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2.1 The Rhizosphere and soil microorganisms

From the microbial point of view soil is a nutritional desert. In contrast, soil adjacent to roots is relatively nutrient rich. This is because as the underground parts of the plant grow through the soil, the microhabitats of the soil in close proximity of root witness a drastic change as nearly about 40% of the photosynthates are lost to the soil through root exudation and this has an impact on the microorganisms residing in the region. Bacteria are the most dominant group of microorganisms in this region of soil and probably equal to one half of the microbial biomass present there in. Population of bacteria in this region is high ranging from $10^{10}$ to $10^{12}$ cells per gram of soil. The most abundant bacterial forms or genera present here are *Pseudomonas, Bacillus, Azospirillum, Enterobacter, Serratia, Arthrobacter, Micrococcus, Flavobacterium, Sarcina, Corynebacterium, Mycobacter* etc. (Mishra 1996; Rangaswami and Bagyaraj 1998; Subba Rao 2000). This area of enhanced activity surrounding living roots that is composed of soil particles and active communities of soil microorganisms is called the “rhizosphere”. This environment is richer in nutrients, and its microbial communities differ from those present in area not influenced by the roots. The width of the zone of soil influenced by the root varies with the plant species its age and cultural conditions, soil conditions, environmental conditions etc. (Rangaswami and Bagyaraj, 1998).

Microorganisms in agriculture soils are known to exert profound influence on fertility and productivity of soil (Barea *et al.*, 2005). Most rhizobacteria remain confined to roots surface, but some enter the root interiors and behave as endophytes (Sturz *et al.*, 2000). The endophytic bacteria can be defined as “those bacteria that can be isolated from surface-disinfected plant tissues or extracted within the plant, and that do not visibly harm the plant (Lodewyckx *et al.*, 2002).” Some bacterial species living in the rhizosphere can affect the plant growth in either a positive or in a negative way. Rhizosphere bacteria having favorable effects on plant growth and yield of commercially important crops are denominated as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroot, 1978). Studies on beneficial effects of rhizospheric bacteria have most often been based on
increased plant growth, faster seed germination, better seedling emergence, enhanced nodulation and nitrogen fixation in leguminous crops and suppression of diseases. As a consequence, PGPR have been further divided into subsets like emergence promoting rhizobacteria (EPR), nodulation promoting rhizobacteria (NPR) and disease suppressing rhizobacteria (DSR). However there can be considerable overlap among these subsets. For instance, PGPR that act through biological control can also enhance germination or growth. In recent years, many reviews have appeared dealing with PGPR isolation, screening, ecology, physiology and their use as agrobiotechnological inoculants (Glick 1995; Lazarovits and Nowak, 1997; Saxena et al., 2000). The science of PGPR is thus relatively young in comparison to nitrogen fixing bacteria and momentarily applications to crop production are limited. The science is developing rapidly and producers and crop production industry wise to keep abreast of developments as they may reach the dealer level in years to come.

2.2 Mechanism of PGPR Plant Growth Promotion

The mechanisms by which PGPR increase crop performance is not well understood. Anyway, plant growth promoting rhizobacteria (PGPR) influence the plant growth and development either directly or indirectly (Persello-Cartieaux et al., 2003)

**Direct Plant growth promotory mechanisms of PGPR**

- Nitrogen fixation
- Production of plant hormones
- Phosphorus Solubilization
- Enhanced iron availability
- Zinc Solubilization
- Ethylene reduction by ACC deaminase enzyme

**Indirect Plant growth promotory mechanisms of PGPR**

- Antibiosis
- Competition for nutrients and niche
- Lytic activities
- HCN production
- Induced systemic resistance

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2.2.1 Biological nitrogen fixation (termed Biofertilization)

Biofertilizers in toto accounts for approximately 65% of the nitrogen supply of the crops worldwide (Lugtenberg et al., 2002). The most exploited PGPR for the purpose are rhizobia including *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* for their ability to fix N\textsubscript{2} in their legume hosts. The other free living and endophytic PGPR are *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Glucanacetobacter diazotrophicus*, *Herbaspirillum*, *Klebsiella pneumoniae*, *Paenibacillus polymyxa* etc. with the ability to fix nitrogen (Hashem, 2001; James et al., 2002; Timmusk, 2003). All of these bacteria form a host-specific symbiosis with leguminous plants.

A practical challenge is to widen the host range of the symbiosis towards major non-leguminous food crops such as rice. Recent results show that rhizobial type-III secretion systems secrete specific proteins and are involved in the establishment of the symbiosis (Viprey et al., 1998) Remarkably, genes encoding type-III secretion systems have also been identified in a plant beneficial *Pseudomonas fluorescense* (Rainey et al., 1999).

Free-living nitrogen-fixing rhizobacteria such as *Azospirillum*, *Herbaspirillum*, *Acetobacter*, *Azotobacter* and *Azoarcus* are also able to fix atmospheric nitrogen. They use a nitrogenase complex that functions under low oxygen conditions and that is not as specific in its interaction with the plant as are rhizobia. *Azospirillum* predominantly colonizes the rhizosphere, whereas the other bacteria are predominantly found as endophytes inside roots, stems and leaves. The genes involved in nitrogen fixation, nitrogen assimilation and nitrogen regulation have been described for *Azospirillum*. Several of the *nif* genes have also been described for the other free-living nitrogen fixers, which all have similar nitrogenase complexes, except for *Azoarcus* which possesses three differently encoded nitrogenase complexes.

2.2.2 Phytohormone production (termed Biostimulants)

Organic substances capable of regulating plant growth produced either endogenously or applied exogenously, are called plant growth regulators, which affect physiological and
morphological processes at very low concentration. Many bacteria have the ability to produce auxins, gibberellins, cytokinins, and ethylene (Arshad and Frankenberger, 1998). Indole-3-acetic acid is involved in root initiation, cell division, and cell enlargement. Most commonly, IAA producing PGPR are believed to increase root growth and root length, resulting in greater root surface area, enabling plants to access more nutrients from soil. Cytokinins are known to promote cell division and enlargement and tissue expansion in certain plant parts. Gibberellins modify morphology by extension of plant tissues. Evidence of GA production by PGPR is rare; however, Gutierrez-Manero et al. (2001) provided evidence that different forms of GA are produced by *B. pumilus* and *B. licheniformis*. Ethylene is a gaseous hormone known as 'wounding hormone' and mediates a range of different plant responses such as seed development, tissue differentiation, formation of root and shoots primordial, flower opening etc.

