

## Endophytic microorganisms: a review on insect control and recent advances on tropical plants

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In the past two decades, a great deal of information on the role of endophytic microorganisms in nature has been collected. The capability of colonizing internal host tissues has made endophytes valuable for agriculture as a tool to improve crop performance. In this review, we addressed the major topics concerning the control of insects-pests by endophytic microorganisms. Several examples of insect control are described, notably those involving the interactions between fungi and grazing grasses from temperate countries. The mechanisms by which endophytic fungi control insect attacks are listed and include toxin production as well as the influence of these compounds on plant and livestock and how their production may be affected by genetic and environmental conditions. The importance of endophytic entomopathogenic fungi for insect control is also addressed. As the literature has shown, there is a lack of information on endophytes from tropical hosts, which are more severely affected by pests and diseases. Having this in mind, we have included an updated and extensive literature in this review, concerning new findings from tropical plants, including the characterization of endophytic fungi and bacteria microbiota from several Amazon trees, citrus and

medicinal plants among others.

The natural and biological control of pests and diseases affecting cultivated plants has gained much attention in the past decades as a way of reducing the use of chemical products in agriculture. Biological control has been frequently used in Brazil, supported by the development of basic and applied research on this field not only in our country but also in South America as it can be found in several reviews (Lecuona, 1996; Alves, 1998; Melo and Azevedo, 1998). In fact, by having vast agriculturable areas and most of its territory in the tropical region, Brazil and all Latin America, show their agriculture severely affected by agricultural pests. The use of agrochemicals, although decreasing the attack of insects and phytopathogenic microorganisms, still represents a high risk to field workers and consumers. In addition, their use is, in certain cases, economically unviable. The control of pests and diseases by means of biological processes i. e., use of entomopathogenic microorganisms or those that inhibit/antagonise other microorganisms pathogenic to plants, is an alternative that may contribute to reduce or eliminate the use of chemical products in agriculture.

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Agriculture by its own nature is antiecolological and, with the use of implements as fertilizers on a large scale, insecticides, fungicides, herbicides and, antibiotics, profound biological modifications have been occurring. These products, such as insecticides and fungicides, aim to the control of pests and phytopathogenic fungi. However, they are responsible for eliminating important species of insects that control other pests and microorganisms that are performing a crucial role in the environment, inhibiting the growth and the multiplication of other microorganisms. One group of microorganisms that is affected by these antropogenic modifications is the endophytes, which includes mainly fungi and bacteria. Endophytic microorganisms are those that inhabit the interior of plants, especially leaves, branches and stems, showing no apparently harm to the host (Azevedo, 1998). In the 70's, endophytes were initially considered neutral, not causing benefits nor showing detriment to plants, but later on they started to be better studied. By that time it was possible to conclude that in many cases, they had an important role in host protection against predators and pathogens.

In the present review we will focus on examples of associations between endophytic microorganisms and plants, especially those that result in insect control. It is also our purpose to update the literature on endophytes with new information coming from tropical plants.

### Endophytic microorganisms

All microorganisms that inhabit, at least for one period of their life cycle, the interior of a vegetable, may be considered as an endophyte. The distinction among endophytes, epiphytes (those that live on the surface of plants) and phytopathogens (those that cause diseases to plants) is of pure didactic meanings. There is a gradient separating them and thus, it comes to a situation where it is a very difficult task to draw limits to discriminate each category. For more details about this subject we suggest the review of Azevedo (1998) and the books of Redlin and Carris (1996), Isaac (1992) and, Fokkema and Van den Heuvel (1986). Mycorrhizae and nitrogen-fixing bacteria also live in an intimate relationship with their hosts and could be considered as endophytic microorganisms. However, mycorrhizae are distinguished from other root endophytes by the fact that they possess external structures as hyphae. Likewise, nitrogen-fixing endophytic bacteria such as *Rhizobium*, which form external structures called nodules, are distinguished from other root endophytic bacteria. These two groups of microorganisms are well known and extensively studied and therefore, will not be further considered in this review, except for a specific topic concerning endophytic diazotrophic bacteria.

Although they were already described in the past century, endophytic microorganisms only received considerable attention in the last 20 years, when their capacity to protect their hosts against insects-pests, pathogens and even domestic herbivores as sheep and cattle was recognized. As

endophytic fungi and bacteria started to be better analysed, it became clear that they could confer other important characteristics to plants, such as greater resistance to stress conditions (i. e. water), alteration in physiological properties, production of phytohormones and other compounds of biotechnological interest (i. e. enzymes and pharmaceutical drugs). In addition to the economical aspects, the study of endophytic microorganisms has strong academic interests, concerning the discovery of new microbial species, mainly when tropical hosts are investigated.

### Earlier reports of endophytic microorganisms in the control of insects-pests

In the early 80's the specialized literature published the first reports showing that endophytic microorganisms, in this case fungi, could play an important role inside plants. It was demonstrated that the presence of these microorganisms in their respective hosts could result in the reduction of insect attacks. Landmark reports on the subject started in 1981, less than two decades ago. From 1981 to 1985, which may be considered a historical period to the field, it was demonstrated the existence of plant protection against herbivore insects given by endophytic microorganisms. The nature of the protection and the variables involved in the process were also initially addressed.

Webber (1981) was probably the first researcher to report an example of plant protection giving by an endophytic fungus, in which the endophyte *Phomopsis oblonga* protected elm trees against the beetle *Physocnemum brevilineum*. It was suggested that the endophytic fungus *P. oblonga* was responsible for reducing the spread of the elm Dutch disease causal agent *Ceratocystis ulmi* by controlling its vector, the beetle *P. brevilineum*. The author associated the repellent effect observed towards the insect to toxic compounds produced by the fungi. This was confirmed four years later by Claydon et al. (1985), who showed that endophytic fungi belonging to the *Xylariaceae* family synthesize secondary metabolites in hosts of the genus *Fagus* and that these substances affect the beetle larvae.

Other earlier observations on the control of insects-pests by endophytic fungi are those of Funk et al. (1983) showing protection of the perennial ryegrass *Lolium perenne* L. against the sod webworm. Gaynor and Hunt (1983) observed in several ryegrasses that high fungi infection is correlated with a decrease in the attack frequency of the Argentine steem weevil, *Listronotus bonariensis*. These authors also showed that these associations may be complex and factors like the level of nitrogen fertilizers may as well affect attack frequency. With the same insect-plant interaction, in New Zealand, Barker et al. (1984) and Prestidge et al. (1984) observed that plants free of endophytic fungi of the genus *Acremonium*, nowadays classified as *Neotyphodium* (in this review both names will be used according to the original reports) are severely

attacked. Lasota et al. (1983) had also correlated in the white spruce *Picea glauca*, death rate of the Homoptera *Adelges abietis* when galls are infected with the endophytic fungus *Cladosporium sphaerospermum*. In *L. perenne* and members of the genus *Cyperus*, weight gain and survival of the insect-pest, *Spodoptera frugiperda*, were affected by endophytic fungi like *Balansia cyperi* (Clay et al., 1985a; 1985b; Hardy et al., 1985). Studies related to this insect-pest affecting different species of *Lolium* were also conducted by Larch et al. (1985b) with two endophytic fungi, *Acremonium lolii* and a member of the genus *Gliocadium*. The authors discussed the benefits and risks resulting from the increase in toxin production to insect control and resulting harm to domestic animals that would feed on these plants. They observed also that *Acremonium* affected aphids feeding on members of the genera *Lolium* and *Festuca* (Latch et al., 1985a). Ahmad et al. (1985) verified similar effects of the same fungi over the grasshopper *Acheta domesticus*. Johnson et al. (1985), using choice tests showed that insects would feed on endophyte-free *Festuca* plants rather than on infected samples.

These examples of earlier reports in the literature show not only the importance of microorganisms in controlling insects-pests in agriculture but also the diversity of control mechanisms displayed by endophytes. The role of endophytic fungi in the control of insects has been the subject of several reviews (Saikkonen et al., 1998; Carroll, 1991; 1995; Breen, 1994; Clay, 1989). In this review the major themes related to the control of insects-pests by endophytes will be addressed, together with the perspective of applying this knowledge to the development of a biological control by means of classical and molecular biology techniques.

### Effects of endophytic microorganisms towards insects

In addition to the earlier reports cited above, a great deal of data has been collected on the effects of endophytic microorganisms towards insects. It was verified that the plant protection process promoted by endophytes is more complex than one might have expected. Endophytes can not be considered, therefore, a magic box for the herbivory control. Although, in most of the published reports on the subject they show a mutualistic interaction, that is, positive in respect to the host and negative to the insects-pests and there are also reports of neutral or even antagonistic interactions. Almost the totality of the research done has been performed with plants from temperate climate, particularly with grazing grasses as the tall fescue, *Festuca arundinacea* Schreb. and *L. perenne* L. They are plants of great importance for countries in the Oceania, Europe and for the USA, where they are widely used to feed livestock. Only in the Southeast of the USA there are 10 million cultivated hectares of *Festuca* (Clay, 1989). On the other hand, intoxication, weight loss, decrease in milk production and other problems in cattle fed on these plants had been

detected a long time ago. Thus, the correlation between detrimental effects on herbivorous domestic animals and insect control became the next step to be addressed.

