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RHIZOBIAL COMPETITION AND ENHANCING RHIZOBIAL COLONIZATION IN THE LEGUME RHIZOSPHERE USING A SYSTEMIC FUNGICIDE

Review article

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Abstract

The inoculation of legumes with effective rhizobia or bradyrhizobia represents an inexpensive alternative to the use of chemical nitrogen fertilizers, whose prices have risen due to the high cost of energy involved in their production. These fertilizers are also pollution hazards. The process of symbiotic biological nitrogen fixation requires that the host crop be adequately nodulated by the specific root-nodule bacteria effective in nitrogen fixation. Not all the strains of *Rhizobium* or *Bradyrhizobium* that can produce nodules on a given host are able to use N_2 rapidly and efficiently. Nonetheless, selection of an effective (i.e. N2-fixing) strain is a prerequisite for any crop to be inoculated. A second important characteristic is the competitiveness of the strain. Unfortunately, effectiveness and competitiveness are generally mutually exclusive and are not dependent upon each other. Little information exists on the effects of systemic fungicides on symbiotic nitrogen fixation or nodulation. It has been reported that the systemic fungicide benomyl increased the relative abundance of nodules formed by the inoculated strain, the number of added rhizobia on the root, the total N content, and the percentage N of soybean plants grown in four soils when the seeds were inoculated with a benomyl-resistant strain of *Bradyrhizobium japonicum*. It was also found that oxamyl (a basipetally translocated fungicide) applied to the seeds, foliage, or both increased the yield, N content, percentage N, and weight of nodules, pods, and grains along with the number of nodules formed by the inoculated strain when soybean seeds were inoculated with oxamyl-resistant *Rhizobium japonicum.*

Introduction

The inoculation of legumes with effective rhizobia or bradyrhizobia represents an inexpensive alternative to the use of chemical nitrogen fertilizers, whose prices have raised due to the high cost of energy involved in their production. These fertilizers are also pollution hazards. The process of symbiotic biological nitrogen fixation requires that the host crop be adequately nodulated by the specific root-nodule bacteria effective in nitrogen fixation. Not all the strains of *Rhizobium* or *Bradyrhizobium* that can produce nodules on a given host are able to use N_2 rapidly and efficiently. Nonetheless, selection of an effective (i.e. N2-fixing) strain is a prerequisite for any crop to be inoculated. A second important characteristic is the competitiveness of the strain. The effectiveness and competitiveness are generally mutually exclusive and are not dependent upon each other. The ability of *Rhizobium* to proliferate in the rhizosphere is necessary for successful nodulation (Harris, 1954), and a positive correlation has been found between failure to nodulate and low *Rhizobium meliloti* numbers in the rhizosphere of *Medicago sativum* L. (Rice *et al.*, 1977, 1984). There are also reports of positive relationship between effectiveness of a strain and nodule occupancy (Athar and Johnson, 1996a, b, c). This effectiveness has also been referred to as competitiveness (Robinson, 1969; Mytton, 1975, Dowling and Broughton, 1986).

The soil environment is very complex. It contains many types of microorganisms. There is inadequate information available on their interactions with each other and with the environment. The poor nutrient status of soil may affect the growth of rhizobia and bradyrhizobia. Because the supply of essential resources, such as nutrients, water, and oxygen available in natural environments is often insufficient to meet all biological need, species have to compete with each other to obtain the essentials for growth (Amargar 2001). The growth rates of bacteria enhance the outcome of competition with rhizobia and nodulation induced by root-nodule bacteria (Li and Alexander, 1986). An organism, such as a species of *Rhizobium* or *Bradyrhizobium*, which grows more slowly than many indigenous species, presumably is at a competitive disadvantage and will not develop extensively.