Three gibberellin-like substances were detected in the cultures of *Azotobacter chroococcum* strain A6 (Brown and Burlingham, 1968). When an inoculum of *Azotobacter* was added to seeds or roots, the later development of tomato plants was altered, possibly because it was taken up by the seedlings at a critical stage of vegetative and reproductive primordial differentiation.

Microbial production of C$_2$H$_4$ can affect plant growth of etiolated pea seedlings. Etiolated pea seedlings when treated with soil fungi *Acremonium falciforme* which produces ethylene, presented a classical triple response, which includes reduction in elongation, swelling of the hypocotyl and a change in the direction of growth (horizontal) (Arshad and Frankenberger, 1998). Further studies by Arshad and Frankenberger (1991) showed production of plant growth regulators (PGRs) by many soil microorganisms in the presence of suitable precursors. In laboratory studies, the microbial biosynthesis of auxins, cytokinins and ethylene in soil was monitored. Dubékovsky et al. (1993) demonstrated the existence of positive effect of bacterial IAA on plants after inoculation of black current soft wood cuttings by a recombinant *Pseudomonas* strain.

A review on the formation of hydrocarbons by microorganisms that while ethylene is a common product of fungi, the soft rot bacterium, *Pseudomonas solanacearum* has also been shown to produce the gas in pure culture. More recently its formation by other
bacteria has been demonstrated. Experimental results on the effect of wild-type and mutant PGPR (*P. putida*) in the rooting of green gram cuttings showed that treated cuttings had a significant higher number of roots compared with cuttings rooted in water. Enhancement of symbiotic nitrogen fixation and plant growth have been reported due to inoculation of Vitamin B12 secreting *Pseudomonas* strain 267 in growth medium (Derylo and Skenipska, 1993).

2.2.3 Solubilization of phosphate

Phosphate solubilizing bacteria produce organic acids that solubilise mineral phosphates, including calcium phosphate in high-pH soils and rock phosphate fertilizers. These bacteria occur in most soils and potentially represent 40% of the culturable population (Richardson 2001). The extent to which P-solubilising rhizobacteria contribute to plant P-uptake in natural systems has not been clearly elucidated (Richardson 2001). The organic acids released by plant roots probably have a greater impact on the solubilisation of P than do the acids produced by the rhizobacteria. However, rhizobacteria consume the organic acids in the root exudates and, therefore, indirectly may moderate solubilisation of P and other immobile elements, such as Fe and Mn. Organic acids are metabolized two to three times faster in the rhizosphere than in bulk soil, typically with 60% being mineralized and the remainder incorporated into microbial biomass.

A range of organic acids with P-solubilising activity are produced by rhizobacteria but gluconic acid and 2-ketogluconic acid appear to be the most active and important (Moghimi and Tate 1978). Some gram negative rhizobacteria have membrane bound enzymes that enable the extracellular conversion of glucose to gluconic acid (Glucose dehydrogenase) and then to 2-ketogluconic acid. The possibility of enhancing P uptake of crops by artificial inoculation with P-solubilising strains of rhizobacteria has been an attractive proposition for research. Plant responses to inoculation in the field have been widely reported but are variable (Richardson 2001).

A majority of agricultural soils contain large reserves of phosphorus, of which a considerable part is accumulated as a consequence of regular applications of P-fertilizer (Richardson, 2001). The phenomenon of fixation and precipitation of P in soil is dependent
on pH. It can reduce efficiency of soluble P fertilizers (Goldstein, 1986). In acidic soils, P is precipitated as Al and Fe phosphates, whereas in calcareous soils high concentration of Ca results in P precipitation. The soil is indeed a habitat for diverse group of organisms which employ variety of solubilization reactions to release soluble phosphorus from insoluble phosphates (Salih et al., 1989; Ilmer and Schinner, 1995; Singh and Kapoor; 1998). The potential of these phosphate solubilizing microorganisms (PSM) have been utilized as bioinoculants for crops grown in soils low in available P and amended with rock phosphate or tricalcium phosphate (Salih et al., 1989; Chabot et al., 1993; Chabot et al., 1996).

In growth chamber studies, Bacillus circulans and Bacillus megaterium var. phosphaticum inoculants increased plant weight and P-uptake of millet and pea respectively (Raj et al., 1981). Datta et al., 1982 found that a P-solubilizing and IAA producing strain of B. firmus, increased the grain yield and uptake of rice grown in P-deficient soil amended with rock phosphate. Increased biomass and P-uptake was observed in wheat inoculated with Penicillium bilaji (Kucey, 1987). Gained and Gaur (1991) reported increased biomass, grain yield, and P and N uptake by green gram inoculated with Bacillus subtilis. Chabot et al. (1993) demonstrated that some PSM isolated from Quebec soils stimulated the growth of maize and lettuce in field trials. Several phosphate solubilizing strains belonging to genera Bacillus and Xanthomonas where found to enhance the growth and yield of canola (Brassica napus L.) but did not influence the P-uptake by plant, (de Freitas et al., 1990). They suggested the production of IAA like hormones by these rhizobacteria to influence the growth and yield of plant.

Organic phosphorus present in soil must be hydrolyzed to inorganic P before it can be utilized by plants. Organic P is catalyzed through hydrolysis of C-O-P ester bonds by phosphatase or phytase, which are very important in the nutrition of plants (Tarafdar and Claassen, 1988). Major mechanism of mineral phosphate solubilization is through the action of organic acid synthesized by soil microorganisms (Rodriguez and Fraga, 1999). Production of organic acids such as gluconic acid extracellularly mineralized mineral phosphate into Pi proton substitution by Ca$^{2+}$ (Goldstein, 1986). It is reported that gluconic acid is produced by phosphate solubilizing bacteria such as Erwinia herbicola and
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*Pseudomonas* whereas 2-ketogluconic acid is produced by *R. leguminosarum, Rhizobium meliloti* and *B. firmus* (Halder and Chakraborty, 1993; Illmer and Schinner, 1995). Strains of *B. licheniformis* and *B. amyloliquefaciens* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Solubilization of organic phosphate is also called mineralization of organic phosphorus. The mineralization of these compounds is carried out by the action of several phosphatases.