### Endophytes and the decrease in the incidence of insects on host plants

In addition to the reports mentioned above (Webber, 1981; Funk et al., 1983; Gaynor and Hunt, 1983; Lasota et al., 1983; Barker et al., 1984; Prestidge et al., 1984; Ahmad et al., 1985; Clay et al., 1985a; 1985b; Claydon et al., 1985; Hardy et al., 1985; Latch et al., 1985a; 1985b; Johnson et al., 1985), that correlate endophytic fungi with negative effects towards insects, other reports kept accumulating in the following years. Some of them will be presented here but we should keep in mind that most of them refer to the host *F. arundinacea* and *L. perenne*. In fact, Clay (1989) stressed that, although many endophytic fungi have already been isolated from around 80 grass species of temperate countries, only six genera are the most frequent and among them *Acremonium*.

The situation, since Clay's review (1989) about ten years ago, has gained minor changes. For example, in *L. perenne*, Ahmad et al. (1986) showed that the blue grass billbug *Sphenophorus parvulus* is less frequent on plants infected with endophytic fungi. Also Ahmad et al. (1987) verified the same results in grasses attacked by the Southern armyworm *Spodoptera eridania*. Saha et al. (1987), studying endophytes in fine fescue, verified that endophytic fungi from the genus *Acremonium* were associated with host resistance to *Blissus leucopterus hirtus* and the same observation was made by Mathias et al. (1990), in *L. perenne*-*A. lolii* interaction. Kindler et al. (1990), verified that *Acremonium* reduced the attacks of the aphid *Diuraphis noxia* in *Lolium* as well as in *Festuca* and the same observations were made by Clement et al. (1990; 1992). In the interaction *F. arundinacea*/*A. coenophialum*, protection against leafhopper and froghopper species was evident, notably in periods of high incidence of insects (Muegge et al., 1991). Eichensner and Dahlman (1992) verified reduced survival and reproduction rates of the aphid *Rhopalosiphum padi* in the same interaction.

Kanda et al. (1994) reported the preference of larvae from the bluegrass webworm *Parapediasia teterrella* for diets with endophyte-free plants of *L. perenne* and *F. arundinacea*, to a point that the larvae would starve to death if only plants infected with *Acremonium* were available. In the field, endophyte-free species were severely attacked by insects, whereas those infected with *Acremonium* stayed almost free of insect larvae.

As it will be seen in more details, endophytic fungi (Clavicipitaceae, Ascomycetes) synthesize alkaloids during plant infection, reducing the survival and the development of *S. frugiperda* in Graminae and Cyperaceae. In the flour beetle *Tribolium castaneum*, diets composed of grinded seeds of *F. arundinacea* and *L. perenne* infected with

endophytic fungi resulted in a reduction of infested seeds and insect growth, notably in *F. arundinacea* (Cheplick and Clay, 1988). The authors concluded that this is a general characteristic of infected grasses, that is, insect resistance and consequential improved performance of plants in nature.

Considering other grasses, the studies are sparse. However, the host protection process displayed by endophytic fungi against insects seems to be general. For example, alcoholic extracts from *Poa ampla* infected or not with *Neotyphodium typhnium* were assayed against mosquito larvae. Only the extracts from the infected plants were effective against the insect, whereas the extract obtained directly from the fungi was not (Ju et al., 1998).

Although plants other than grasses can be protected by endophytes, the data on these cases are not so conclusive nor numerous. Sherwood-Pike et al. (1986) described in oak (*Quercus* sp.), the role of the endophytic fungus *Rhabdocline parkeri* against the insect *Contarinia* sp. Petrini et al. (1989) found that the endophytic fungi *Phyllosticta* sp. and *Hormonema dematioides* were dominant in the balsam fir *Abies balsamea*. The first fungus colonized preferentially galled needles attacked by *Paradiplosis tumifex*, being a candidate for biological control. In the black spruce *Picea mariana*, Johnson and Whitney (1994) found that 21 hyphae extracts out of 100 were toxic to *Choristoneura fumiferana*, and extracts of fungi isolated from older leaves were more effective than young ones in cell culture. The dry weight and development of larvae fed on hyphae extracts were reduced.

The results described above show the efficiency of some endophytic fungi in reducing host damage provoked by insect attack. The examples given so far include researches performed in field and lab conditions, as well as comparisons between field and lab experimental data. Choice tests on diets composed of endophytes-free and infected plants are often used along with several choice combinations. Although, the majority of the works cited above are quite conclusive, the results do not always finish up in the control of insects by endophytes. As it will be shown, there are cases of neutrality or even improvement of insect performance in plants colonized by endophytic microorganisms.

### **Endophytes not reducing insect attack to their hosts**

Although in all of the given examples there has been insect control resulting from the presence of endophytic fungi in the hosts, this is not always obvious. In 1986, Kirfman, studied the relationship between endophyte infestation and insect abundance in *F. arundinacea*. Some Cicadellidae species and the Chrysomelidae *Chaetocnema pulicardia*, decreased in numbers with the consecutive increase in endophytes. However, there were a concomitant increase in abundance of another Cicadellidae species (*Exitianus*

*exitiosus*) and an insect from the genus *Bruchomorfa*. The authors suggested that the elimination of toxic endophytes from grasslands would provoke a biological imbalance, resulting in higher incidence of certain insects-pests and decrease of others. In the first half of the 90's, Breen (1993a; 1993b) developed a wide study with endophytes of the genus *Acremonium* in several species of turfgrasses in relation to the attack of three aphid species and, two Lepidoptera species, *S. frugiperda* and *S. eridania*. Although, in most cases it was observed a decrease in insect survival, each case was considered different from the others. This suggests that the plant genotype, the endophyte and the insect must be analysed separately because, depending on the conditions, control may not be achieved or even, an opposite effect can occur, such as an increase in pest incidence in the presence of the endophyte.

Therefore, the presence of endophytes, even of those belonging to groups where some members are known to control insects, do not always result in an effective pest control. In oak, studies performed during a four-year-period showed that there are seasonal differences concerning the presence of endophytes. In this case, the incidence of the Lepidoptera leafminer *Cameraria* sp. was not altered by the endophyte and, in some cases, there was higher incidence of the pest, coinciding with the increase of endophytes. These results suggested that the leafminer promotes the entry and colonization by the endophytic fungi (Faeth and Hammon, 1996; 1997a).

Experiments with oak plants infected or not with the fungus *Asteromella* sp. did not show significant differences in size and in the survival period of insect larvae. In addition to *Asteromella*, other endophytes like *Plectrophomella* sp. and a filamentous yeast were also analyzed and, in these cases, larvae fed on leaves colonized by two of these fungi showed a slower development (Faeth and Hammon, 1996; 1997a; 1997b). In *Betula pubescens*, it was not detected any effect of endophytes on the control of the leaf beetle *Phratora polaris* (Lappalainen and Helander, 1997) Murphy et al. (1993) analyzed in *F. arundinacea* the influence of endophytic fungi towards species of billbug (genus *Sphenophorus*), responsible for reducing turf covering up to 25% in the absence of endophytes. In pests like sod webworms and scarabeid grub larvae, host protection given by the fungi was observed in some cases and in others the differences observed between endophyte-free and infected plants were not significant.

On the other hand, in some cases there were no protection at all against the Japanese beetle *Popillia japonica* and *Cyclocephala lurida* when preference tests were carried out with diets containing endophyte-free and infected plants (Crutchfield and Potter, 1994). Lewis and Clements (1986) also observed no increase in resistance against larvae of the fruit fly *Oscinella* spp. in grasses, when both types of plant diets were tested. Situations in which control was not achieved may be explained by the insect behaviour on the plant. In extremely mobile insects, such as the third instar

of the Hemiptera hairy chinch bug *Blissus luciopterus*, an intense movement towards Kentucky bluegrass plants not infected with *Acremonium* decreased the endophyte's potential as a pest control (Carriere et al., 1997). In other cases, the small amount of alkaloids accumulated in the host plants may explain the lack of differences in weight, larvae survival and, adult emergence when insects were fed on plants infected or not by the fungus. This is the case reported by Lewis and Vaughan (1997), when studying *Tipula* spp. larvae fed on *L. perenne* infected with the fungus *Neotyphodium lolii*.

It seems that the initial overestimated perspective of endophyte-directed insect control is progressively being constrained. For example, in four strains of wild barley infected with the fungus *Neotyphodium*, two showed decrease in the aphid *Diuraphis noxia* densities, whereas the other two showed no differences from the endophyte-free plant (Clement et al., 1997). In the same manner, Tibbets and Faeth (1999) assayed the effects of *Neotyphodium*, introduced into two types of *Festuca*, against the leafcutter ant *Acromyrmex versicolor*. Experiments where the ants could choose between endophyte-free and infected plant diets were performed. Assays where only infected plants were offered were also conducted to determine the fungus effects on pest survival and development. In the preference tests, endophyte-free plants of only one type were preferred. In experiments that presented only infected plants, the queens did not survive more than six weeks. However, the effects were highly variable and dependent on the fungal genotype and host, in addition to environmental factors (Tibbets and Faeth, 1999).

