbionts. Specific flavonoids are necessary to stimulate nodulation and \( N_2 \) fixation (e.g., Zhang and Smith, 1995; Day et al., 2000). Some of these signal compounds have also been shown to stimulate mycorrhizal colonization directly (Siqueira et al., 1991; Tsai and Phillips, 1991; Bécard et al., 1992; Poulin et al., 1993; Xie et al., 1995). Furthermore, there has been some indication that flavonoid root accumulation is enhanced in response to AMF (Larose et al., 2002) and rhizobia (Schmidt et al., 1994). However, we found no reports that described the effects of individual flavonoids in the context of the tripartite symbiosis of \textit{Bradyrhizobium}, AMF, and soybean. In this chapter we focus on details of the communication between the three symbionts prior to establishment of the tripartite symbiosis. Based on evidence from the current literature and our recent findings a description for the mechanism regulating the tripartite symbiosis is proposed. We point to some gaps in the current understanding of the tripartite symbiosis and provide some insight into the possible use of flavonoids in agriculture.

THE RHIZOBIAL TRANSDUCTION PATHWAY

The symbiosis between leguminous plants and rhizobia results in the formation of the nodule, a new organ where bacteroids, as many as 20,000 per infected cell, have a suitable environment to convert \( N_2 \) into the plant-usable ammonia. As a rule of thumb, the heavier the nodule, the greater the rate of \( N_2 \) fixation. The process is catalyzed by the enzymatic complex nitrogenase, which requires as much as 25 to 30 mol of ATP per mol of \( N_2 \) fixed (see Marschner, 2002). It is therefore understandable why nodules provide a strong sink for P once they start functioning. Soybean nodule formation can be seen shortly after emergence but \( N_2 \) fixation does not begin until the second or third trifoliolate leaf is unrolled (approximately 3 to 4 wk after emergence under field conditions) (Ritchie et al., 1994). For the first 7 to 10 d, the nutrients and C are supplied by the cotyledons, which lose 70% of their weight. The loss of both cotyledons during emergence can lead to a yield reduction between 8 to 9%, which highlights the significance that early effects can have at later stages of growth.

The formation of nodules requires expression of bacterial nodulation (\textit{nod}) genes. The colonization process has been studied extensively and the \textit{nod} genes are fully identified and characterized (e.g., Hirsch, 1992; Göttfert, 1993; Fisher, 1994). They are usually categorized as “common” \textit{nod} genes, \textit{nodA}, B and C, “regulatory” \textit{nodD} genes and genes that determine host specificity. \textit{NodABC} are indispensable for nodulation and any mutation of their sequences produces a \textit{Nod\textsuperscript{-}} phenotype (Dudley et al., 1987). In many rhizobia, \textit{nod} genes are organized in several operons which are usually preceded by the \textit{nod box}, a promoter that contains highly conserved DNA regions (Schofield and Watson, 1986). The biochem-
ical functions of nod gene products are the synthesis and transport of the primary early signal molecule perceived by the host plant, the so-called Nod factor. These molecules consist of lipochitooligosaccharides (LCOs), in which a -1,4-linked N-acetylglucosamine (GlcNAc) backbone of three to five residues is modified at both the reducing and nonreducing ends in a species-specific-manner (Day et al., 2000). Although some progress has been made (Niebel et al., 1999), it is not known to which plant receptors a specific LCO binds. However, it is known that this chitin-related compound induces a cascade of responses prior to bacterial entry into the plant root, such as deformation and curling of root hairs and the division of cortical cells. An increase in the synthesis and exudation of flavonoids is among those responses (Schmidt et al, 1994; Xie et al., 1995). Empty nodule structures develop when roots are exposed to nanomolar concentrations of LCOs (Spaink, 1996). Nod factor also elicits the expression of several early nodulin (ENOD) genes, such as ENOD12 and ENOD40 (van Rhijn et al., 1997). Nodulins are plant-encoded proteins that are expressed during nodule development. Once the process has been switched on, some legumes seem to acquire all functions necessary for nodulation.

Rhizobia usually carry only one nodD gene, which follows nodABC in the DNA chain. However, many rhizobial species, as is the case for B. japonicum, have extra nodD genes. After it was discovered that nodD were the only nod genes expressed in the absence of the host, studies to determine the factors needed to trigger the expression of other nod genes followed. The protein product of nodD is a transcriptional activator that binds to the nod box, but it only induces the expression of nodABC genes in conjunction with a plant flavonoid. Flavonoids have also been shown to induce nodD genes (Banfalvi et al., 1988). Specificity plays an important role in host-range determination as each rhizobium is adapted to recognize the flavonoids (e.g., flavones, chalcones, isoflavones) exuded by its compatible host (Day et al., 2000). For example, luteolin is the principal flavonoid involved in nod gene expression in Sinorhizobium meliloti (Dangeard) De Lajudie et al., whereas genistein is required for B. japonicum (Graham, 1999).