2.2.4 Siderophores mediated iron acquisition

Siderophores are produced by PGPR under iron-limited conditions. Iron is an essential growth element for all living organisms. Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion (Whipps *et al.*, 2001). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (O'Sullivan *et al.*, 1992). Some PGPB strains go one step further and draw iron from heterologous siderophores produced by cohabiting microorganisms (Castignetti and Smarelli, 1986; Lodewyckx *et al.*, 2002; Loper and Henkels, 1999; Whipps, 2001).

Iron is a cofactor of most of the enzymes needed for growth in all organisms, but the availability of solubilized Fe$^{3+}$ in soils is limited at neutral and alkaline pH, leading to Fe$^{3+}$ limitation to both plant and microbes. The ability to produce specific siderophores and/or to utilize a broad spectrum siderophores may contribute towards the root colonizing ability of *Pseudomonas* strains. The ability to produce efficient siderophores is sometimes combined with the ability to take up related siderophores from other organisms (Koster *et al.*, 1995). The ability to scavenge iron under Fe$^{3+}$ limitation provides the biocontrol organisms, a selective advantage over pathogens or deleterious organisms that possess less efficient iron binding and uptake systems. Thus, production of high affinity siderophores, which scavenge more iron from rhizosphere and thereby depriving pathogens from iron, may result in growth inhibition of pathogens (Kloeper *et al.*, 1988).

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Under aerobic soil conditions, most of the iron exists in insoluble ferric form, which is unavailable to plant and microbes (Hersman et al., 2001). Under this iron limited situation, most soil microorganisms produce siderophores. These are low-molecular weight metabolites with a high affinity for Fe^{3+} and facilitate transport of ferric-siderophore complex into bacterial cell and plants coupled with specific siderophore receptor protein (Neilands, 1981). *Pseudomonas* species have been used as provider of heterologous siderophores for plants (Carrillo-Castanenda et al., 2002; Sharma et al., 2003).

Siderophore biosynthesis is generally tightly regulated by iron-sensitive Fur proteins, the global regulators GacS and GacA, the sigma factors RpoS, PvdS, and FpvI, quorum-sensing autoinducers such as N-acyl homoserine lactone, and site-specific recombinases (Cornelis and S. Matthijs 2002; Ravel and P. Cornelis. 2003). However, some data demonstrate that none of these global regulators is involved in siderophore production. In addition, GrrA/GrrS, but not GacS/GacA, are involved in siderophore synthesis regulation in *Serratia plymuthica* strain IC1270, suggesting that gene evolution occurred in the siderophore-producing bacteria (Ovadis et al., 2004). A myriad of environmental factors can also modulate siderophores synthesis, including pH, the level of iron and the form of iron ions, the presence of other trace elements, and an adequate supply of carbon, nitrogen, and phosphorus (Duffy and Défago. 1999). Leeman et al. (1996) reported that LPS of *P. yuorescens* strains WCS 374 and WCS 417 are the major determinants of ISR under iron-replete conditions but under iron-limited conditions, LPS of these bacteria were not involved in ISR in radish against *Fusarium* wilt. They also found that pyoverdin-type pseudobactin, siderophore, produced by these bacteria was responsible for ISR. Application of purified pseudobactin alone, isolated from strain WCS 374, to the roots of radish induced resistance. Thus, different bacterial determinants in inducing systemic resistance in radish vary depending upon iron availability. Induction of ISR by LPS and siderophores seems to be complementary rather than additive and full induction of resistance by one determinant masks contributions by other(s).

Pyoverdine, the well-known yellow-green fluorescent pigment characteristic of the fluorescent *Pseudomonas* species (Elliott, R. P. 1958), is the major siderophore of these bacteria (Meyer, J.-M., and J.-M. Hornsperger. 1978). In the structure of pyoverdine, there
is a quinoleinic chromophore which imparts the color and fluorescence to the molecule, associated with a peptide chain of L-, D-, and uncommon amino acids, such as $\alpha$-N-hydroxyornithine and $\alpha$-hydroxyaspartic acid (Budzikiewicz H. 1997). Both parts of the molecule participate in the complexation of the iron (III) ion, as illustrated in Fig. 2 for the poverties of *P. aeruginosa* ATCC 27853 (Tappe *et al.*, 1993). The acyl chain (R group) could be succinic acid, succinamide, or glutamic acid (Tappe *et al.*, 1993).

**Figure 2 Structure of the iron complex of the *P. aeruginosa* ATCC 27853 pyoverdine**

### 2.2.5 Zinc and Potassium solubilization

Zinc, one of the eight trace elements, is essential for the normal healthy growth, and reproduction of plants. In soil, the most ubiquitous zinc form is zinc sulphide followed by zinc carbonate, zinc oxide and zinc phosphate (Alloway, 2004). Rhizobacteria like *Bacillus* and *Pseudomonas* have the ability to produce organic acid especially gluconic acids which can solubilize inorganic insoluble zinc phosphate, zinc oxide, and zinc carbonate into soluble form (Saravanan *et al.*, 2003).

Besides weathering of K reserves (mica and feldspars) by physical and chemical means, many microorganisms in the soil are able to solubilize ‘unavailable’ forms of K bearing minerals such as mica, illite and orthoclases, by excreting organic acids which either directly dissolve rock K or chelate silicon ions to bring K into solution. Microorganisms with such a property are called K solubising microorganisms (KSM). Production of carboxylic acids like citric, tartaric and oxalic acids was associated with...
feldspars solubilization by Bacillus mucilaginosa and Bacillus edaphicus. Recently, Sheng (2005) reported that inoculation of potassium releasing bacterial strain B. edaphicus NBT was found to increase root and shoot growth of cotton and rape.