In an interesting study, Wilson and Carroll (1997) investigated a system where an endophytic fungus provokes mortality of the gall-forming insect *Besbicus mirabilis*, but not of *Bassetia ligni*, both feeding on the same plant. It was noticed that to form galls, *B. mirabilis* avoids leaf parts where the endophyte is more frequently found. However, due to selection pressure of grazing herbivores and developmental constraints, the insect was forced to form galls on leaf parts other than those endophyte-free, resulting in the observed mortality. However, *B. ligni* occupies a highly infested area in the leaf sheet, where the endophyte has no growth activity. In addition, gall emergence for *B. ligni* occurs as the fungus reaches its higher infection density, so this specie does not come into contact with highly infected tissues for long.

Carroll (1995), in a review concerning endophytes from forest essences, questioned most works on the effects of endophytic fungi as mutualists in the control of herbivorous insects. According to him, more convincing proofs should be acquired on these effects and, for this, there is the need to show that the fungi inhabit tissues that are preferentially attacked by the insect. There should be also a correlation between endophyte infestation and decrease in herbivory or insect death and finally, endophyte-free and infected plants

and the insect destiny in both cases must be compared. He also argued the need to prove that the fungi produce toxins.

On most works done on this subject, some but not all of these proofs mentioned above were observed. Furthermore, he highlighted the fact that most of the data were obtained with an insect, an endophyte and, a host whereas in nature the problem is much more complex, involving interactions between endophytes, several herbivorous, host and other plants cohabiting in the same space. Other problems are the difficulties to succeed in inoculating endophyte successfully and in proving toxin production by forest endophytes *in vivo*. Carroll (1995) issued the factor coevolution considering the interactions of endophyte-host-insect. These three components have long evolutionary histories and, according to the author, they should live in harmony, except in unusual and artificial conditions as those of the modern agriculture. That makes the factor coevolution not so important, so in new conditions the interactions may be modified. We should remember that agriculture practices have only 10 thousand years, compared to millions of years of coevolution. Thus, agrochemical use, climate changes and, modifications in soil and cultural practices turn the interaction of the three components not much predictable.

### Mechanisms of insect control displayed by endophytic fungi

Since the pioneer works in the field, the capacity of endophytic fungus to repel insects, induce weight loss, growth and development reduction and even to increase pest death rate, was correlated with toxin production. In several cases, it was shown that the mode of action of certain fungi was based on the capability to render the plant unpalatable to several types of pests like aphids, grasshoppers, beetles, etc (Carroll, 1988; Clay, 1988a; 1988b).

Bacon et al. (1977) established for the first time a correlation between an endophytic fungi, *Epichloë typhina* and the toxicity of its host, *F. arundinacea*, to herbivorous domestic mammals. It is now a fact that several toxins are produced by endophytic fungi and that these substances confer host protection against different herbivorous. We suggest for more details on toxins the review of Bacon and Hills (1996) in which the most important toxins found in *L. perenne*, like ergot alkaloids of two types, ergopeptine and clavine and neurotoxines called lolitrems are described. In opposition to the ergot toxins that are isolated directly from the endophytic fungi, the neurotoxins produced by endophytes like *A. lolii* are only precursors of toxins like, for example, paxiline. It is not known if the precursor is converted in lolitrems by the plant or if the fungus is not capable of synthesizing it in pure culture but is able to do so while inside the plant.

Examples of toxin production by endophytic fungi, notably those colonizing grasses are considerably abundant in the

literature. For instance, in 1986, Miller showed that the protection of Canadian fir that against the spruce budworms resulted from the production of toxic secondary metabolites by endophytic fungi. In 1988, Prestidge and Gallagher established a relationship between the presence of the fungus *A. lolii* in *Lolium perenne* and the growth, survival and feeding behaviour of *Listronotus bonariensis* larvae. In this case, the reduction in insect attacks towards infected plants was due to a strong toxin, lolitrem B, also toxic to mammals. This toxin, once added to insect diets, reduced insect growth and survival. Its assimilation occurs by ingestion but not by absorption through the insect integument.

In certain cases, the production of toxin by the endophytic fungi was a plausible explanation for interactions resulting in natural insect control. Thus, Clark et al. (1989) showed in *Abies balsamea* and red spruce *Picea rubens*, that from 900 samples of fungal isolates, five produced toxic substances and three of those produced powerful toxins that, once extracted and given to insects, caused death and decreased development rate of *C. fumiferana*. Many other cases of insect control involving toxins produced by endophytic microorganisms can be found in the literature. Siegel et al. (1990) verified the presence of the alkaloids N-formil, N-acetyl loline, peramine, lolitrem B and, ergovaline during plant attack by aphids. Several grasses infected with *Acremonium* spp. and *E. typhina* were analysed. These fungi, generally produce alkaloids, mainly peramine and ergovaline. Peramine, lolitrem B and ergovaline were found in *Lolium* and *Festuca* infected with *A. coenophialum* and *A. lolii* and in *Festuca longiflora* infected with *E. typhina*. Individuals of *Rhopalosiphum padi* and *Schizaphis graminum* did not survive in grasses containing the alkaloid loline. On the other hand, ergovaline did not affect both insect species.

The methanolic extracts of *F. arundinacea* infected with *A. coenophialum*, contain lolines of fungal origin, which are able to alter feed behaviour and weight of insects-pests. Diets amended with extracts containing loline derivatives reduced weight and altered certain behaviours of *S. frugiperda* and *O. nubilalis*, notably in the first insect (Riedell et al., 1991).

Patterson et al. (1992) observed in *Lolium* and *Festuca* that the production of alkaloids by *Acremonium* reduced attacks of the Japanese beetle *Popilla japonica*. Other works find that ambient variations may affect toxin production by endophytic fungi. Breen (1992) verified that changes in temperature and seasons modify toxin levels in the plant. This is the case of the peramine toxin, produced in *L. perenne* by *A. lolii*. The antixenosis towards the aphid *S. graminum* is dependent on peramine and the endophyte concentration so, a natural consequence is that the effect on the insect-pest will also be modified. The specificity between certain endophytic fungi and host plants led Leuchtman (1992) to suggest a further study on the occurrence of physiological races in endophytic fungi, that

could be important to the development of new biological controls.

Surveys have been carried out aiming to the discovery of new toxins useful to insect control. Two new active toxins against the Spruce budworm *C. fumiferana* were found in an unidentified endophytic fungus infecting the wintergreen *Gaultheria procumbens* (Findlay et al., 1997). Alkaloids from *N. lolii* and *L. perenne* are capable of altering insect behaviour. Several of these alkaloids were added to the diet of adult individuals of the Coleoptera *Heteronychus aratur*. Peramine, lolitrem B, lysergol-type alkaloids, festuclavine and lisergic acid showed no effects on the insect. Ergovine showed moderate effects whereas ergotamine, ergovaline from the ergot-type alkaloid family seem to be responsible for the plant resistance (Ball et al., 1997b).

Recently, Miles et al. (1998) showed that endophytic isolates of *Neotyphodium* sp. produce N-formilonine and a paxiline analogous in the host *Echinopogon ovatus*. These compounds show insecticidal activity against *L. bonariensis* and other insects.

The majority of works related to toxin production was performed in grasses. However, Calhoun et al. (1992), for the first time, identified toxic products synthesized by endophytic fungi in woody plants and that were able to modify growth and death rates in larvae of the spruce budworm *C. fumiferana* feeding on balsam fir. The endophytes in this case were identified as *Phyllosticta* and *Hormonema dematioides* and the toxic compounds were mainly heptelidic acid and rugulosine. Bills et al. (1992) also detected the existence of tremorgenic toxins in tropical woody plant infected with an endophytic fungus from the genus *Phomopsis*.

### Genetic and environmental modifications influencing insect control by endophytes

The expression of insect resistance may be affected by several factors, i. e. active amounts of allelochemicals, plant genotype (Breen, 1993a; 1993b), endophyte concentration (Breen, 1992), soil fertility and endophyte genotype (Breen, 1992; Christensen et al., 1991; Bacon, 1988). Hydric stress, temperature, soil pH, insect-pest resistance and other factors may also affect the endophyte concentration and toxin production. Breen (1994), idealized a model with all of these interactions affecting the increase or decrease in plant resistance to insects-pests in the presence of endophytes. Likewise, as mentioned before, in each case variable results may be found. Some generalization may be drawn like, for instance, that endophytic control of insects is more effective in pests feeding on leaves or that results obtained with *L. perenne* are more variable than those obtained with *Festuca* because the last has less variability. For example, in plants of *F. arundinacea* and other grasses, infected or not with *A. coenophialum*, attacks of two Coleoptera were less frequent in the first insect instars, but afterwards there were

variations indicating that many factors play a role in the insect control (Potter et al., 1992).

Concerning alterations due to nutrients in the soil, especially nitrogen fertilizers, there are studies showing the existence of this interaction, which is very significant. Arechavaleta et al. (1989) had already verified in the *F. arundinacea* endophyte *A. coenophialum* that the production of ergopeptide alkaloids increased in soil amended with nitrogen fertilizers and during a moderate hydric stress, which consecutively altered insect-pest control.

A study about the interaction between nitrogen fertilization, pests and the endophytic fungus *A. coenophialum* showed that in a general manner, insects like *S. frugiperda* developed better in nitrogen fertilized plants not infected by the endophyte. However, considering this and other insects, the occurrence of *A. coenophialum* blocks insect development. The results, therefore, are quite variable and do not allow us to draw any general correlation between nitrogen fertilizer and endophyte-mediated pest control in *F. arundinacea* that will work in all cases (Davidson and Potter, 1995). In the same manner, Bultman and Conard (1998) verified interactions among several factors like nutrient levels and plant damage during endophytic fungi control of *S. frugiperda* in the host *F. arundinacea*. The insect pupal mass was negatively influenced by the endophyte in low nutrient levels but the effect was negligible in plants highly fertilized. This study showed again that these interactions are complex and influenced by several factors, in this case by fertilizer application.