Failure of root-nodule bacteria inoculated into soil or onto seeds is generally associated with their poor survival and poor competitiveness with other soil microorganisms (Bohlool and Schmidt, 1973; Boonkerd *et al.*, 1978; Bohlool *et al.*, 1984). Alexander (1971) defines competition as" a condition in which there is a suppression of one organism as the two species struggle for limiting nutrients, oxygen, or any other common requirements." Many workers have reported the effect of several factors, such as the type of soil (Moawad and Bohlool, 1984; Dowling and Broughton, 1986; Kosslak and Bohlool, 1985), drought (Athar and Johnson, 1996a, Serraj *et al.*, 1999; Zahran, 1999), temperature (Hardarson and Gareth-Jones, 1979; Juwarker and Rewari, 1988), extreme conditions (Bordeleau and Prevost, 1994; Bottomley, 1992; Graham, 1992; Kulkarni *et al.*, 2000) host variety (Diatloff and Brockwell, 1976; Ruiz-Argüeso *et al.*, 1977; Pueppke *et al.*, 1999; Belachew and Pant, 2010), autoregulation (Heron and Pueppke, 1987; Bhuvaneswari *et al.*, 1988; Cregan *et al.*, 1989) bacterial motility (Aguilar *et al.*, 1988; McDermott and Graham, 1989) or composition of culture media (Fernandez-Flouret and Cleyet-Marel, 1987) on the competition. Recently Brophy *et al.* (2011) described a novel methodology for evaluating competition among strains of Rhizobium bacteria which can be found naturally occurring in or can be introduced into soil. Rhizobia can occupy nodules on the roots of legume 'fix' atmospheric nitrogen. Their model defines competitive outcomes for a community (the multinomial count of nodules occupied by each strain at the end of a time period) relative to the past state of the community (the proportion of each strain present at the beginning of the time period) and incorporates this prior information in the analysis. Their approach for assessing competition provides an analogy to multivariate methods for continuous responses in competition studies and an alternative to univariate methods for discrete responses that respects the multivariate nature of the data.

It has been reported that improved nodulation is possible if a large number of rhizobia is present in the inoculum (Kapusta and Rouwenhorst, 1973; Weaver and Frederick, 1974; Fernandez-Flouret and Cleyet-Marel, 1988), which suggests that the extent of nodulation is directly proportional to the number of infective rhizobia in the rhizosphere. For example, an increase in the number of nodules on the upper tap root of the subterranean clover has been reported to be associated with the presence of a greater abundance of *Rhizobium trifolii* in this region of the root (Chatel and Parker, 1973; Hanus *et al.*, 1981). The extent of nodulation, however, remains poor even after inoculating several times with a large number of rhizobia (Dube *et al.*, 1975), and usually no more than 5% of the nodules are formed by the inoculated strain (Döbereiner, 1977).

The large, heterogeneous, and metabolically active community in the rhizosphere is well adapted to that environment. It has been postulated that rhizosphere bacteria may affect the growth of *Rhizobium* and *Bradyrhizobium* strains differentially by possibly altering the competition and nodulation among strains of root-nodule bacteria by direct or indirect means (Fuhrmann and Wollum, 1989a). Although organic compounds excreted by growing roots sustain the community, competition in this environment must still be intense in view of the size and diversity of the community. An inoculated species, therefore, must be able to overcome competitive stress from other native or indigenous organisms already present in the environment and which are better adapted and can grow faster than a newly introduced species. Microbial antagonism may also contribute to problems encountered with colonization of the host rhizosphere, nodulation of the host, and the establishment, and survival of the *Rhizobium* introduced in the soil. The term "microbial antagonism" refers to any kind of relationship in which one organism inhibits the growth or restricts the activities of another organism. "Antibiosis" has been defined as the suppression of one species by a second as a result of toxin production (Alexander, 1977). The occurrence of antibiotic-producing microorganisms effective against rhizobia in the host rhizosphere or on the host root surface, as well as on the nodule surface has been well documented (Smith and Miller, 1974; Shukla and Dwivedi, 1979; Pugashetti *et al.*, 1982).

Two types of competition are encountered by rhizobia introduced into a soil in which legumes have been grown; intraspecific and interspecific competition. Intraspecific competition involves different strains of the same species and interspecific competition involves competition between different species (Alexander, 1977; Dowling and Broughton, 1986).