2.2.6 ACC deaminase mediated ethylene reduction

ACC is a precursor of ethylene production in plants. ACC deaminase is an enzyme which breakdown ACC into ammonia and a-ketoglutarate (Honma and Shimomura, 1978). The enzyme has been reported in many bacteria such as Alkaligenes xylosoxidans, B. firmus, B. pumilis, Enterobacter cloacae, P. chloroaphis, P. fluorescens, P. putida, R. leguminosarum and R. hedysari etc. (Belimov et al., 2001, 2002, 2007; Ghosh et al., 2003; Ma et al., 2003a&b).

It has been proposed that uptake and cleavage of ACC by plant growth promoting bacteria can lower down plant ethylene levels in developing for stressed plants (Glick et al., 1998). In order to maintain equilibrium between internal and external ACC levels, plants exude more ACC. As a consequence, decrease the level of ACC inside plant cell and thereby reduce the production of ethylene. The reduced level of ethylene in plant root enhances better root enhances better root growth and development.

Indirect PGPR action

2.2.7 Antibiotic production

Various rhizospheric bacteria are potential microbiological pesticides, which are able to protect plants against diseases and improve plant yield. Among these, pseudomonads and bacilli are the most common resident of rhizosphere and help suppress diseases by various mechanisms (Moenne-Locoz and Defago, 2004; McSpadden-Gardener, 2004; Bergsma-Vlami et al., 2005). Most pseudomonads suppress soil borne fungal pathogens by producing diverse array of secondary metabolites with antifungal properties such as hydrogen cyanide, pyoluteorin, pyrrolnitrin, phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol (DAPG), zwittermycin, kanosamine, anthranilate, iturin etc. (Nowak-Thompson et al., 1999; Delany et al., 2001; Yu et al., 2002; Haas and Defago, 2005). Bacillus species produce a variety of antibacterial antifungal peptide antibiotics.
(Heins et al., 2002; Pinchuk et al., 2002; Yu et al., 2002). Recently production of iturin a lipopeptide (antibiotic) by B. amyloliquefaciens and B. subtilis for control of Rhizoctonia solani was reported by Yu et al. (2002). The mechanism of antibiotic action in soil is poorly understood.

2.2.8 Competition for nutrients and niches

The other mechanism to inhibit fungal pathogens operates through competition for nutrients and suitable niches on the root surface (Elad and Baker, 1985; Elad and Chet, 1987). Growth of soil microorganisms is highly dependent upon nutrient present in the rhizosphere or at the plant root exudates are probably significant in most interaction between PGPR and pathogens. Population of bacteria already established (pre-emptive competitive exclusion) on a plant root could act as sink for nutrients in the rhizosphere, thus reducing nutrient availability for pathogen stimulation and/or subsequent colonization of the root. The ability of PGPR to establish themselves in niches or rapidly compete in the use of nutrients that are shared by the pathogen is thought to be a general mechanism for antagonistic activity. In this way, biocontrol bacteria have the function as probiotic. Competition for nutrients such as carbon, nitrogen or iron is one of the mechanisms through which biocontrol strains can reduce the ability of fungal pathogen to propagate in the soil (Alabouvette, 1986). Failure of a pathogen to compete effectively with the biocontrol strain and use the available nutrient sources at those shared niches can disable the pathogen propagation. A classical example of niche exclusion is the control of leaf frost injury caused by P. syringae, which exhibits ice nucleation.

2.2.9 Lytic activity

Bacteria and fungi are capable of producing lytic enzymes such as chitinase, β-(1,3)-glucanase, cellulose, lipase and protease. Some of these enzymes are involved in the breakdown of fungal cell wall by degrading constituents such as glucans and chitins, resulting in the destruction of pathogen structures and propagules, Biocontrol bacteria producing chitinase, proteases, cellulase, and β-glucanase were shown to suppress pathogens (Moenne-Locoz and Defago, 2004). The degradation products released can be
used by the biocontrol agent to proliferate. Lytic enzymes can also act synergistically with other antifungal compounds (Duffy et al., 1996; Fogliano et al., 2002).

2.2.10 Hydrogen cyanide (HCN) production

HCN by certain rhizospheric microorganisms influences root and soil borne pathogens (Voisard et al., 1989). The mechanisms of plant growth inhibition and phytopathogens are mediated through inhibition of cytochrome C oxidase of respiratory chain in mitochondria and cell’s ATP consequently becomes depleted (Taiz and Zeiger, 2003). Several producers that reduce seed germination, seedling vigor, and subsequent plant growth have been isolated from root of seedling of various weeds (Kremer and Kennedy, 1996). Some cyanogenic rhizobacteria are typically host specific and remain associated with the roots of the host plant. Therefore, HCN produced in the rhizosphere of seedling by selected rhizobacteria is a potential and environmentally compatible mechanism for biological control of weeds and helps in minimizing potential deleterious effects on growth of the desired plants (Kremer and Souissi, 2001).

2.2.11 Induced systemic resistance (ISR)

Induced protection of plants against various pathogens by biotic or abiotic agents has been reported since 1930s when Chester (1933) proposed the term `acquired physiological immunity. Since then several terms have been used to describe the phenomenon of induced resistance such as `systemic acquired resistance (Ross, 1961), `translocated resistance (Hurbert and Helton, 1967). Induced resistance is defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection by a pathogen is called induced systemic resistance (ISR) or systemic acquired resistance (SAR) (Hammerschmidt and Kuc, 1982). The induction of systemic resistance by rhizobacteria is referred as ISR, whereas that by other agencies is called SAR (Van Loon et al., 1998). SAR is expressed to a maximum level when the inducing organism causes necrosis (Cameron et al., 1994) whereas ISR by PGPR typically do not cause any necrotic symptoms on the host plants (Van Loon et al., 1998). Both SAR and ISR are the activation of latent resistant mechanisms that are expressed upon subsequent or challenge

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inoculation with a pathogen (Van Loon, 1997). A degree of dependence on plant genotype is observed in the generation of these ISRs. Elucidation of the plant factors involved in the pathways leading to ISR and SAR has shown that induced disease resistance can be enhanced by the simultaneous activation of ISR and SAR pathways (Wees et al., 2000). In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Wei et al., 1991). The utilization of natural PGPR strains as inducers of plant defense responses may increase the chance of their applicability and offer a practical way to deliver immunization.