Phytohormones, the synthesis of which is stimulated by intrinsic or external factors, have a powerful effect on plant development. The five different groups of major phytohormones have been involved in the process of nodulation (e.g., Stougaard, 2000; Ferguson and Mathesius, 2003). Auxin (indoleacetic acid, IAA) was shown to be involved in nodule formation through its stimulation of cell division and regulation of root differentiation (Dudits et al., 1993). Incubation with auxin transport inhibitors resulted in development of empty nodule-like structures (pseudonodules) on the roots of some legumes (e.g., alfalfa [Medicago sativa L.], sweetclover [Melilotus sp. P. Mill.]) and expression of LCO-responding genes ENOD12, ENOD40, and ENOD2 during early phases of nodulation (Fang and Hirsch, 1998). The LCO signal was reported to be responsible for inhibiting auxin transport during nodulation. Mathesius et al. (1998) observed that external application of LCO leads to inhibition of acropetal auxin transport in clover roots. Inhibition of auxin transport by flavonoids was also described by Jacobs and Rubery (1988). Moreover, Mathesius et al. (1998) found that specific flavonoids, such as formononetin [7-hydroxy-4′-methoxyisoflavone], accumulated in the vacuoles of the same cells that later underwent division for nodule commencement and which
also contained elevated levels of auxin. Therefore, it seems that regulation of the phytohormone auxin by accumulation of specific flavonoids is a further important mechanism in nodule development. Cytokinins are involved in the process of nodulation in addition to auxin. The cytokinins mimic the Nod factor effect when externally supplied to roots of alfalfa. ENOD12, ENOD40, and ENOD2 genes are all up-regulated by cytokinins (Fang and Hirsch, 1998).

In summary, N₂ fixation is a high energy process which begins 2 to 3 wk after the nodules are first established. Different types of flavonoids are exuded by roots of seedling legumes and are involved in chemotactic responses and the activation of nod genes of rhizobia. Flavonoids play a role in host-range determination. Phytohormones and LCOs are also involved in the rhizobial transduction pathway.

**SPECIFIC FLAVONOIDS AS INDUCERS OF B. JAPONICUM NOD GENES: PROSPECTS FOR THEIR USE IN AGRICULTURE**

Flavonoids are phenylpropanoid derivatives with a basic C₆-C₃-C₆ structure and the various groups of flavonoids are classified according to the substitution patterns of ring C (Fig. 11–1) (Bohm, 1998). Isoflavones have ring B in position 3 relative to the chromane ring C. The primary isoflavones in soybean seeds are genistein [5,7,4'-trihydroxyisoflavone] and daidzein [4',7-dihydroxyisoflavone]. There are also small amounts of a third isoflavone, glycitein [7,4'-dihydroxy-6-methoxyisoflavone] (Messina, 2002). Formononetin, the 7-methyl ether of daidzein, and coumestrol [2-(2,4-Dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid-_lactone], a coumestan, have also been found in soybean plants and, as described later, are worth of being evaluated within the context of the mycorrhizal and rhizobial symbioses (see Antunes, 2004).

Flavonoid compounds from roots of legume plants have the potential to either induce or repress nodulation (Firmin et al., 1986). Djordjevic et al. (1987) hypothesized that regulation of the synthesis of inducing and inhibiting flavonoids could be a mechanism by which legumes prevent overnodulation. Genistein, daidzein, and coumestrol, which were found to be exuded from soybean seedlings into the rhizosphere, act as stimulatory signal molecules that induce the transcription of nodulation genes in B. japonicum (e.g., Kossokl et al., 1987; Banfalvi et al., 1988; Peters and Verma, 1990; Rao and Cooper, 1995). These compounds were found to act as plant factors when a reporter gene, lacZ, was used; the transcription of this gene can be detected by assaying for _-galactosidase activity. In this procedure, a gene responsible for _-galactosidase synthesis, lacZ, was taken from Escherichia coli (Migula) Castellani and Chalmers bacteria and inserted into a nodC gene of Rhizobium meliloti Dangeard located in plasmid pRmSL26. As those flavonoids function in conjunction with the product of nodD genes to induce nodABC (in this case nodABC-lacZ) transcription, an increase in _-galactosidase activity would indicate a cause-and-effect relation. As stated earlier, B. japonicum has two nodD copies, nodD1 and nodD2 (Göttfert et al., 1992). NodD1 protein up-regulates nod gene expression only in the presence of key flavonoids (e.g., Mergera et al., 1997). The NodD1 gene itself is also inducible by these (Banfalvi et
NodD2 appears to down-regulate expression of nod genes. Although NodD1 alone appears to be sufficient to allow soybean nodulation, *B. japonicum* has also a secondary independent system for flavonoid recognition and therefore colonization establishment which involves *nodVW* genes (Day et al., 2000).