The first published study on intraspecific interactions affecting *Rhizobium* was performed by Nicol and Thoronton (1941). These researchers showed that being dominant in competition determines which of the strains will form the most nodules when more than one strain is present in the surroundings of their host root system. Competition masks the relative infectivity of a strain and results in poor nodulation even if the inoculated strain has been proven to possess excellent infectivity and effectiveness in pure culture. Many other workers have also addressed this subject of competition (Amarger, 1981, 2001; Amarger and Lobreau, 1982; Barnet *et al.*, 1988; Fernandez-Flouret and Cleyet-Morel, 1988; Hicks and Loynachan, 1989). Competition between closely related strains may be particularly intense because of their identical or nearly identical needs to proliferate in soil. For example, inocula containing more than one strain of *B. japonicum* produced fewer nodules per plant than the inocula composed of a single strain (Fernandez-Flouret and Cleyet-Morel, 1988). A similar situation was reported by Franco and Vincent (1976) with *Rhizobium* strains capable of nodulating *Macroptilium atropurpureum* and *Stylosanthes guianesis*. The plants were inoculated with pairs of *Rhizobium* strains, and a competitive index for each strain was calculated. In five out of seven cases, successful nodulation was directly related to the proportion of each strain present in the inoculum. The ability of an individual *Rhizobium* strain to compete for nodule formation was not related to any characteristic of the legume-*Rhizobium* symbiosis.

Nodulation failure following the use of a single-strain inoculant is usually associated with the presence in soil of ineffective but more competitiveness native strains. Holland (1970) found that certain native strains of *Rhizobium* isolated from different species of *Trifolium* growing in northern California range soils nodulated subterranean clover, but the association was ineffective. Nonetheless, these indigenous *Rhizobium* strains were able to out-compete the inoculated strains provided in the commercial inoculum. Effective nodulation by a desired strain was only achieved by an application of 7.5 x 10^4 CFU of rhizobia per seed; four times the recommended rate. Similarly, it has been reported in other studies that even if the *Rhizobium* used as an inoculant effectively nodulates the host by out-competing indigenous strains in the first growing season, the inoculated rhizobia does not persist to form nodules on the plants in the following season (Ikram and Broughton. 1981; Meade *et al*., 1985; Rao *et al*., 1985).

Interspecific interactions that deleteriously affect colonization of the legume rhizosphere by rhizobia have not received much attention. In one study, however, the lack of response in the field of subterranean clover to seed inoculation with *Rhizobium trifolii* was attributed to an unspecified type of antagonism that prevented colonization of root surface by *R. trifolii* (Hely *et al*., 1957). Studies by Harris (1953) on *R. trifolli* indicated that species of *Fusarium*, *Sclerotium*, and *Hormodendrum* decreased the number of nodules or prevented nodulation of white clover grown on agar, although the antogonists did not produce antibiotics acting on *R. trifolii*. Similarly, strains of *Azospirillum* that did not produce toxins against *R. trifolii* did inhibit nodulation of sunterranean and white clover (Plazinski and Rolfe, 1985). Further evidence supporting the significance of interspecific competition comes from a study in which suppression of indigenous bacteria by the addition of antibacterial antibiotics permitted extensive growth of species of *Rhizobium* and *Bradyrhizobium* in soil, whereas suppression of eucaryotes resulted in less enhancement (Pena-Cabriales and Alexande, 1983). Similarly, bacteria in the rhizosphere of alfalfa were suppressed, and the growth of a streptomycin-resistant strain of *R. meliloti* was enhanced as a result of the addition of streptomycin to non-sterile soil in which alfalfa was grown. Inoculation of a bacterial mixture into sterile soil inhibited colonization by *R. meliloti* and its nodulation of alfalfa roots (Li and Alexander, 1986). Pseudomonas have been known to effect competition among *B. japonicum* and decrease the extent of nodulation. Iron availability may be an important factor modifying interactions involving soybean plants, *B. japonicum*, and associated microorganisms in the soybean rhizosphere (Pugashetti *et al*., 1982; Leong, 1986; Fuhrmann and Wollum, 1989b).

The movement of root-nodule bacteria along the root system from the site of inoculation is important for successful colonization. The bacteria applied should be capable of migrating or of being moved far along the growing roots of the host plant. Since inoculation of the whole root system is impractical, movement or migration is important to ensure the colonization of the root system (Bowen and Rovira, 1976; Suslow, 1982; Bashan. 1986).

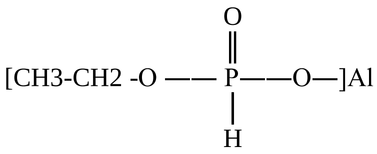
Movement of bacteria in soil has been demonstrated extensively for rhizobia inoculated onto legumes rather than for any other beneficial rhizosphere bacteria. The first study was done by Kellerman and Fawcett (1907). They reported horizontal movement at a rate of 2.5 cm in 48 h in saturated sterile soil and a lower rate in barely moist soils. Migration distances from several centimeters to several tens of centimeters have been reported (Frazier and Fred, 1922; Hamdi, 1971; Wong and Griffin, 1976; Madsen and Alexander, 1982), but the extent of movement is uncertain due to differences in the results.