More recent studies, in hydroponic conditions, conducted by Richardson et al. (1999) verified that the Chewings fescue *F. rubra* subspecies *commutata* showed differences in alkaloid contents in respect of endophyte colonization and amounts of several nitrogen sources. Fluctuation in the contents of the alkaloids ergovaline and peramine in colonized plants was verified, which will consecutively alter insect resistance.

Clay (1996), in a study concerning ambient changes and plants colonized by alkaloid producing clavicipitaceus fungi, considers the interaction plant-endophyte-herbivorous i. e. insects, showing that the fungi may aid the host to compete with other plants and therefore, altering plant community structures and dynamics.

Other types of interactions, taking into account a main host grass (*Festuca* or *Lolium*) and others grasses living in a common space, were conducted by Clay et al. (1993), who analyzed changes resulting from these plant-plant interactions in insect control and presence of endophytic fungi. They analysed the effects of a member of the genus *Acremonium* in relation to attacks of *S. frugiperda* larvae to the tall fescue *F. arundinacea* and the red fescue *F. rubra* grown separately or mixed with other grasses. In general, insect attacks were reduced in hosts colonized by

endophytes, because the plants were less chosen by the pests. However, different degrees of insect attacks were observed according to the plant associations analysed. These studies demonstrated that plant interactions would change the effects of endophytic fungi in insect control.

## Endophytic entomopathogenic fungi and their inoculation into plants

It is widely known the existence of fungi and bacteria able to cause disease in insects. Fungal species like *Metarhizium anisopliae*, *Beauveria bassiana* and others are often used in the biological control of agriculture insects-pests. Examples may be found in Lecuona (1996), Alves (1998) and Melo and Azevedo (1998). If these microorganisms inhabit plant tissues as endophytes, they will obviously be able to act in insect control. Even in situations where they are not present in the plant, artificial inoculation techniques may circumvent this obstacle. As we will see in this review, endophytic fungi that do not colonize insects and cause disease, may be used as a natural insect control as well as an effective insect control if they are artificially inoculated into plants and display characteristics against the pests.

Unfortunately, works concerning this promising line of research are scarce and, when were conducted, involved a temperate climate system, as usual, where the pest control problem is not so urging as in tropical cultures.

Clay (1989), in a review on the potential of insect control by endophytic fungi, stressed that insect biocontrol may be improved by the development of artificial inoculation techniques, as those developed by Latch and Christensen (1988) and Leuchtman and Clay (1985).

As endophytic fungi, notably members of the genus *Acremonium*, are able to control some insects-pests, it is important to know if they can be inoculated in endophyte-free plants and, if they can transmit to the host the capacity to resist to certain pests. That was done with success by Koga et al. (1997), by infecting *F. arundinacea* and *L. perenne* seeds with the endophytic fungus *Acremonium* and, as a result, obtained plants resistant to the bluegrass webworm *P. teterrella*.

In the same manner, Pereira et al. (1999), using a tropical host, the banana tree, inoculated artificially an endophytic fungus tagged with a genetic marker for resistance to fungicides. It was observed that not only the inoculation worked well, but also the mutant fungus was kept in competition with wild type ones. These results demonstrated that inoculation of valuable strains for biological control will not only be used with success but, modified microorganisms, once inoculated into a host, will be able to coexist and even overcome wild type strains of the same species.

An important point, at least in grasses, resides in the fact that several fungi species may be transmitted by seeds, where the biocontrol becomes able to be passed to the next

generation. There are works showing the importance of endophytic fungi colonizing seeds in insect control. Knoch et al. (1993) observed in *F. arundinacea*, in which the endophytic fungus *A. coenophialum* is transmitted by seeds, that ants often prefer to collect endophyte-free seeds, avoiding those infected. Even seeds infected with the fungus are later discarded, favouring seed dispersion and germination.

Entomopathogenic fungi can be placed inside plants. This procedure was done in corn (Bing and Lewis, 1992a; 1992b; 1991) to verify the potential of these fungi as endophytes to control the European corn bore *Ostrinia nubilalis*. *Beauveria bassiana* in this case was inoculated by injection and aspersion and was detected endophytically, colonizing several corn tissues and blocking the insect attack in some of its developmental stages. Injection resulted in 95% colonized plants and aspersion in 98,3%.

An interesting finding was that in 33,3% of the control plants, therefore not treated, the fungus was found, indicating that the fungus is a natural endophyte. It was not determined the process by which the fungus invades the plant, but the movement was shown to be passive, occurring in the vascular system. It was also showed that the infected insect does not transmit the fungus because plants free of attacks could contain the fungus in an endophytic state. Bing and Lewis (1993), also refer to the fact that the entomopathogenic fungus *B. bassiana* has been used for a long time in the control of the European corn borer and this fungus, as mentioned before, is isolated as endophyte from corn. This could explain the pest suppression in certain seasons, correlated with the presence of *B. bassiana* in the corn senescent phase (Lewis and Bing, 1991). Other entomopathogenic fungi have been isolated from other plants, such as from the *Carpinus caroliniana* bark (Bills and Polishook, 1991).

### **Other endophytic interactions affecting insect control**

It would be naive to suppose that endophytic fungi interact with the host and the insect-pest in a simple manner and independently of environmental conditions, genotypes and other factors mentioned before. Therefore, different indirect effects may occur and some of them will be addressed in the following discussions.

### **Endophytic fungi affecting parasites and ectoparasites of domestic animals**

Kirfman et al. (1986) suggested that the elimination of endophytic fungi that produce harmful toxins to domestic animals and insects feeding on infected plants, would have beneficial effects on cattle but, would also have negative effects, for example, an increase in some insects-pests and decrease in others, with unpredictable biological imbalance. Therefore, it is important to verify if endophytes that reduce insects-pest can also interfere with the latter's biological

controls. This analysis was carried out in *Microctonus hyperodae*, a Hymenoptera that controls *L. bonariensis*, (Barker and Addison, 1997; 1996). In fact, *A. lolii* infecting *L. perenne* did not affect *M. hyperodae*, facilitating its performance as a biological control agent of the insect-pest that was fed also on infected plants. In some cases, however, the development of the parasite larvae was retarded in hosts that had been fed on artificial diets containing diterpenes, alkaloids usually produced by *A. lolii*. In the same manner, Bultman et al. (1997) verified in the *F. arundinacea*-*A. coenophialum* interaction, that there was interference of alkaloids produced by the fungus not only on the fall armyworm *S. frugiperda*, but also on two parasites that control this pest, *Euplectrus comstockii* and *E. plathypenae*. It was observed that the fungus has only a moderate negative effect on the parasites that feed on *S. frugiperda* larvae. The parasites' pupal mass suffered decrease, but the development and survival rates were normal.

Other indirect effect of endophytic fungi with applied interests is the control of ectoparasites in domestic animals. *Haematobia irritans* larvae of horn fly, which is a cattle ectoparasite, were killed when cattle manure was amended with seed extracts containing lolines from plants infected with *N. coenophialum* (Dougherty et al., 1998). These effects will probably also occur on insects that feed on manure from animal fed on *F. arundinacea* infected with the endophyte *N. coenophialum*.

### **Indirect effects in pest control: insect-vector elimination and gall formation by insects-pests**

The first work showing the importance of endophytic fungi to the control of agriculture pests, was curiously an indirect demonstration that the endophyte, by blocking insect gall formation, also was able to control a disease caused by a fungus (Webber, 1981; Carrol, 1986). Several other examples were analysed in which endophytic fungi block or alter insect's larvae installation and, by doing so, repress potential vectors to disseminate diseases.

Hata and Futai (1995) studied the endophytic mycobiota of *Tecodiplosis japonensis* galls in *Pinus*. The endophytic fungus *Phialocephala* sp. was the most frequent, however young galls did not contain the fungus, indicating that the endophytes was not inoculated by the insect. In this case, it was possible to verify that the insects do not behave as vector for endophytic fungi but, on the other hand, do not avoid the transmission of plant pathogenic fungi.

Other interesting effect of endophytes over insect galls was seen in oak, where apparently dormant endophytic fungi become active with gall formation and, by destroying the leaves, also kill the insect, protecting, therefore, their hosts. This process was described in three associations between endophytes and insects, one of them involving the fungus *Gloeosporium quercinum* and the insect *Neuroterus numismalis* (Butin, 1992).



## Effects on seed dissemination and plant protection

As mentioned before, endophytic fungi may indirectly affect seed dissemination by insects, especially ants. In *Festuca*, seeds infected with certain endophytes are discarded after being collected and, therefore, favour plant dissemination (Knoch et al., 1993).

In other case (Valentine et al., 1992) involving seeds of *L. perenne* containing 88% of *A. lolii* and others with only 1%, significant differences in experiments carried out in green house concerning the number of eggs, larvae and adults were shown. They were found in higher numbers in seeds containing few endophytes. Therefore, once the seeds were colonized, the endophytes protect the host against the Argentine stem weevil *L. bonariensis*.