Aside from acting as inducers of nodulation genes, flavonoids are also chemoattractants and growth stimulants for rhizobia (Phillips, 1992). After this was recognized, their significance as promoters of nodulation began to be investigated. It is now established that nodulation can be limited by availability of these nod gene inducers. Kosslak et al. (1987) showed a concentration dependence up to 5 µM for daidzein and genistein to stimulate *B. japonicum* nodABC-lacZ fusions. Coumestrol has also been shown to promote the growth of *B. japonicum* (d’Arcy Lameta, 1987). Sutherland et al. (1990) observed that genistein and daidzein had their greatest inducing ability at concentrations of about 10 µM. Zhang and Smith (1996) monitored genistein accumulation in soybean roots under different temperatures and found that cool root zone temperatures reduced genistein accumulation and nodulation; supplementing with genistein under cool conditions was beneficial. Parmar and Hume (unpublished data, 1997) showed that inoculating soybean seeds with *B. japonicum*, strain 532C, and treating these seeds with different concentrations of genistein (from 5–20 _µM_ ) significantly increased nodule numbers and shoot and root biomass. Zhang and Smith (1995) also reported that addition of genistein increased soybean nodulation and that it resulted in a shorter period between inoculation and root hair curling, leading to the earlier onset of _N_2 fixation.

One can therefore conclude that adding exogenous genistein and likely other flavonoids such as daidzein and coumestrol to commercial soybean inoculants may have the potential to increase further the benefits from bradyrhizobial symbiosis. The mechanisms behind these processes are however not fully understood and even more so when this is linked to the study of mycorrhizal fungi. Although, as will be described below, AMF do not induce typical defense responses of the host plants, there is some evidence that AMF may stimulate the exudation of flavonoids by the root at an early stage. Taking this into consideration, and given the fact that commercial isoflavones are expensive and their fates in soil uncertain, effective management of AMF might be used as a means of reducing the need to use commercial flavonoids for the enhancement of _N_2 fixation. In support of this statement, and before outlining the processes involved in the tripartite symbiosis, current literature linking mycorrhizal fungi and flavonoids is now reviewed.

**ARBUSCULAR MYCORRHIZAL FUNGI COLONIZATION, AND FLAVONOIDS**

As in the symbiosis with rhizobia, colonization by AMF involves a number of phases (Gianinazzi-Pearson and Gianinazzi, 1989) in which exchange of signals between the symbionts must take place (Peterson and Farquhar, 1994). Starting with the germination of AMF spores, hyphal growth through the soil and host recognition (appressorium formation), all take place before root colonization is achieved, and each step may be influenced by the plant (Douds and Nagahashi, 2000). Spore germination occurs when environmental soil conditions such as ma-
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...tric potential, temperature, and carbon dioxide (CO₂) levels are favorable. Therefore, Douds and Schenck (1991) suggested that root exudates are unlikely to affect this developmental stage. However, the germ tube growth ceased, before consumption of the spore reserves if a host root was not present (Bécard and Piché, 1989) and germination of AMF spores was enhanced in vitro by root exudates and flavonoids (Gianinazzi-Pearson et al., 1989; Tsai and Phillips, 1991). It is certain that root exudates stimulate the growth and morphology of AMF hyphae in the soil prior to colonization (e.g., Pinior et al., 1999). Hyphae branch profusely in the rhizosphere allowing the fungus to colonize the plant more efficiently. Various experiments have shown that the hyphal branching pattern is influenced by exposure to different concentrations of root exudates (e.g., Nagahashi et al., 1996; Buée et al., 1998), suggesting that some signal molecules, which are synthesized and secreted by roots, are likely involved. Several authors have suggested that specific compounds contained in the root exudates, namely flavonoids, play an important role in the hyphal growth of different AMF genera (e.g., Elias and Safir, 1987; Mosse, 1988; Bécard and Piché, 1990; Chabot et al., 1992; Fries et al., 1997; Vierheilig et al., 1998). Bel-Rhlid et al. (1993) isolated several flavonols and flavones from roots of carrot [Daucus carota L.] and proposed that flavonoids are fundamental regulating factors during the early events of AMF symbiosis establishment. Giovannetti et al. (1996) observed that the hyphal branching response was triggered by indeterminate compounds with a molecular mass below 500 Da. Flavonoids have a molecular mass lower than this, making them strong candidates for being a branching signal. In contrast, Bécard et al. (1995) suggest that flavonoids are not absolutely required as stimulatory compounds for AMF. Moreover, Buée et al. (2000) isolated a root extract of unknown chemical structure, which they called ‘branching factor’ for mimicking the presence of root on the fungal response. Using chalcone-synthase deficient mutants of maize, it was determined that the ‘branching factor’ was unlikely a product of the flavonoid pathway. The last step prior to colonization, the formation of an appressorium, seems to be a contact recognition between the plant and the fungus, and does not require a chemical signal (Nagahashi and Douds, 1997). However, not only can the AMF penetrate the plant through root hairs without forming an appressorium, but also, there might be regulatory mechanisms associated with plant hormones at this point (see Guinel and Geil, 2002).