Current practices in legume production include inoculation of seeds with rhizobia and concurrent treatment of the seeds with fungicides or other pesticides to reduce seed rot, damping-off, and other seed-borne or soil-borne problems resulting from pests. Many of these chemicals are toxic to rhizobia, and some reduce the amount of nitrogen fixed (Diatloff, 1970; Fisher, 1976; Staphorst and Strijdom, 1976). The use of rhizobia resistant to particular chemicals used as seed protectants has been studied by several workers (Odeyemi and Alexander, 1977; Ramirez and Alexander, 1980; Chao and Alexander, 1981). Broad-spectrum antimicrobial chemicals applied to the soil or seed not only have an effect on the root-nodule bacteria but also may influence the interaction among soil microorganisms and rhizobia. Hence, a fungicide resistant *Rhizobium* or *Bradyrhizobium* might proliferate more extensively in the legume rhizosphere and nodulate more frequently should its potential antagonists be inhibited by the added chemicals.

Little information exists on the effects of systemic fungicides on symbiotic nitrogen fixation or nodulation. Hossain and Alexander (1984) reported that the systemic fungicide benomyl increased the relative abundance of nodules formed by the inoculated strain, the number of added rhizobia on the root, the total N content, and the percentage N of soybean plants grown in four soils when the seeds were inoculated with a benomyl-resistant strain of *Bradyrhizobium japonicum*. They also found that oxamyl (a basipetally translocated fungicide) applied to the seeds, foliage, or both increased the yield, N content, percentage N, and weight of nodules, pods, and grains along with the number of nodules formed by the inoculated strain when soybean seeds were inoculated with oxamyl-resistant *Rhizobium japonicum*. Most of the available information on the other hand, concerns the residual effect of such systemic fungicides used against a crop other than legume. For example, the fungicide triadimefon, which is widely used against cereal pathogens, was shown to reduce nitrogen fixation by clover when present in soil at concentrations somewhat greater than those likely to be encountered in practice (Fisher *et al*., 1979; Fisher and Hayes, 1981).

The word "systemic" implies movement inside the plant system. This term may be misleading because most systemic fungicides are only partially systemic in plants and are carried only with the transportation stream over long distances via the xylem. This mode of translocation is referred to as apoplastic. Fungicides translocated by this mode of translocation in the plants are termed acropetal fungicides. Leaves are the primary transpiring organs and movement within plants is from the soil to the expanded leaves. Very young leaves, flowers and fruits do not transpire significantly, and therefore receive only minute quantities of fungicides applied to soil or seed. Some synthetic chemicals, which are termed "basipetal", are translocated long distances in the phloem and in the direction of assimilate transport, i.e. towards roots. This mode of translocation is called symplastic. Synthetic chemicals which are translocated by both systems are designated as ambimobile (Edginton *et al*., 1980; Martin and Edgington, 1981).

The systemic fungicide Fosetyl-Al (aluminum tris-O-ethyl phosphonate), known under the trade name of Aliette (Rhŏne-Poulenc Agrochimie, Lyon, France) is used worldwide to control diseases caused by members of Peronosporales, especially root and crown rot caused by various *Phytophthora* species and foliar diseases such as downy mildews. Aliette is systemic in both basipetal and acropetal directions. Ethyl-phosphonate degrades to ethanol and phosphonic acid (synonym: phosphorous acid, H_3PO_3) in plants and soil. Phosphonic acid, at physiological pH, is ionized primarily to phosphonate (synonym: monohydrogen phosphite, HPO_3^{-2}) in plants and soil due to its pKa values of 1.3 and 6.7 for the hydroxyl groups (Williams *et al*., 1977; Saindrenan *et al*., 1985; Cohen and Coffey, 1986; Ouimette and Coffey, 1988). The HPO_3^{-2} anion has been shown to be up to 15 times more active against various *Phytophthora* species than ethyl-phosphonate (Fenn and Coffey, 1984, 1989; Cohen and Coffey, 1986). Some properties and the molecular formula of aliette are shown in Figure 1.



$$[CH3\text{-}CH2\ \text{-}O - \overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle H}{|}}{P}} - O -]Al$$

Aluminum tris -O- ethyl phosphonate

Trade name:	Aliette
Common name:	Fosetyl-Al (BSI)
Other names:	LS 74-783, EPLA EXP 1695, RP 32545
Solubility at 20°C:	120 g/L in water
Some other characteristics:	Systemic (Ambimobile) Non-corrosive Non-flammable Non-explosive

Fig. 1. Some properties and structure of aliette.