Recently, work on the spectrum of PGPR-mediated ISR against deterrent pathogens in different crop plants has been gaining importance (Wei et al., 1996; Liu et al., 1995a). Seed-treatment with P. yuorescens strain WCS 417 has protected radish through induction of systemic resistance not only against the fungal root pathogen F. oxysporum f. sp. raphani, but also against the avirulent bacterial leaf pathogen P. syringae pv. tomato and fungal leaf pathogens Alternaria brassicola and F. oxysporum. This implies that the same PGPR strain can induce resistance against multiple pathogens in the same crop. ISR by P. putida strain 89 B - 27 and S. marcescens strain 90-166 against anthracnose of cucumber was established by Wei et al. (1991). Seed-treatment of S. marcescens strain 90-166 has shown ISR in cucumber against anthracnose, cucumber mosaic virus, bacterial angular leaf spot and cucurbit wilt diseases (Kloeper et al., 1993; Liu et al., 1995b). Parallel experiments have shown that the P. yuorescens strain Pf1 induces resistance against different pathogens in different crops, viz., Rhizoctonia solani (Nandakumar, 1998), Colletotrichum falcatum in sugarcane (Viswanathan, 2001) and Pythium aphanidermatum in tomato (Ramamoorthy et al., 1999). The broad spectrum of PGPR-mediated ISR is more rewarding than narrow spectrum of disease protection. Hence selecting a suitable strain having potential to induce systemic resistance against multiple pathogens and pests is the most important task in the delivery of microbial agents to the field.

PGPR bring about ISR through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defense chemicals against the challenge pathogen. There are several bacterial determinants involved in the induction of systemic resistance by PGPR the most
important being lipopolysaccharides present in the outer membrane of bacterial cells, siderophore, salicylic acid production (Van Loon et al., 1998) and benzo thiadiazole (Sticher et al., 1997).

Lipopolysaccharides present in the outer membrane of PGPR are the major determinants of ISR in certain PGPR strains. LPS of *P. fluorescens* strain WCS 417 has induced systemic resistance in carnation against Fusarium wilt caused by *F. oxysporum* f. sp. *dianthi* (Van Peer and Schippers, 1992). Similarly, LPS of *P. fluorescens* strains WCS 374 and WCS 417 have induced systemic resistance in radish against *F. oxysporum* f. sp. *raphani* (Leeman et al., 1996). They also established that mutant of *P. yuorescens* strain WCS 417, lacking the O-antigen side chain of the LPS, has not induced resistance in radish indicating the O-antigen side chain of the LPS might have served as a signal or trigger in the induction of defense mechanism in plants. In contrast, LPS of *P. putida* strain WCS 358 having o-antigen side chain do not induce systemic resistance in radish. In another study, LPS of WCS 417r and mutant of WCS 417r lacking O-antigen side chain of LPS elicit defense mechanism in *Arabadopsis* (Van Wees et al., 1997). This indicates that ISR by LPS of PGPR varies with different host plant and lipopolysaccharide is not the only trait in determining the ISR. Other traits of PGPR are also involved in ISR.

2.3 Development of PGPR Inoculants

Development of PGPR inoculants is not refined and very laborious. This definitely has contributed to the lack of reliable inoculants available to market. The steps involved in bringing a PGPR to market are outlined in Figure 2. Current means of delivery of inoculants include peat, granular, liquid formulations and are not unlike present delivery mechanisms for legume inoculants. However, growth promotion is often inconsistent in the field compared to greenhouse or growth-chamber studies and is the dominant barrier to widespread use of PGPR. A major determinant of growth promotion is degree of colonization of the surface or interior of roots. The ability of a PGPR to establish in the rhizosphere is referred to as rhizocompetence. A big part of the laboratory and greenhouse screening is to obtain types of PGPR with the most rhizocompetence. However in field soil, environmental conditions and competition or displacement by the myriad of
organisms present in the rhizosphere limit colonization. Improving delivery as well as selection of PGPR to thrive on specific crop varieties and specific soil types promises more consistent crop performance response.

2.3.1 PGPR as endophytes

Endophytic bacteria are defined as bacteria which reside within the living plant tissues without doing substantive harm or gaining benefit other than residency. In addition to rhizosphere and rhizoplane colonization, certain PGPR are reported to be endophytes localized in the intercellular spaces of the root epidermal cells and vascular tissue (Chen et al., 1994; Benhamou et al., 1996a, b; M'Piga et al., 1997). Several factors favour endophytic bacteria as potential agents of ISR. Endophytes have a natural and intimate association with plants. The internal tissues of plants provide a relatively uniform and protected environment when compared with the rhizosphere and rhizoplane (Chen et al., 1994), where ectophytic bacteria must compete for nutrients with other microbes and endure fluctuations of temperature and moisture, as well as exposure to ultraviolet radiation on above ground surfaces. In spite of these advantages, the potential of bacterial endophytes has only been explored to a limited extent. Application of endophytic bacteria by stem injection in cotton plants reduced root rot caused by *Rhizoctonia solani* and vascular wilt caused by *F. oxysporum* f. sp. *vasingectum* (Chen et al., 1995). These bacteria move upward and downward from the point of application and by colonizing the internal tissues, can exclude the entry of a pathogen in the vascular stele. Endophytic bacteria have brought about significant control against *F. solani* in cotton and *Sclerotium rolfsii* in beans (Pleban et al., 1995). In pea, colonization of epidermis, cortex and vascular tissue in roots by endophytic bacteria prevented entry of mycelial growth of fungus or restricted the growth of mycelium to the epidermis. (Benhamou et al., 1996a). Seed treatment of tomato with endophytic bacterium *Bacillus pumilus* strain SE 34 prevented the entry of vascular wilt fungus *F. oxysporum* f. sp. *radicis-lycopersici* into the vascular stele and the mycelial growth was restricted to the epidermis and outer root cortex (Benhamou et al., 1998). Similarly application of *P. yuorescens* strain 63-28 restricted the growth of *Pythium ultimum* in pea (Benhamou et al., 1996a) and *F. oxysporum* f. sp. *radicis-lycopersici* in tomato (M'Piga et al., 1997). The use of an endophytic strain for inducing systemic
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resistance is more beneficial in vegetatively-propagated crops, like banana, sugarcane, and tapioca, for example Viswanathan and Samiyappan 2001 revealed the utility of endophytic P. yuorescens strain EP1 isolated from stalk tissues of sugarcane in inducing systemic resistance against red rot (Colletotrichum falcatum). Further, when the endophytic bacteria are introduced into the vegetatively propagated seed, the bacteria survives and moves in the vegetative part and subsequently the propagative seed will also have the introduced bacteria, thus minimizing the need for frequent application of bacterial strains.