The morphological aspects of AMF colonization are well documented but relatively little is known at the molecular level (see review by Hirsch and Kapulnick, 1998). The stimulatory effect of AMF on the production and release of flavonoids is little understood. The biochemical compounds likely to serve as elicitors that stimulate transcription for enzymes involved in the synthesis of flavonoids are small polysaccharides, glycoproteins, and proteins (e.g., chitin-related compounds) of fungal or bacterial origin (Ebel and Greisebach, 1988). Although plant colonization by AMF typically occurs without strong induction of plant responses (Gianinazzi-Pearson, 1996; Mohr et al., 1998), a build-up of phenylpropanoids has been observed at early stages in the development of the symbiosis (e.g., Harrison and Dixon, 1993; Volpin et al., 1994). Larose et al. (2002) reported that the application of AMF tissue to uncolonized roots of alfalfa affected the synthesis of different flavonoids. More recently, Zakhia et al. (2003) detected in vitro
accumulation of flavonoids in localized areas of root tissue of *Medicago laciniata* (L.) P. Mill where inocula of *Glomus* sp. had been applied. This suggests that some defense mechanisms of the plant may initially be involved in the AMF symbiosis. Salzer and Boller (2000) proposed that chitin-related elicitors are released from the fungal cell walls into the rhizosphere where they are enzymatically inactivated by extracellular constitutive chitinases released by the host plant. However, at an early stage of mycorrhizal formation, plant constitutive chitinases may only partially cleave the fungal elicitors and the plant defense response is induced. The remaining AMF chitin elicitors bind to a yet unknown receptor in the plasmalemma of the root cortical cell and the expression of proteins related to pathogenicity results. Albrecht et al. (1998) proposed the chitin elicitor to be called ‘Myc factor’, equivalent to the rhizobial Nod factor. Vierheilig and Piché (2002) emphasize that there is no direct evidence for the presence of a Myc factor so far. Nevertheless, it is reasonable to hypothesize that flavonoid compounds are released into the rhizosphere as a response to Myc factors in a mechanism of positive feedback for AMF.

The involvement of plant hormones in the transduction pathway used for arbuscular mycorrhizal colonization is also a possibility as these fungi have been shown to have the capacity to synthesize plant hormones (Gogala, 1991). If auxin is synthesized by the fungus, then a general down-regulation of enzymes related to chitin breakdown could occur, leading to the synthesis of plant flavonoids.

It therefore appears that mycorrhizal chemotaxis, growth, and spore germination benefit from the release of some flavonoids onto the rhizosphere and that release may be influenced by the presence of the microsymbiont prior to root colonization. The question of the existence of specificity between individual flavonoids and their effect on AMF remains open, but we will return to this later.

**COMMONALITIES IN ARBUSCULAR MYCORRHIZAL FUNGI AND RHIZOBIAL TRANSDUCTION PATHWAYS**

The symbiosis between AMF and plants is considerably more ancient than the rhizobial symbiosis with legumes. The oldest fossil records of AMF date from the Silurian period, more than 400 million years ago (MYA) (Smith and Read, 1997). In the case of rhizobia, analyses of evolutionary changes in highly conserved bacterial genes suggest that they evolved 500 MYA but it is not certain when nodulation capacity was acquired. However, legumes only appeared between 125 and 171 MYA (Hirsch et al., 2001). One can therefore argue that the evolution of rhizobia towards becoming a symbiont with legumes benefited from taking place in an environment where appropriate pathways to overcome plant defense responses had already been “opened” by mycorrhizal fungi. Given the similarities between the two symbioses, LaRue and Weeden (1994) proposed that the process of nodulation evolved from mechanisms associated with the AMF symbiosis. Usually, but with some exceptions, as in the case of soybean, Nod− legumes are also Myc−, implying that in some legume species the same genes are critical for both symbioses to develop. It could also be argued that the genes involved in each symbiosis underwent converging mutation. However, in support of
the first possibility van Rhijn et al. (1997) showed that early nodulin genes such as ENOD2 and ENOD40 are also expressed in alfalfa colonized by mycorrhizal fungi. As described earlier, these genes can be upregulated by cytokinins. Since the levels of cytokinins are elevated during nodulation and in mycorrhizal roots, this hormone could be one component of the signal transduction pathway that mediates induction of these genes during the formation of each symbiosis (Harrison, 1999). Also, when mycorrhizal colonization of legumes is well established, genes similar to those encoding for nodulins (e.g., leghemoglobin) are expressed (Frühling et al., 1997).