Phosphorus compounds applied to the plants could be used as fertilizer sources by the plants. MacIntire *et al*. (1950) evaluated certain phosphorus compounds as fertilizers in pots using red clover, rye grass, alfalfa, brown-top millet, and soybeans as test plants. They did not find any response during the first year with phosphonic acid (H_3PO_3) as the P source, but they did find beneficial effects during the second year of plant growth. Presumably plants and microorganisms cannot readily oxidize HPO_3^{-2} to HPO_4^{-2} to use the former as a P source. They attributed the second year response to oxidation of HPO_3^{-2} to HPO_4^{-2} by microorganisms in soil.
Siddiqui and Alexander (1991) studied enhancing legume root colonization by added strains of *Rhizobium* and *Bradyrhizobium*. An effort was made to inhibit the rhizosphere bacteria which compete with these inoculated strains and render them less abundant. Specific concentrations of aliette to which root nodule bacteria were made resistant and at which other soil bacteria were not able to grow were coated on the seeds and in some cases also applied as a foliar spray.

Biological Nitrogen Fixation: The consequences of using chemical fertilizers over the decades have translated into increased incidence of chronic human diseases. These chemicals impair almost every human body system from neuro to nephro by altering the functions of the specific organs. Biofertilizes are lucrative alternatives to elude these chemical insults encountered from the environment. These biofertilizers are environmentally safe, economically viable, enhance biomass residual effects and ensure slow and constant integrated nutrient supply not only during the life cycle of a current crop but also for the subsequent crops. They also release growthpromoting substances like hormones and vitamins and increase soil fertility. In addition, they hasten seed germination, flowering and maturity in a natural way. They conserve the soil naturally by preventing salinity caused by chemical fertilizers and reduce the chemical run off from the agricultural fields to the rivers, lakes and other water bodies of economic and social importance.

Biofertilizes are living cells of different macro/microorganisms that can mobilize or convert nutritionally important elements from non-available to available form by different natural biochemical processes. Some examples of important biofertilizers include different species of *Azospirillum*, phosphobacteria, rhizobia, blue green algae, Azolla-anabena, and mycorrhiza.

Conclusion

The last half of the 20th century saw a surge in research relating to BNF, azolla –anabaena, mycorhiza, plant residue composting and other symbiotic and non-symbiotic systems collectively termed as biofertilizers contributing strongly for low cost and fertilizer free food production. Many of these processes are still under investigation, as they are still not completely understood. The knowledge and information obtained so far has not yet either been exploited to it maximum potential to decrease our dependency on chemical fertilizers.

Ever shrinking resources of energy world wide and increasing higher cost associated with the use of energy for the production of fertilizers concomitantly coupled with the reduction and in most cases abolishing the government subsidiaries for food production in almost all countries of the world are forcing the farmers to leave the farm for a better and economically more rewarding alternative careers. Sustained, low cost food production using biological fertilizers and resources in accordance with the developed and developing technologies will help stabilize the farm and the farmer economically and environmentally. These practices will bring long lasting conservations to our soils, lakes, rivers and air environment.

Deterioration of soil and ground water as a consequence of over use synthetic chemical fertilizers can be avoided. Soils can be made more healthy and productive simultaneously reducing the dependence of crop production on chemical fertilizers and financial burdens on farmers incurred on these fertilizers. The multi facet benefits of reducing financial and chemical inputs for food production and conserving the soils and water resources from run off and leeching of these chemicals will greatly improve the ecosystem and quality of food. The healthy organic food thus produced will help eliminate chemical contamination controversies to some extent.

The productive food yield may decrease to some extent as a result of a switch over to use biofertilizers. It will, however, will not be significant compared to the current cost of food production using extensive and expensive synthetic chemical fertilizers. As the equilibrium in the soils would be established in few years after continuous use of biofertilizers, the food produced would be more natural, organic and healthy. The chemical contamination caused by the use of excessive synthetic fertilizers will reduce in due time and will also help politically to eliminate or at least reduce the controversies of adverse health effects on consumers.

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