2.3.2 PGPR formulation and methods of application

An important area of microbiological research with regard to biocontrol is the development of formulations that would preserve microbial activity for a period long enough to enable delivery of a selective product for field application. P. fluorescence can be applied in the form of a powder formulation (Kloepper and Schroth, 1981). It is desirable from the consumer's perspective to formulate and package PGPR in ways similar to chemical pesticides. Mass multiplication of PGPR in a suitable medium and development of a powder formulation was first carried out in 1980.

2.3.3 Limitations in the use of PGPR

There are several limitations to the use of PGPR for commercial use. Primarily efficacy is not reliable for most PGPR. This is because the mechanism of action of the PGPR in promoting growth is not well understood. Research needs also to be conducted determining if and how variations in soil type, management practices (e.g. agrochemical use, rotations) and weather affect PGPR efficacy. Research into PGPR is increasing, attempting to deal with these issues. From about 1988 when the term PGPR first became coined, research activity as gauged by scientific publications has steadily increased.

Secondly, hurdle in PGPR development is the lack of field testing. This because conducting field trials is laborious, costly and progression is slow as usually one crop can be grown in a year. In light of this, researchers resort to laboratory and greenhouse testing. Though valuable field testing is must be conducted repeatedly for selection of vigorous PGPR organisms as well as to demonstrate efficacy using different crop varieties, soil types and weather conditions. Of special note, bogus and unfounded efficacy claims that
abound in the PGPR inoculant industry in the United States, hampers product development. Companies amounting to being basically snake-oil salespeople tarnish the industry and prevent legitimate companies from raising capital required to bring effective products to market. Regulatory bodies in the United States (Environmental Protection Agency) and Canada (Canadian Food Inspection Agency and the Pest Management Regulatory Agency) have established procedures for the registration of PGPR. Demonstration primarily of efficacy and safety are required prior to PGPR registration. Producers should not use PGPR unless the product is registered and approved to be a Biofertilizer or Bioprotectant in Canada. Currently there are no such product registered in Canada and only a handful in the United States.

Recent advances in the molecular techniques also are encouraging in that tools are becoming available to determine the mechanism by which crop performance is improved using PGPR and track survival and activity of PGPR organisms in soil and roots. The science of PGPR is at the stage where genetically modified PGPR can be produced. PGPR with antibiotic, siderophore, and phytohormone can be made. However until GMO-PGPR is accepted by regulators in response to public will, such products will not be available commercially.

2.4 Need for Molecular Methods

Plant pathologists are faced with the important challenge to discern plant pathogenic variants within the *fluorescent Pseudomonas* species. Each *Fluorescent Pseudomonas* species is currently further subdivided at the intraspecific level into pathovars. Pathovars within each species cannot be reliably distinguished by their cellular metabolism or other phenotypic characteristics (Dye *et al.*, 1962; Palleroni, 1984; Van Zyl and Steyn, 1990). Therefore they are classified on the basis of their distinctive pathogenicity to one or more host plants (Young *et al.*, 1991). Unfortunately, identification based on pathogenicity test can be inconclusive and open to alternative interpretations (Gabriel *et al.*, 1992; Gabriel *et al.*, 1989). Several attempts have been made to classify pathovars and strains using alternative features of the pathogen. Serologic testing (Benedict *et al* 1989), fatty acid profiling (Stead, 1992), genomic and plasmid DNA analysis (Berthier *et al*., 1993; Denny
et al., 1988; Pecknold and Grogan, 1973) and protein analysis (Van Zyl and Steyn, 1990) have been used to classify pathovars and strains of different species.

2.4.1 DNA Fingerprinting methods

2.4.1.1 Random Amplified Polymorphic DNA (RAPD) Fingerprinting

One approach used for developing suitable species- or strains-specific probes for the detection of bacteria and fungi is based on the random amplified polymorphic DNA technique (RAPD) (Williams et al., 1990). The RAPD is a variation of conventional PCR where one primer of arbitrary sequence is used, and the annealing temperature is low (usually 35 °C). Species or strain-specific RAPD fragments are selected; sequenced, and suitable primers are devised to amplify the specific fragment in conventional PCR reactions. Such SCAR markers have been successfully used to develop species-specific probes for a number of Fusarium species (Nicholson et al., 1996; Nicholson et al., 1998; Schilling et al., 1996; Young et al., 2001), particularly fumonisn-producing fusaria (Geisen 1998), and for Aspergillus fumigatus (Brandt et al., 1998). Murillo et al., (1998) developed a primer pair based on the sequence of a random genomic clone for the detection of Fusarium moniliforme.

The random amplified polymorphic DNA (RAPD) fingerprinting assay detects small inverted nucleotide sequence repeats throughout the genomic DNA (Welse and McClelland 1990; Williams et al., 1990). In RAPD-PCR, amplification involves only single primers of arbitrary nucleotide sequence. The principle of RAPD assays is discussed in detail by Hadrys et al., (1992) and Tingey and Del Tufo (1993). In brief, a single primer binds to the genomic DNA on two different priming sites in an inverted orientation. Amplification between these points results in a discrete product. As each primer can be expected to amplify several discrete loci in the genome the final result is a profile of amplification products generally of varying sizes. In addition, at the primer attachment stage in the amplification the annealing temperature is kept low which also encourages a degree of primer-mismatching, and increases the potential number of amplification products. There are many advantages of this assay: (1) no prior information for DNA sequence is needed. The protocol is relatively simple and quick and only nanogram
quantities of DNA are required to give a PCR product, (2) the technique is pregerred when the genotypes of a large number of species, population or pathotypes has to be discriminated. RAPD markers can also be used to analyze the genotypes of fusion products and parents at different taxonomic levels, (3) this PCR-based assay is a good tool for creating genetic maps and has proved as an efficient method for the identification of molecular markers (Tingey and del Tufo 1993) and (4) the technique is suitable for studying population genetics and has been successfully used to differentiate among species and strains within species of plants, bacteria, animals and fungi (Williams et al., 1990).