The passage of a fungus from generation to generation through seeds is of great importance to the control of insects-pests, as already mentioned by Clay (1989), and by us in this review. An endophytic fungi inoculation, as it is done for nitrogen fixing bacteria and mycorrhizic fungi may open new perspectives to apply endophytic microorganisms to control agriculture pests. There are already studies in this field and which will be addressed in this review in the topic concerning the use of the recombinant DNA technology in endophytes as a way of improving pest control.

## Effects on the host plant physiology with impact on insect control

In certain plants, toxin production is not solely a fungal function. There are fungus-plant interaction factors acting on this production and toxins may be produced and dissipated throughout the host tissues. Evidently, it may result in insect control even if the endophyte is not present in the attacked sites. The development and nutrition of the moth *Plutella xylostella* larvae were studied in brassicas. Larvae were affected by the ingestion of leaves, before the endophytic fungus *Acremonium alternatum* reached these leaves. There were also differences in feed efficiency of males and females. The results may be related with the phytoesterol metabolism in the plants and indicate that endophytic fungi in the soil, even through a tenuous interaction, alter insect-plant interactions (Raps and Vidal, 1998). In addition, levels of alkaloids and other toxins may be altered not only quantitatively but also qualitatively, depending on the plants' physiological state.

Contrasting with their utility in insect control, toxin and mycotoxin produced by endophytic fungi may cause problems to cattle feeding on colonized plants. In *L. perenne* infected by *N. lolii*, peramine production plays a major role in controlling the Argentine stem weevil *L. bonariensis*. It was verified that with plant ageing, the amount of peramine decreases in leaves and reaches the lower levels during the inflorescence phase in eight fungus-

plant interactions (Ball et al., 1997a). Other seemingly simple interactions may occur like the increase of stroma induced by the endophytic fungus *E. typhina* in host plants, resulting in an increase in stroma-mediated transpiration and consecutively reduction in herbivorous insect attacks (White et al., 1993).

## Oriented use of endophytic microorganisms: molecular biology and the recombinant dna technology

As we were able to see in the extensive literature cited above, the presence of endophytic microorganisms in plants may be of extreme importance to host protection against insect attacks and, in the control of plant diseases, caused by bacteria and fungi. In relation to insects, some uses of endophyte inoculation have been mentioned before, such as the introduction into seeds or plants of, for example, entomopathogenic microorganisms that will protect the plant and disseminate among the pests, which will decrease in number. It is also possible to monitor the existence of toxins inside plants by observing the presence of insects, especially aphids (Eichenser and Dahlman, 1992). Finally, improvement programs have been conducted, where there is an association between improved plant varieties with endophytic microorganisms that act efficiently in pest control but do not cause any harm to cattle fed on these plants (Saha et al., 1987). These applied aspects, as in the past, are being timidly investigated by research groups and are concentrated only on hosts from temperate countries.

Evidently, more promising results should be expected when applied to plants from tropical regions that show much more diversity and are affected by several pests. More recently, the recombinant DNA technology has been applied as a tool to improve endophytic microorganisms, aiming to the introduction of new characteristics of agronomic interests as biological control of pests.

Probably, Fahey (1988; 1991) described the first work directed at the introduction of a heterologous gene in an endophytic microorganism with the purpose of insect control. As a member of the biotechnological company Crop Genetics International, he described the major steps in the construction of an endophytic bacterium with the purpose of insect control. This was achieved through the secretion of an insecticidal toxin in the host plant. He used the endophyte *C. xyli* subsp. *cynodontis*, a gram positive, xylem-inhabiting bacterium, capable of colonizing several plant species. The commercial product received the designation INCIDE.

This bacterium received a gene from other bacterium, *Bacillus thuringiensis*, which is able to produce the  $\delta$ -endotoxin active against insects in nature, especially against Lepidoptera and Coleoptera. Therefore, the genetically modified bacterium is able to secrete toxin inside the plant, protecting it against attacks of target insects. The author described several advantages of inoculating this bacterium

into corn plants as a way of protecting it against insects: a) the product requires only one application in seeds or aspersions over young plants; b) there is no need for a large amount of the product because the bacterium multiplies after inoculation; c) the genetically modified bacterium stays restricted to the inner parts of the plant and thus, there is no dissemination to other plants; d) the process does not yield toxic compounds; e) this and other similar products require a shorter time to be technologically developed because it is easier to modify a microorganism than a plant; f) the modified microorganism is not transmitted to progeny seed of inoculated plants and therefore, from a commercial point of view, it must be acquired constantly; g) the process has wide applicability because it can be used for other plant species; h) bacteria multiplication is high inside the plant, resulting in up to  $10^8$ /gram of inoculated plant.

As we can see, endophytic bacteria may be found inside hosts at high densities. Consequently, false positive results may be observed in plant transformation protocols involving, for instance, enzymatic activity detection. This was the case of Tor et al. (1992), while transforming plants of the genus *Discorea* with a vector expressing *Gus* ( $\beta$ -glucuronidase). Although, *Gus* activity was observed in putative plant transformants, this activity was due to endophytic bacteria inhabiting the plant material.

Following the work of Fahey (1988), several other researchers, belonging to the same company, published more detailed reports describing the construction of the insect biocontrol. Turner et al. (1991) showed that a plasmid carrying two copies of the *B. thuringiensis* subsp. *kurstaki* *cryIA(c)*  $\delta$ -endotoxin gene and containing a genomic DNA fragment of *C. xyli* subsp. *cynodontis* could be integrated into the chromosome of *C. xyli* subsp. *cynodontis* by homologous recombination. However, the engineered bacterium exhibited insecticidal activity in artificial diets but not *in planta*. Lampel et al. (1994) used an improved integrative vector that, although showed some instability, resulted in toxin production *in planta* after all.

These transformed bacteria were then inoculated in corn crops and showed to reduce damages caused by the European corn borer *Ostrinia nubilalis* (Tomasino et al., 1995). Uratani et al. (1995) constructed different secretion cassettes for *C. xyli* subsp. *cynodontis* using several *Streptomyces* signal sequences. The *Escherichia coli* alkaline phosphatase gene, lacking the native promoter and signal sequence, was placed under the control of the *Bacillus*  $\phi$  phage and the secretion was driven by the different signal sequences. Alkaline phosphatase secretion was detected *in planta*, proving that *Streptomyces* signal sequences work well in *C. xyli* subsp. *cynodontis*. Using a similar approach, Haapalainen et al. (1998) constructed a *C. xyli* subsp. *cynodontis* strain, able to secrete endoglucanase into the xylem sap of corn, with the purpose of controlling fungal diseases. Tester (1992) inoculated 18-day-old corn plants with *C. xyli* expressing the *B. thuringiensis* subsp. *kurstaki*  $\delta$ -endotoxin gene and used as control plants

inoculated with the wild type bacteria. It was verified that in both plants the nitrogen content was increased, but in plants with the engineered bacteria, the water content was higher. In soil, the remains of both plants showed no differences. It was also verified that the engineered bacterium was not capable of surviving outside the plant, which is a desirable characteristic from a biosafety point of view.

Endotoxin gene from *B. thuringiensis* has also been introduced into nitrogen-fixing bacteria from the genus *Bradyrhizobium*. In this case, the engineered bacterium was introduced into roots of *Cajanus cajan*, improving nitrogen fixation and protecting the host against *Rivelia angulata* larvae (Nambiar et al., 1990). Other reviews on the use of genetically modified endophytes may be consulted for more details on the subject (Azevedo, 1998; Hallman et al., 1997; Di Fiore and Del Gallo, 1995).

As we can see, the reports concerning engineered endophytes for biocontrol are concentrated on bacteria, mainly *C. xyli*. In endophytic fungi, this type of study is restricted to the development of transformation systems, introduction of reporter genes and inoculation into plants for colonization studies. Already at the beginning of the 90's, in a review on endophytic fungi from the genus *Acremonium*, Van-Heeswijk and McDonald (1992) launched the idea of using engineered endophytic fungi in the control of insects and diseases affecting *L. perenne*. Murray et al. (1992) introduced into a lolitrem B-deficient strain of *Acremonium* (designated 187B) a  $\beta$ -glucuronidase (*Gus*) gene. The transformation protocol yielded 700-800 transformants/mg DNA and transformants were successively introduced into *Lolium* where *Gus* activity was observed. In a similar manner, Tsai et al. (1992) developed a transformation system for *A. coenophialum*.

Several genes from endophytic fungi and related to toxin production are now being cloned and studied in depth. Wang et al. (1999) cloned a gene coding for a DMATSynthase (dimethyltryptophan synthase) that is responsible for the first step in the synthesis of ergot by the fungus *Claviceps purpurea*. Genes related to the latter were identified in the endophytic fungi *Balansia obtecta* and *Neotyphodium* spp., known to produce, respectively, ergobalansine and ergovaline. Tudzynski et al. (1999) also cloned a gene (DMATS) of *C. purpurea* involved in the ergot synthetic pathway *in planta*. Young et al. (1999) found in *Epichloë* a cluster containing genes for the synthesis of mycotoxins like paxiline and lolitrem B.

In our laboratory in the Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo at Piracicaba, for some years we have been developing studies with endophytic microorganisms from banana, corn, citric plants, among others. Efficient transformation systems have been established for some of these endophytic microorganisms as well as protocols for reintroducing these microorganisms into plants. These studies are among the

first involving genetic manipulation of endophytic microorganisms from tropical plants (Longo, 1996; Pamphile et al., 1997; Glienke-Blanco, 1999; Pereira et al., 1999; Gai et al., 1999).