Another point of similarity between the two symbioses is related to LCOs. Nod factors are related to a type of molecule more common in fungal cell walls than in gram-negative bacteria. Xie et al. (1995) observed that rhizobial nod factors stimulate mycorrhizal colonization of nodulating and non-nodulating legumes. However, it was only those Nod factors which had an effect on flavonoid secretion that produced such a response in AMF, suggesting that communication between the two microbial symbionts prior to colonization is mediated through the plant defense mechanisms. As indicated by legume mutants that are Myc− and Nod−, the products of the expression of common genes seem to encode proteins that perceive both Nod and Myc factors. Since nonleguminous plants and the majority of legume mutants are Myc+ and Nod−/H11002, it is tempting to speculate that legumes have protein receptors for Nod factors that can also perceive Myc factors; and that any mutation capable of affecting these receptors will more easily impair the perception of Nod factors than of Myc factors. A recent study by Kosuta et al. (2003) showed that the Nod factor-inducible gene MtENOD11 is expressed differently in roots of Medicago truncatula Gaertner depending on the presence of Myc or Nod factors suggesting that these compounds although sharing chemical similarities are not identical. In three Myc−/Nod− mutants, MtENOD11 was only induced in presence of AMF and was blocked in response to Nod factor (Kosuta et al., 2003). Investigation of whether chitinaceous fragments from cell walls of different AMF act as signals for plant recognition and stimulation of the symbioses would clarify some aspects involved with the establishment of the tripartite interaction.

Chitinase isoforms appear to have a function in mutualistic symbioses. Their activity is at a low level in plants but is induced in response to a wide variety of stimuli from fungi, bacteria, virus, etc. (Salzer et al., 2000). Roots infected by AMF show a transient stimulation of chitinase activity followed by suppression (e.g., Spanu et al., 1989; Volpin et al., 1994; Dumas-Gaudot et al., 1996). Lambais and Mehdy (1996) suggest that variation in suppression of defense-related gene expression, especially chitinases, is one factor contributing to different colonization levels of plant roots by AMF. In the context of rhizobial symbiosis, chitinases released by the plant into the rhizosphere have been shown to rapidly hydrolyze the Nod factors by cleaving the LCO backbone (e.g., Parniske et al., 1994; Stachelin et al., 1995; Ovtysna et al., 2000). Salzer et al. (2000) monitored the expression of eight chitinase genes in Medicago truncatula during mycorrhiza formation, nodulation, and pathogen infection. They observed that the same genes were induced in nodulation and pathogen infection but the pattern of chitinase gene expression in mycorrhizal roots was markedly different. Although the role of chiti-
nases in mutualistic symbioses is not yet clear, the fact that AMF can alter the pattern of their expression may cause a reduction in the ability of the plant to control nodulation when AMF are present.

Within the complex processes implicated in the interaction between rhizobia and mycorrhizal fungi, the response to flavonoids seems to play an important part. Xie et al. (1995) observed that various plant flavonoids increased mycorrhizal colonization and postulated that AMF and rhizobia may have evolved functionally similar recognition systems for those signal compounds. This response appears to be obligatory in the case of the rhizobial symbiosis and, although it may not be essential for the AMF symbiosis, a number of flavonoids have the potential to stimulate hyphal growth and branching.

As stated above, one other point where the transduction pathways may converge involves the hormone auxin. If auxin is synthesized and exuded by AMF then the synthesis of flavonoids as well as the enhanced cell division and root differentiation during nodulation can occur. The possibility of a localized AMF-induced auxin influence on flavonoid synthesis by the plant cannot be excluded and that can also have consequences for the rhizobial symbiosis. However, it seems likely that this mechanism is more critical at a later stage of the tripartite symbiosis, when the auxin precursors (e.g., tryptophan) are supplied by the plant (see review by Salzer and Boller, 2000).