RAPD-PCR assays have been used extensively to define fungal populations at specific, intraspecific, race and strain levels. In general, most studies have concentrated on intra-specific grouping, although others have been directed at the species level. Particularly in the determination of distinct intraspecific groups such as anastomosis groups in Rhizoctonia solani and pathogen groups (Levy et al., 1991; Bidochka et al., 1994; Yates-Siilata et al., 1995). In general, most studies have concentrated in intraspecific grouping, although others have been directed at the species level. Some examples of RAPD-PCR at species level include the production of species-specific probes and primers from RAPD data for Fusarium oxysporum f. sp. dianthi, Phytophthora cinnamoni, Tuber magnatum and Glomus mosseae. In some RAPD-PCR studies, band patterns have been used to differentiate both within and between individual species, as exemplified by species of Metarrhizium and Candida (Bridge et al., 1997a).

Single simple repetitive primers have been designed to amplify the microsatellite regions of fungal chromosomal DNA (Meyer et al., 1992; Bridge et al., 1997b). In most applications these primers have been used to group fungi at intraspecific levels (e.g. Bridge et al., 1997a). However, in some instances micro satellite-primed PCR has been used to generate species-specific patterns, and one recent example of this is the work on morels by Buscot et al., (1996) who found considerable homogeneity from both mono- and poly-sporic isolates of individual species.

Randomly Amplified Polymorphic DNA (RAPD) is a PCR technique that yields genetic markers without the need to obtain prior nucleotide sequence data (Williams et al.,
1990). Random nucleotide sequences are annealed to the template DNA under low stringency. This is followed by PCR amplification and electrophoresis to produce a DNA fingerprint. The RAPD technique is relatively easy, fast and requires only a minimum amount of starting material. However, the low stringency of the annealing process can produce PCR artifacts.

**Rep-PCR Fingerprinting**

The identification and classification of bacteria are of crucial importance in environmental, industrial, medical and agricultural microbiology and microbial ecology. A number of different phenotypic and genotypic methods are presently being employed for microbial identification and classification (see Fig. 2 and Louws et al. 1996). Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, biovar to the strain specific level (Fig. 3). Moreover, each method has its advantages and disadvantages, with regard to ease of application, reproducibility, requirement for equipment and level of resolution.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Subspecies</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sequencing</td>
<td>16S rDNA sequencing</td>
<td>ARDRA</td>
<td>DNA-DNA reassociation</td>
<td>rRNA-PCR</td>
</tr>
<tr>
<td>ITS-PCR</td>
<td>RFLP DDEA PFGE</td>
<td>Multilocus isoenzyme</td>
<td>Whole cell protein profiling</td>
<td>AFLP</td>
</tr>
<tr>
<td>RAPD’s APCR</td>
<td>rep-PCR</td>
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**Figure 3 Levels of phylogenetic classification, from the genus, species, subspecies, biovar to the strain specific level.**
Generally, DNA-based methods are emerging as the more reliable, simple and inexpensive ways to identify and classify microbes. In fact, the assignment of genera/species has traditionally been based on DNA-DNA hybridization methods and modern phylogeny is increasingly based on 16S rRNA sequence analysis (Woese 1987). Here, we describe a method referred to as rep-PCR genomic fingerprinting, a DNA amplification based technique, which has been found to be extremely reliable, reproducible, rapid and highly discriminatory (Versalovic et al. 1994; Louws et al. 1996).

Rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock 1992). Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensuses (ERIC) sequence, and the 154 bp BOX elements (Versalovic et al. 1994). These sequences appear to be located in distinct, intergenic positions around the genome. The repetitive elements may be present in both orientations, and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX, in the polymerase chain reaction (PCR) (Versalovic et al. 1994). The use of these primer(s) and PCR leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively, and rep-PCR genomic fingerprinting collectively (Versalovic et al. 1991; 1994). The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint (Versalovic et al. 1994; see Fig. 4). These fingerprints resemble "bar code" patterns analogous to UPC codes used in grocery stores (Lupski 1992).
The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies and strain level.

![Diagram](image)

**Figure 4 Overview of rep PCR genomic fingerprinting**

Rep-PCR genomic fingerprinting protocols have been developed in collaboration with the group led by Dr. J.R. Lupski at Baylor College of Medicine (Houston, Texas) and have been applied successfully in many medical, agricultural, industrial and environmental studies of microbial diversity (Versalovic et al. 1994). In addition to studying diversity, rep-PCR genomic fingerprinting has become a valuable tool for the identification and classification of bacteria, and for molecular epidemiological studies of human and plant pathogens (Louws et al. 1996 and Versalovic et al. 1993).

Analogous rep-PCR derived genomic fingerprints were generated from purified genomic, colonies on agar plates, liquid cultures, and directly from lesions on infected plants. REP-, ERIC- and BOX-PCR generated fingerprints of specific *Pseudomonas* strains were found to yield similar conclusions with regard to the identity of and relationship between these strains. This suggests that the distribution of REP-, ERIC- and BOX like is a reflection of their genomic structure. Thus, the rep-PCR technique appears to be a rapid, simple and reproducible method to identify and classify *Pseudomonas* strains, and it may be a useful diagnostic tool for these important microorganisms.
16S rDNA Amplification

Ribosomal DNA Gene Cluster.

The DNA sequences that encode for rRNAs have been extensively used to study the taxonomic relationships and genetic variations in fungi (e.g. Bruns et al., 1991; Hibbett 1992). The ribosomal RNA gene cluster is found both in nuclei and mitochondria, and consists of both highly conserved and variable regions (White et al., 1990). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved sequences found in the large subunit and small subunit genes have been exploited to study the many relationships among distantly related fungi (Bowman et al., 1992; Bruns et al., 1991). The spacer regions between the subunits, called the internal transcribed spacers (ITS), and between the gene clusters, called the intergenic spacers (IGS), are considerably more variable than subunit.