## Endophytes isolated from tropical hosts

### Importance and first studies

As mentioned several times in this review, most of the researches on endophytes have been carried out using hosts from temperate countries, mainly from the Northern Hemisphere and New Zealand. The data available from tropical regions are scarce. However, these data are showing that tropical plant hosts contain a great diversity of endophytic microorganisms, many of them not yet classified and possibly belonging to new genera and species. Potentially, they are of biotechnological importance as new pharmaceutical compounds, secondary metabolites, agents of biological control and other useful characteristics could be found by further exploration of tropical endophytes. Also a better understanding of plant-endophyte relationships in tropical conditions can be achieved from these studies.

The first reports related to the isolation of endophytic fungi from tropical host plants are those of Petrini and Dreyfuss (1981) and Dreyfuss and Petrini (1984) with Araceae, Bromeliaceae and Orchidaceae from French Guiana, Brazil and Colombia, all in South America. After that, some groups of plants were investigated for the presence of endophytes mainly palms and fruit-trees.

### Endophytes from tropical palms

Rodrigues and Samuels (1990) isolated, for the first time, endophytic fungi from a tropical palm tree growing in the rainforest of Queensland, Australia. From this palm, which belongs to the species *Licuala ramsayi*, eleven fungi were isolated. One of them was described as a new species designated *Idriella licualae*. Also an unusual isolate of *Fusarium aquaeductum* was found. The other endophytes found were mostly Xylariaceae fungi. This research showed that tropical plants could be a repository of new fungal species, which may have valuable biotechnological properties. Other three new species of *Idriella* (*I. euterpes*, *I. assaicola* and *I. amazonica*) were isolated from *Euterpe oleracea* another palm tree, near the town of Belem, in the Brazilian Amazon forest.

Xylariaceous fungi were the most frequent ones (Rodrigues, 1991; Rodrigues and Samuels, 1992). A detailed study using 81 isolates and 15 *Xylaria* species from *E. oleracea* was carried out by Rodrigues et al. (1993) and the presented data showed high degree of intra and interspecific diversity among species when isozyme variation was examined. *E. oleracea* is an Amazon palm tree, which has been acquiring economic importance, with its fruits being used for the production of juices and ice-

cream. Rodrigues (1994) carried out a study for a two-year-period, recovering 57 species and six familial taxa from this host plant. Ascomycotina and Deuteromycotina were frequently isolated and *Xylaria cubensis* and *Letendraeopsis palmarum* were the most common endophytic species. As expected, few isolates were recovered from younger leaves in comparison to the endophytic isolates from older leaves. The endophyte *L. palmarum* was described as a new genus and species (Rodrigues and Samuels, 1994).

As reported by Rodrigues (1996), few studies have been carried out on the effects of the fungus-host interactions in tropical regions. The only published paper about toxic effects of endophytes in livestock is concerned with trichothecene toxins produced by strains of *Phomopsis* spp. isolated from *Baccharis* in Brazil (Jarvis et al., 1991). Rodrigues (1996) made a review on endophytic communities of palm leaves, mainly from *E. oleracea*. This species is common in the floodplains of the Amazon Basin.

All taxa found and their relative isolation frequencies were presented in this review and showed that Xylariaceae species were very common. The role of this group and other endophytes from palm trees and tropical hosts is still unknown but it can be speculated that they could play a role against pathogenic microorganisms and phytophagous insects (Rodrigues, 1996). The occurrence of fungal pathogens in healthy plant tissues was also a common feature found in *E. oleracea* as it was shown to occur also in other plant hosts (Pereira et al., 1999; Pereira et al., 1993; Sinclair, 1991).

Other palm species were also investigated by Sothcott and Johnson (1997), who isolated endophytes from *Sabal bermudana*, an indigenous palm from Bermuda and from *Livistona chinensis*, an introduced palm in Bermuda. Two species of *Idriella* were the most common endophytes found as well as *Aspergillus*, *Phomopsis*, *Wardomyces*, *Penicillium* and an unidentified fungus. Endophytic growth patterns within the leaves of the palm were different from the two species but there was no difference in the infection frequency between them. Endophytic fungi were isolated from *Trachycarpus fortunei*, a temperate palm species outside its natural geographical range. Endophytes were isolated from this host growing in Australia, Switzerland and two sites in China. *Glomerella cingulata* and *Phomopsis* spp. were the dominant species but a total of 75 fertile species were also recorded. The endophytic assemblages showed more affinity with temperate hosts than with tropical palm hosts (Taylor et al., 1999).

Fungi occurring in the aerial parts of six individual palms of the genus *Licuala* in Australia and Brunei Darussalam (Borneo) were isolated. As a whole, 189 species were found, plus 53 mycelia sterilia, giving a total of 242 taxa. Previous estimates of 5.7 fungal species per host, give the figure of 1.5 million species of fungi occurring in the world (Hawksworth, 1991). If a more realistic figure of 33 fungi

species per host is considered, the global fungal diversity requires revision upwards, being at least 5 times greater than 1.5 million (Frohlich and Hyde, 1999).

## Endophytes from tropical fruit-trees

### Endophytes from citrus

In the Department of Genetics, "Escola Superior de Agricultura Luiz de Queiroz", University of São Paulo, in Brazil, we have been conducting several researches since 1995, aiming to the isolation and characterization of endophytes from citric plants. The choice for this host resides in the fact that Brazil is the major world producer of orange juice and 85% of this production is derived from the State of São Paulo. Several insect-pests and diseases affect citrus production in Brazil. Endophytic microorganisms could, in several ways, be useful in the prevention and control of these pests. Endophytic fungi and bacteria were isolated from healthy tangerine, citrus plants and rootstocks. Although no endophytes were found in seeds, from leaves of the tangerine species *Citrus deliciosa*, *C. reticulata* and hybrids between *C. reticulata* x *C. sinensis*, 407 isolates of endophytic fungi were obtained. The two most frequent genera were *Colletotrichum* and *Guignardia*.

About 100 isolates of the species *Guignardia citricarpa* were further studied. This fungus is the causal agent of the disease "black spot of citrus". As they were isolated from healthy plants, based on morphologic and genetic variability detected via RAPD markers, two hypotheses were postulated. The first suggests that they were non-pathogenic endophytes and the second hypothesis suggests that they were latent pathogens. One year after the isolation of *G. citricarpa*, the disease started to occur in the same orchards from which the postulated endophytic isolates were obtained.

The fungus was then isolated from lesions and genetic, cytologic and morphologic studies were conducted to observe possible differences between *G. citricarpa* isolated from healthy leaves as endophytes and from black spot lesions (pathogens). RAPD analysis from these two groups produced 343 bands, one of them found exclusively from DNA of pathogenic isolates. This band was cloned and sequenced and a pair of primers was synthesized based on the obtained sequence. These primers were used with success to discriminate pathogenic isolates from endophytic ones. Morphologic tests also discriminated the two groups in terms of sporulation. Electrophoretic karyotypes of most isolates revealed no differences between the two groups and that the fungus has a genome of at least 21.5 Mb, with 8 chromosomes. Transformation was also achieved in *G. citricarpa* (7 transformants/mg of DNA) using germinating spores (Glienke-Blanco, 1999; Glienke, 1995).

Endophytic bacteria were isolated from 10 citrus rootstocks. No endophytes were recovered from seeds but 530 bacterial isolates were obtained from leaves, 64% of

them being gram positive and the remainder gram negative. The most frequent genus was *Bacillus*, with several species being isolated. Some species resembling *Bacillus* were isolated, but did not produce spores. It was suggested that being endophytes and living inside the plant host, they would have lost the capability to produce spores.

Other genera found were *Alcaligenes*, *Enterobacter*, *Erwinia* and *Pseudomonas*. Plasmids were detected in some endophytes as *Erwinia herbicola*. Variability among 34 isolates of *E. herbicola* was limited and this species was found in all rootstocks. A sort of syntrophism was found between an endophytic fungus from citrus, *G. citricarpa* and isolates of *E. herbicola*. The fungus is able to stimulate the *E. herbicola* growth. On the other hand, some endophytic fungi from citrus have inhibited the growth of some bacteria from the genus *Bacillus*, isolated as endophytes from the same host. In this case, fungi and bacteria do not colonize the same regions in the interior of the host or some of the *Bacillus* species are in fact epiphytic, entering occasionally the plant (Araujo, 1996). These relationships may be important to distinguish endophytes from epiphytes and to understand the maintenance of a necessary equilibrium between endophytes and latent pathogens, avoiding the emergence of diseases.

In Brazil, the plant pathogenic bacterium *Xylella fastidiosa* is provoking great damage to the citrus industry, causing the disease known as Variegated Chlorosis of Citrus (CVC). This disease is of particular interest to the economy of the State of São Paulo, where losses of over US\$ 200 millions have occurred over the past three years, in a sector that accounts for more than 400,000 jobs. An interesting finding is that in affected orchards composed of plants with the same genotype a few individuals show resistance to the disease, exhibiting vigorously growth. Therefore, we started in 1996, another wide study on the endophytic microbiota of citrus plants, characterizing the endophytic populations of three types of sweet orange (*Citrus sinensis*): healthy; CVC-affected and resistant plants and also *Citrus reticulata*, known to exhibit natural resistance to the disease. Our working hypothesis is that different endophytic populations would account for the resistance observed. The results obtained with endophytic fungi are still preliminary but on the other hand, the work done with bacteria has yield promising results and will be presented here.