A WORKING MODEL FOR THE ESTABLISHMENT OF THE TRIPARTITE SYMBIOSIS

Based on the current knowledge regarding the initial colonization steps involved in mycorrhizal and rhizobial symbioses, a working description of the establishment of the tripartite symbiosis is provided (Fig. 11–2). The AMF hyphae branch as they approach the root, being chemically attracted by C compounds. Flavonoids already present in the rhizosphere can be an additional stimulus in the process. During hyphal progression through the soil, chitinaceous fragments are released from the cell walls into the rhizosphere. Some of these fragments are cleaved by plant constitutive chitinases and the remaining fragments serve as elicitors (Myc factor), binding to protein receptors in the root. The binding triggers a transient defense response with synthesis and/or release of flavonoids into the rhizosphere. These not only have the potential to generate a positive feedback on mycorrhizal hyphae growth and branching but also to further activate transcription of nod genes with the necessary synthesis of Nod factor (LCO) by the rhizobial bacteria. When AMF Myc factors are present, the rhizobial Nod factor has less chance to be cleaved by constitutive plant chitinases and nodulation is enhanced. Nod and Myc factors bind to receptors in the plasmalemma of epidermal cells of the root. The more occupied the binding sites are because of the presence of both colonization factors, the greater the plant response in terms of synthesis and/or release of flavonoids to the rhizosphere. The process likely occurs over a short period of time because the interaction can be readily observed as early as 10 d after plant emergence. The key for the magnitude of beneficial effects observed on the plant due to the tripartite symbiosis is likely in the initial levels of AMF and rhizobia
Fig. 11–2. A working model for the establishment of the tripartite symbiosis. 1. Flavonoids and chitinases already present in the rhizosphere may be involved in the transduction pathways used by rhizobia and AMF. 2. Chitinaceous fragments are released into the rhizosphere from the cell walls of rhizobia (Nod factors), and from mycorrhizal hyphae (Myc factors) as they progress through the soil. 3. Some of these microbial factors are cleaved by constitutive plant chitinases while others bind to protein receptors in the root. 4. The binding triggers a transient defence response with synthesis and/or release of flavonoids into the rhizosphere. 5. These flavonoids, not only have the potential to generate a positive feedback on mycorrhizal hyphae growth and branching but also act to stimulate further the transcription of nod genes.

present within the rhizosphere. Moreover, AMF show a considerable variation in colonizing strategies; such variations seem to be taxonomically based at the family level (Hart and Reader, 2002). Therefore it is important to emphasize that the types of AMF families present in soil may induce different plant responses, not only depending on eventual chemical specificities of the Myc factor involved but also on the life strategy associated with each AM fungal family. Larose et al. (2002) observed variable accumulations of flavonoids in alfalfa colonized by different AMF.

Once the symbioses are established, the extent to which the root is colonized is regulated by plant control mechanisms. Shifts in chitinase activity in the case of
AMF (Salzer et al., 2000) and root exudation patterns for both mycorrhizal (Pinior et al., 1999; Guenoune et al., 2001) and rhizobial (Caetano-Anollés and Gresshoff, 1991) symbioses have been reported. The enhancement of the tripartite symbiosis due to the interaction between the two microsymbionts is likely to continue at this stage mainly through plant-mediated systemic mechanisms. Increased P supply by the fungus is then likely to be the main factor regulating the tripartite symbiosis. Other factors may involve hormones as in the case of auxin and cytokinins described earlier. Vierheilig and Piché (2002) indicated that inoculation of one-half of a split root system with rhizobia strongly reduced nodulation on the other half of the root system. Vierheilig et al. (2000), also working with split root systems, verified that colonization of one side of the root system with Glomus mosseae (Nicolson and Gerdemann) Gerdemann and Trappe was suppressed when various AMF were already colonizing the other half. It would be of interest to inoculate half of a split root with rhizobia and the other half with AMF at different phases of colonization by both symbionts and measure hormone and flavonoid levels in various parts of the split-root system over time.