![Diagram of rDNA structure](image)

**Fig. 5.** General physical map of rDNA in fungi. The complete repeat unit is represented with genes location and spacer regions. ITS – Internal transcribed spacers, IGS – Intergenic spacers, ETS – External transcribed spacers, rDNA – Ribosomal DNA.

Diagram of rDNA unit is illustrated in the Fig. 5 includes three rRNA genes: the small nuclear 18S and the large nuclear 28 S genes. In one unit, the genes are separated by two internal transcribed spacers (ITS1 and ITS2), and two rDNA units are separated by the intergenic spacer (IGS). These genes have been used widely on studies on the relationships among species within a single genus or among interspecific populations (Klassen and Buchko 1990; Nazar et al., 1991; Baura et al., 1992; Lee and Taylor 1992; Kim et al., 1992; O’Donnell 1996; Buscot et al., 1996; Arora et al., 1996). The last rRNA gene (5S)
may or may not be within the repeated unit. Numerous sequence data are now available and allow the determination of primer sequences for the PCR amplification of different parts of the nuclear rDNAs (White et al., 1990). There are now many examples of the use of either RFLP or sequence differences in different spacer regions for discriminating between closely related species within a fungal genus.

**Intergenic Transcribed Spacer (ITS)**

The ITS region has been most frequently used as target for species-specific detection of fungi. The ITS consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit, and the large subunit rRNA genes. The ITS region is a particularly useful area for molecular characterization studies in fungi for four main reasons: (i) the ITS region is relatively short (600-800bp) and can be easily amplified by PCR using universal single primer pairs that are complimentary to conserved regions within the rRNA subunit genes (White et al., 1990), (ii) the multicycope nature of the rDNA repeat makes the ITS easy to amplify from small, dilute or highly degraded DNA samples (Gardes and Bruns 1993), (iii) the ITS region may be highly variable among morphologically distinct species (Gardes and Bruns 1991; Gardes et al., 1991; Baura et al., 1992; Chen et al., 1992; Lee and Taylor 1992; Gardes and Bruns 1993), ITS-generated RFLPs restriction data can be used to estimate genetic distances and provide characters for systematic and phylogenetic analysis (Bruns et al., 1991) and (iv) PCR generated ITS species-specific probes can be produced quickly, without the need to produce a chromosomal library (e.g. Sreenivasaprasad et al., 1996) and many researchers have selected sequences from the ITS region to develop species-specific probes because the sequences occur in multiple copies and tend to be similar within and variable between fungal species.

This region was targeted for the detection of spoilage yeast including Zygosaccharomyces sp. and *Torulaspora delbrueckii*, Saccharomyces (Arlorio et al., 1999), *Alternaria* sp. (Zur et al., 1999) and Penicillia (Boysen et al., 2000). was developed ITS-based primer pairs for the detection of fumonisin producing *Fusarium* species. Hendolin et al., (2000) developed a PCR technique coupled with multiplex liquid hybridization based on ITS specific primers for the detection of a number of fungi in
clinical specimens. PCR-amplified rRNA ITS sequences have been used for the characterization, identification and detection of Verticillium albo-atrum and V. dahliae (Nazar et al., 1991). In this study the identification of distinct clusters of non-homologous nucleotides in both the ITS1 and ITS2 regions enabled the design of specific primers that provides a reliable identification/detection method of these two important plant pathogens (Nazar et al., 1991). The same principle was used by Moukhamedov et al., (1993) who used sequences from amplified regions of the 5.8-28 S ITS regions to differentiate V. tricorpus from other species of Verticillium. The genus Rhizoctonia consists of a taxonomically diverse group of species that differ in many significant features, including their sexual and asexual stages (Sneh et al., 1996). Within the important phytopathological species R. solani, further intra-specific groups have been designated on the basis of anastomosis (anastomosis groups; AGs). Originally, RFLP analysis of nuclear rDNA was undertaken with probes and Southern blotting (Jabaji-Hare et al., 1990; Vilgalys and Gonzales, 1990), and more recently PCR-amplified rRNA has been found to be useful in examining the genetic relatedness within different AGs of R. solani and binucleate species of Rhizoctonia (Kanematsu and Naito, 1995 Liu et al., 1995b; Vilgalys and Cubeta, 1994; Hyakumachi et al., 1998). These workers identified six subgroups within AG1 and five within AG2 on the basis of their ITS-RFLPs.

Edel et al., (1996) differentiated several strains of F. oxysporum at the species level by RFLP analysis of a region of ITS and a variable domain of the 28S rDNA. Recently, Schilling et al., (1996) evaluated sequence variation in the ITS regions of F. avenaceum, F. culmorum and F. graminearum in order to distinguish between the three species. They found that the ITS sequences of F. culmorum and F. graminearum were not polymorphic enough to allow the construction of species-specific primers; however, sufficient sequence variation was found in the ITS1 and ITS2 regions of F. culmorum and F. graminearum to distinguish them from F. avenaceum.

Kageyama et al., (1997) have used species-specific primers derived from ITS sequences to detect P. ultimum in naturally infected seedlings. Bunting et al., (1996) used ITS1 sequences to examine the relationship of Magnaporthe poae to other species in the genus that has similar growth or phytopathogenic characteristics. Poupart et al., (1993)
used amplified ITS regions in their characterization of *Pseudocercosprella herpotrichoides* isolates. PCR-RFLP of ITS has also allowed the descrimination of *Tuber* species (Carbone and Kohn 1993), the identification of species within the *Gaeumannomyces-Phialophora* complex (Ward and Akrofi, 1994), *Sclerotinia* species (Carbone and Kohn, 1993) and *Pencillium* species (Lobuglio et al., 1993). One further example of a genus where extensive use has been made of the ITS region of the species level is *Collectotrichum*. Sherriff et al., (1994) compared a range of isolates of *Colletotrichum* species on the basis of a 886 bp region of the LSU and the ITS2 regions, and were able to use this information to distinguish between individual species. Further extensive species characterization has been undertaken in this genus, leading to the development of a number of species-specific primers (Sreenivasaprasad et al., 1996).