The most frequently bacteria found as endophytes in the examined citrus plants were *Pantoea agglomerans* (sin. *Erwinia herbicola*), *Enterobacter cloacae*, *Methylobacterium extorquens* and *Curtobacterium flaccumfaciens*. Seasonal fluctuation in population densities was observed for most bacteria but not when geographical location was considered. Differences in *M. extorquens* and *C. flaccumfaciens* isolation frequencies were observed between healthy and resistant plants and affected ones. For each genus, RAPD analysis showed low genetic diversity

among isolates from different areas of the State of São Paulo. All bacteria have been inoculated into citrus seedlings and showed colonization of most tissues (leaf, root, and stem). The first three have been transformed by electroporation at a reasonable frequency and stable vectors, based on cryptic plasmids, have been developed for *P. agglomerans* and *E. cloacae*. These results show that endophytic bacteria may play a role in the resistance observed in some citrus trees, making them potential candidates for biological control of *X. fastidiosa*. Furthermore, with the aid of molecular biology techniques, we were able to develop stable transformation systems that will allow us to construct genetically modified endophytic bacteria. These bacteria may, in the future, carry new properties into citrus plants as a way of controlling pests and diseases.

### Endophytes from other fruit-trees

From banana trees (*Musa acuminata*) growing in three localities of State of São Paulo, Brazil, 16 fungal taxa were isolated and *Xylaria* sp. was the most frequent genus, followed by the species *Colletotrichum musae* and *Cordana musae* (Pereira et al., 1999). The presence of endophytic *Xylaria* as mentioned before seems to be a common feature in tropical hosts. Spontaneous resistant mutants to two fungicides were obtained from the endophytic *C. musae*. Equal amounts of mutants and wild-type isolates were reintroduced in axenic banana plantlets. They were not only successfully reintroduced but also the experiment showed that at least one of the mutants exhibited selective advantage in competition with the wild-type isolate (Pereira et al., 1999).

Studying these endophytic isolates of *Colletotrichum musae*, Maccheroni Jr. and Azevedo (1998) identified at least four individual phosphatase activities under several growth conditions and that may play an important role in phosphate acquisition during the plant colonization. This was the first characterization of the phosphatase system in an endophytic fungus. Isolates of *C. musae*, after being analysed for benlate susceptibility, were transformed using a *ben* resistant gene derived from the fungus *Neurospora crassa*.

Only 3-6 transformants/mg DNA were produced and stable strains were introduced in axenic banana plantlets in competition with wild-type isolates. After 20 days of inoculation with equal amounts of the wild-type and benlate resistant transformants, the proportion between them varied from 8:2 to 4.5:5.5 (Longo, 1995). The impact of the endophytic fungus *C. musae* upon the photosynthetic capacity of *Musa acuminata* was investigated. Endophyte-free plants were obtained from tissue culture and reinfected with the fungus. Measurements of total chlorophyll revealed very little changes between endophyte-free and infected plants. After 45 days of growth, infected plants showed around 15% reduction in maximum photochemical capacity when compared with endophyte-free plants. Apparently, the

reduction observed was caused by toxins produced by the fungus and the effect seems to be the impairment of electron transport in the thylakoid membranes. Despite the limitation in the photosynthesis, there were no macroscopic effects indicating disease symptoms (Rodrigues et al., submitted for publication).

Medeiros (1988) isolated endophytic and epiphytic fungi from leaves of cashew tree (*Anacardium occidentale*) growing in four Brazilian Northeastern States. Twenty-one species of endophytic fungi were reported, with some quantitative and qualitative differences found for different localities. *Colletotrichum gloeosporioides*, *Pestalotia* sp., *Fusarium solani* and *Phomopsis* sp. were the predominant endophytes. Although *C. gloeosporioides* is a pathogenic fungus for cashew tree, it was found as endophyte, as also reported by other authors studying different hosts. A research was carried out using several other fungi isolated mainly as epiphytic in an attempt to control the pathogen *C. gloeosporioides*, being a *Trichoderma* strain the most promising one. Rodrigues and Samuels (1999) isolated endophytes from *Spondias mombin*, an Anacardiaceae host, collected in the States of Rio de Janeiro and Pará, Brazil. Leaves and rachises were investigated. From 13 taxa isolated, three were recovered from rachis. Unidentified *Guignardia* was the dominant endophyte, followed by an also unidentified *Phomopsis* species.

In mango (*Mangifera indica*), several pathogenic fungi occur as endophytic, prior to inflorescence emergence. *Dothiorella* spp. and *Phomopsis mangifera* were found more frequently in trees not sprayed with copper. Endophytic colonization of inflorescence and pedicel tissue was considered to be a primary route of infection for fruits that develop rot stem end during ripening (Johnson et al., 1992).

### Endophytic from other tropical hosts

In Colombia, an endophytic *Phomopsis* sp. was isolated from the woody host *Cavendishia pubescens*. The fungus produced paspalitrems A and C. Such compounds were only recorded at the time as being produced from sclerotia of *Claviceps paspali*, causing neurological disorders in livestock (Bills et al., 1992). In *Atriplex vesicatoria*, the bladder saltbush from Eastern Australia, 71 species from 40 genera of fungi were found and *Fusarium* was the most frequent genera. Several new genera and fungal species were recorded for the first time as endophytes. There was no apparent relationship between a periodic widespread death of bladder saltbush, which occurs in this region, with the presence of endophytic fungi (Cothier and Gilbert, 1994).

In a rain forest of Costa Rica, Central America, fungi were isolated from leaf-litter using a simplified particle-filtration procedure that eliminates most of soil and saprophytic fungi. The number of species/sample ranged from 78 to 134 in pulverized and washed leaf-litter. Endophytes and plant

pathogens were recovered and a high proportion of rare species was found (Bills and Polishook, 1994). The richness of the mycobiota from tropical areas was once more observed. Fisher et al. (1995) isolated 42 different fungal taxa from the host *Gynoxis oleifolia* (Compositae) in Ecuador. Twenty-one of them occurred in frequencies higher than 10% but, in this case, new taxa were not found. Pereira et al. (1993) isolated endophytic fungi from young and old leaves of *Stylosanthes guianensis*, a leguminous genus widely distributed in the tropical and subtropical regions of South America and used as a forage plant. At least thirteen endophytic species were found. Most of them were rare isolates and *Glomerella cingulata*, *Phomopsis* sp. and *Xylaria* sp. were the most frequently found. The frequency of infection of leaves, as expected, increases with the increase of the plant age. The genus *Xylaria* is reported to be frequent among endophytes from tropical hosts, as already mentioned.

The fungus *G. cingulata*, the teleomorph of *Colletotrichum gloeosporioides*, was not found in leaves of plants regenerated from callus culture, thus suggesting that the use of regenerated plants may be an effective means of eliminating latent pathogens as *C. gloeosporioides* from *Stylosanthes*. Rodrigues and Dias (1996) isolated endophytic fungi from two active pastures of tropical grasses named *Brachiaria brizantha* cv. Manrandú and *B. humidicola*. Several species, mainly imperfect fungi were obtained from both grasses. However, *Acremonium*-like fungi were isolated only from leaf sheaths of the first species. Although endophytic fungi had already been isolated from maize (*Zea mays*), these endophytes were obtained from temperate regions (Fisher et al., 1992). A series of investigations were carried out, starting with the isolation of endophytic fungi from populations of *Z. mays* hybrids from tropical regions. Silva (1997) found 17 different taxa in seeds, roots, stems and leaves of maize, besides some unidentified yeast, filamentous fungi and mycelia sterilia. *Fusarium* was the most common fungi isolated from seeds. RAPD analysis of fungi isolated from maize leaves was also carried out (Ribeiro, 1995). Pamphile (1997) also isolated *Fusarium* from maize seeds and 21 *F. moniliforme* isolates were analysed via RAPD, showing that they could be grouped according to the maize populations tested. Genetic transformation was achieved in *F. moniliforme* based on nitrate reductase gene from *F. oxysporum* with frequencies of 30-60 transformants/m g DNA.

Transformants for the *Gus* gene were introduced into maize plantlets and it was possible to observe gene expression inside the host roots. Finally, although transposons were not found in endophytic *F. moniliforme*, it was possible to transfer the *Impala* transposable element from *F. oxysporum* to endophytic *F. moniliforme*. As mentioned above for *M. acuminata*, Rodrigues et al. (submitted for publication) also investigated the impact of endophytic *F. moniliforme* upon the photosynthetic capacity of *Z. mays*. In this case, there was a 50% reduction of this capacity in

infected plants compared to endophyte-free ones. However, the maximum photochemistry capacity was not limited. The reduction of the photosynthetic capacity was due to a reduction in the chlorophyll content, leading to a decrease in the electron transport components and consequent reduction in carbohydrate synthesis. As it occurs in infected banana-trees, no macroscopic effects were detected in maize infected with *F. moniliforme*. Endophytes were isolated from leaves of *Rhizophora apiculata* and *R. mucronata*, two typical mangrove plants growing in the Pichavaram mangrove of Tamil Nadu, Southern India. Leaves were sampled during dry and rainy months and, quantitatively, more endophytes were isolated during the rainy period. Hyphomycetes and sterile forms were the most commonly isolated endophytes (Suryanarayanan et al., 1998).