TRIPARTITE SYMBIOSIS OF ARBUSCULAR MYCORRHIZAL FUNGI, BRADYRHIZOBIUM, AND SOYBEAN

Based on the current understanding of the tripartite symbiosis, it can be postulated that the greater the mycorrhizal potential in soils, as in the case of an undisturbed environment with an intact hyphal network, the greater the number of contact points between developing plant roots and active fungal hyphae there will be at an early phase. As described previously, the timing and extent of AMF colonization affect nodule growth, N2 fixation, and nutrient uptake by soybean. Information is needed to establish the extent to which flavonoids are involved in affecting the regulation of the tripartite symbiosis at a macroscopic scale, taking account of all flavonoids present in soybean. As Harrison and Dixon (1993) emphasize, it is difficult to extrapolate from the observed effect of a single compound in vitro to the symbiotic relationship involving a whole living root, where complex combinations of metabolites may have synergistic or antagonistic effects. Many of the experimental observations documented earlier provided a better understanding of the details involved in the system but were produced in highly artificial conditions that are not necessarily comparable to the complex situations found within the rhizosphere of a real soil–plant environment. Carrying out studies in which the experimental conditions consider the whole plant–soil system both in space and in time is the only way to clarify the importance and precise role of flavonoids in the mycorrhizal–rhizobial interaction. Two fundamental hypotheses clearly need to be tested: first, that flavonoids participate in the establishment of the tripartite symbiosis between soybean, Bradyrhizobium, and AMF; second, that changes in flavonoid accumulation resulting from the development of one microsymbiont can stimulate colonization of soybean roots by the other. For that, soil disturbance treatments to vary the rate of AMF colonization of soybean varieties known to have different concentrations and arrays of the isoflavones genistein, daidzein, and glycitein in the seed can be used (see Antunes, 2004). It is unlikely that isoflavones
from seeds of different soybean cultivars are unique or dissimilar from one another to the extent that they control cultivar specificity to distinct rhizobia strains. However, different cultivars present to the bacteria quantitatively diverse signalling environments which consequently produce different expressions of \textit{nod} genes (Pueppke et al., 1998). Chabot et al. (1992) showed that flavonoids can be both stimulatory and inhibitory to \textit{Gigaspora margarita}. Genistein acted as an inhibitor of hyphal growth with \textit{Gigaspora margarita}. Morandi et al. (1984) reported that daidzein and coumestrol accumulated in mycorrhizal soybean roots. From the commercial flavonoids used by Xie et al. (1995) to study their effect on mycorrhizal colonization in soybean, genistein was the only one that did not produce a significant stimulatory outcome, whereas daidzein had the greatest effect on the percentage of root length colonized by \textit{Glomus mosseae} when compared with a control which had no flavonoids applied. Flavones such as the three main aglycones in soybean and coumestrol have been shown to possess estrogenic activity and are also called phytoestrogens (Gilani and Anderson, 2002). However, they are less active than estrogens and different flavones bind to estrogen receptors to various extents. Genistein is considerably more estrogenic than daidzein, probably because of the influence of the additional hydroxyl group in position 5 of the A ring (Fig. 11–1). Hydroxyl groups are important for binding affinity to estrogen receptors (Ganora, 2003). Interestingly, when estrogen 17\textsubscript{-} estradiol was applied to the AM fungus \textit{Glomus intraradices} (Schenk and Smith), it stimulated hyphal growth, although to a smaller extent than did the isoflavone biochanin A [5,7-dihydroxy-4\textsuperscript{\prime}-methoxyisoflavone] (Poulin et al., 1997). Vierheilig et al. (1998) suggested the existence of variation in binding sites for flavonoids in different AMF. It is tempting to hypothesize that the presence of an extra hydroxyl group may not influence the stimulation of AMF hyphal branching, and that there may be more binding sites for daidzein and coumestrol than for genistein in AMF.

Signalling specificity may be an issue when it comes to tripartite symbioses. Based on the existent literature and the proposed hypotheses, the part of the mechanism for its establishment is viewed as schematized in Fig. 11–3. In it, specific flavones exuded by the root in response to the presence of the micro-symbionts have different targets. Genistein acts only on rhizobia, whereas daidzein and coumestrol play a role in the establishment of both microbes. Very little is known about certain signal compounds (e.g., glycitein) and some are still a matter of debate. Formononetin, for example, accumulates in the vacuoles of the nodule pro-genitor cells; which also contain high levels of auxin (Mathesius et al., 1998). Auxin was shown to have an effect on nodule formation through stimulation of cell division and regulation of root differentiation (Dudits et al., 1993). Formononetin levels were increased in P-treated alfalfa inoculated with \textit{Glomus intraradices} (Schenk and Smith), even in the absence of any root colonization (Volpin et al., 1994). In contrast, when the plants were inoculated with the same fungal species, but under low P conditions, formononetin was reduced (Larose et al., 2002). This isoflavone, which has also been shown to be present in soybean plants (e.g., Landini et al., 2003), appears to act as a plant mediator in the control of AMF colonization. Larose et al. (2002) reported that when the root colonization by AMF was well established many arbuscules collapsed and the root concentration of formononetin increased. Daidzein is a precursor of formononetin and the
Fig. 11–3. (Legend on facing page).
levels of both signal compounds were shown to be inversely proportional. Therefore, it was suggested that such a mechanism might be associated with an autoregulation of the fungal colonization.

CONCLUSIONS

Interactions among participants in the tripartite symbiosis have a significant impact on N$_2$ fixation (e.g., Goss and de Varennes, 2002; Antunes, 2004). Such impact is likely to depend significantly on the timing of early colonization events by both symbiotic microbes due to their use of common transduction pathways. The fact that the rhizobial symbiosis is host-specific implies that signal specificities must be taken into consideration when studying the tripartite symbiosis in different legumes. This detail is of particular importance if the use of chemical signals in agriculture is considered. As an example, genistein, daidzein, and coumestrol are involved in the establishment of *B. japonicum* but only daidzein and coumestrol seem to have an effect on AMF. Daidzein and coumestrol are therefore excellent candidates to be used in studies regarding the tripartite symbiosis not only in soybean but also in other legumes and nonlegumes. Daidzein and coumestrol were found to accumulate in roots of alfalfa when mycorrhizal colonization was halted at the appressorium formation stage as well as when mycorrhizas were fully established (Harrison and Dixon, 1993). Interestingly, nonmycorrhizal plants, such as the ones belonging to the Brassicaceae family (Harley and Harley, 1987), seem to sense the presence of AMF by altering the pattern of chitinase activity (Vierheilig et al., 1994). Since these plants appear to have the receptor for Myc factor but do not have the capacity to synthesize all necessary compounds for mycorrhization, it is tempting to hypothesize that adding specific flavonoids may induce mycorrhizal hyphae branching, a requirement for root colonization.

Mycorrhizal plants, likely due to modifications of the exudation of certain compounds (e.g., jasmonates) by the root to regulate the symbiosis, are more resistant to pathogenic microorganisms (e.g., Norman and Hooker, 2000; Talavera et al., 2001; Vierheilig and Piché, 2002). The same is true for nodulating legumes (e.g., Tu, 1978; Chakraborty and Chakraborty, 1989). No data exist to show whether the tripartite symbiosis affects other soil organisms. This is likely due to the difficulty in working with AMF because of the obligatory nature of their sym-

Fig. 11–3. Schematic diagram of the establishment of the tripartite symbiosis between AM fungi-*Bradyrhizobium japonicum* and soybean plants. 1. Genistein, daidzein, formononetin, glycitein, and coumestrol together with constitutive chitinases are exuded into the rhizosphere. Genistein, daidzein, and coumestrol will induce bradyrhizobial nodD and, in conjunction with its protein products, they activate nodABC. Only daidzein and coumestrol promote the AMF branching necessary for colonization. Glycitein and formononetin are still a matter of debate. 2. Nod and Myc factors are released into the rhizosphere. 3. Those Nod and Myc factors that are not hydrolyzed by constitutive chitinases bind to receptors on the cell membrane of the root epidermal cells. 4. The synergistic effect due to the presence of chitin elicitors from both microsymbionts triggers an enhanced plant response with synthesis and/or exudation of more flavonoids. 5. Daidzein and coumestrol stimulate AMF colonization and are also involved in the bradyrhizobial transduction pathway although to a lesser extent than genistein.
biosis with plants. It has been shown that flavonoids accumulate in cells undergoing development into a root gall induced by parasitic nematodes (Hutangura et al., 1999). Therefore, in future endeavors, it would be interesting to determine possible effects of tripartite symbiosis on pests such as the soybean cyst nematode \textit{Heterodera glycines} (Ichinohe).

The healthy properties ascribed to soybean foods are due to their high phytoestrogen content (Messina, 2002). Gilani and Anderson (2002) have published extensive reviews on the benefits and disadvantages of using phytoestrogenic compounds such as coumestrol and daidzein in human health. The industry has interest in soybean seeds with different concentrations and compositions of flavonoids. Soymilk for babies should be low in flavonoid concentration contrary to what is desirable for soymilk produced for adults. It would be interesting to investigate whether ‘Myc’ and Nod factors have an effect on either the flavonoid content of the sprouts or the seed at maturity in distinct soybean lines.

The initiation and functioning of the tripartite symbiosis is complex and despite the progress achieved in the last decade there are still many unknowns. It is essential to consider such complexity by conducting research that takes an holistic approach to assessing the effects of the tripartite symbiosis on plant health and nutrition. “Darwin’s revolution of biology could not have occurred without the unrestrained view he had of the organisms around him” - Hirsch et al. (2001).

**ACKNOWLEDGMENTS**

We thank the Foundation for Science and Technology of the Portuguese Ministry of Science for the grant that supports P. Antunes.

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