A new genus and species of fungus named *Seimatoantlerium tepuiense* was isolated as an epiphytic of *Maguireothamnus speciosus*, a rubiaceous plant endemic to the tepuis of Southeastern Venezuela. The fungus produces taxol, an anticancer compound produced by several endophytic fungi (Strobel et al, 1999).

### Endophytes from sub-tropical and temperate regions of South America

Although isolated from sub-tropical and temperate areas in South America, some examples of endophytes isolated in our Continent will be presented in this review, mainly because hosts of importance in tropical regions were used. For instance, in Southern Chile, from *Dactylis glomerata* in pastures, a new species of endophytic fungi named *Acremonium chilense* was described. This species is highly aggressive and is intercellular as other *Acremonium*, but can become intracellular and conidia is abundantly produced *in vitro* (Morgan-Jones et al., 1990). Moreover, an endophytic fungus from the genus *Pseudocercospora* was isolated from the forage grass *Trichachne insularis* from Brazil, Argentina and Chile (White et al., 1990). In the South of Brazil, the endophytic fungus *Ceratopycnidium baccharidicola* was isolated from two populations of the host plant *Baccharis coridifolia*. This was the second record of the species in the world and the first in Brazil (Bertoni, 1994). Previously, Bertoni and Cabral (1991) had isolated the endophytic fungus *C. accharidicola* in Argentina from the same host.

In Uruguay, the endophytic mycobiota of two species of *Eucalyptus* was characterized. The diversity of the endophytes isolated was low as was the number of host-specific species (Bettucci et al., 1999). In Argentina, endophytic fungi of *Eucalyptus viminalis* was studied (Faifer and Bertoni, 1988; Bertoni and Cabral, 1988).

### Endophytic diazotrophic bacteria

As already mentioned, endophytes as nitrogen-fixing bacteria would not be included in the present review, since

they are widely known and many reviews are already available concerning them (Baldani, 1999; Baldani et al., 1998; Baldani et al., 1997; Döbereiner et al., 1995; Döbereiner, 1992a; 1992b). However, due to their importance in tropical regions, endophytic diazotrophic bacteria will be briefly referred here. More than four decades ago, Döbereiner and Ruschel (1958) isolated nitrogen-fixing bacteria from rhizosphere of sugarcane grown in tropical soils of Brazil. However, it was only after the publication of Döbereiner and Day (1975) on the bacterium *Azospirillum* that a growing interest in diazotrophic bacteria associated with graminaceous plants was manifested all over the world.

Baldani et al. (1997) reviewed the main features of the development of the research on diazotrophic bacteria and pointed out that, in the last two decades, other nitrogen-fixing bacteria besides the genus *Azospirillum* were isolated as the genera *Herbaspirillum*, *Acetobacter* and *Azoarcus*. Surprisingly, it was only about eight years ago that Döbereiner (1992a) introduced the term endophytic diazotrophic bacteria to designate all diazotrophs able to colonize primarily the root interior of graminaceous plants, even though it had been already demonstrated that these type of bacteria occur inside plants. More recently, Baldani et al. (1997; 1998) suggested the term associative or "facultative endophytes" for strains that are able to colonize surface and root interior and to survive well in soil. On the other hand "obligate endophytes" do not survive well in soil but colonize root interior and aerial parts of plants.

A list of facultative and obligate endophytic diazotrophs colonizing non-leguminous plants includes several bacteria from the genus *Azospirillum* (*A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. irakense*, among others) and the endophytes *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Acetobacter diazotrophicus*, *Azoarcus* spp. and *Burkholderia* spp. These bacteria were isolated from sugarcane, palm trees, forage grasses, tuber plants, cereals, sweet potato and other hosts and are described in the reviews of Döbereiner et al. (1994) and Baldani et al. (1997). The list of hosts has been increasing year by year and it is expected, as it has occurred with endophytes in general, that almost all studied plants will bear nitrogen-fixing endophytes.

The results obtained from the studies with nitrogen-fixing endophytic bacteria, which were first isolated in tropical regions of Brazil, are of great importance from the agronomic point of view. For instance, *Acetobacter diazotrophicus* was isolated from sugarcane and the results have shown that the bacterium concentration varied from  $10^3$  to  $10^7$ /gram of sugarcane material. An improved methodology for isolation of this bacterium and confirmation of its endophytic state was provided by Reis et al., (1994). *A. diazotrophicus* from different sugarcane cultivars growing in Mexico and Brazil were shown to have limited genetic diversity with predominance of one clone. Plasmids were also found in this species (Caballero-

Mellado and Martinez-Romero, 1994). However, Loganatham et al. (1999) isolated *A. diazotrophicus* from the host *Eleusine coracana* cultivated along the coast of Tamil Nadu in India. The endophytic nature of the bacterium was demonstrated using molecular approaches. The isolates fell into two distinct genetically related groups when compared to the strain type from the American Type Culture Collection. (ATCC). This endophytic bacterium is important for the region because could supply part of the nitrogen required by the crop. The bacterium *A. diazotrophicus*, isolated for the first time from sugarcane, was detected inside cortical cells of stems and inside xylem vessels. However, bacterial quantification have shown that in plants fertilized with high nitrogen levels, there was a severe decrease in the bacterial numbers compared to the numbers found inside plants with low nitrogen levels. High nitrogen fertilization of fields might be a threat to the maintenance of endophytic associations occurring naturally (Fuentes-Ramires et al., 1999).

Endophytic diazotrophic bacteria from the genus *Herbaspirillum* occur frequently in roots, stems and leaves of plants, mainly Gramineae (Olivares et al., 1996). In a review, James and Olivares (1998) related that agriculturally important grasses, such as sugarcane, rice, wheat, sorghum, maize, and pastures contain diazotrophic bacteria as *A. diazotrophicus*, *Herbaspirillum* spp. and *Azospirillum* spp. The first two are spread from plant generation to plant generation via seeds, vegetative propagation, dead plant material and possibly by sap-feeding insects. On the other hand, *Azospirillum* enters host plants via seeds or wounds.

The role of these bacteria related to their agricultural importance was discussed in the review. However, it is noteworthy that, as frequently found for endophytic fungi, in some cases there is only a thin line separating endophytes and pathogens in symbiosis. For example, the endophytic bacteria *Herbaspirillum seropedicae* and *H. rubrisubalbicans* were inoculated into *Sorghum bicolor* and plants examined 5 and 14 days after inoculation. Plants inoculated with *H. rubrisubalbicans* expressed symptoms of "red stripe disease". On the other hand, plants inoculated with *H. seropedicae* did not express the disease or showed very mild symptoms (James et al., 1997).

Molecular studies with *A. diazotrophicus* revealed that nitrogen fixation genes and regulatory genes (*nif* and *fix* genes) have similarities with *nif* genes of other nitrogen-fixing bacteria (Lee et al., 1998; Sevilla et al., 1997). Mutants unable to fix nitrogen could colonize sugarcane plants. As expected, under nitrogen deficiency conditions, they do not show growth stimulation in sugarcane plantlets. However, when nitrogen was not limiting, both wild-type and *nif*<sup>-</sup> mutants stimulated growth of inoculated plantlets, when compared to bacteria-free control plants, suggesting other possible beneficial effects of *A. diazotrophicus* to sugarcane in addition to nitrogen fixation (Sevilla et al., 1998). Kirchhof et al. (1998), using a species-specific

oligonucleotide probe and PCR, demonstrated the presence of *A. diazotrophicus* in plant tissues and in field-grown sugarcane. Plasmids in *Azospirillum brasilense* isolated from rhizosphere and as an endophytic of sugarcane were first detected by Lemos et al. (1981). More recently, examined isolates have shown to contain 5 to 8 replicons and some of them were strongly hybridized to 16S rDNA genes as a 1700 kb megareplicon. Others, as the 910 or 980 kb replicons hybridize only slightly to 16S-rDNA genes, suggesting that the species is composed of multiple minichromosomes instead of a single circular chromosome (Caballero-Mellado et al., 1999). Finally, it is worth mentioning the work which is being done by Baldani and Salles (1998) transferring the *cry* gene from *Bacillus thuringiensis* to diazotrophic endophytic bacteria in an attempt to control sugarcane insect-pests as *Migdolus fryanus* and *Diatraea saccharalis*. The *cry* gene was placed under the control of *nif* promoters. Preliminary results have shown some mortality of *Migdolis* sp. larvae treated with the toxin from transgenic bacteria.

### Other bacterial endophytes: actinomycetes

Besides nitrogen-fixing bacteria, which are quite well studied in tropical regions, there are only few data on other endophytic bacteria isolated from tropical hosts. In maize, although nitrogen-fixing diazotrophic bacteria from the tropics have already been isolated as cited above, other endophytic bacteria studies with this host come from temperate regions (Fisher et al., 1992). Souza (1996) obtained 189 bacterial isolates from two populations of *Zea mays* and a hybrid between them, from two localities in Brazil. The main genera observed were *Bacillus*, *Pseudomonas*, *Clavibacter* (*Corynebacterium*), *Micrococcus* and *Erwinia*. Assis et al. (1998) isolated endophytic bacteria from cabbage growing in the State of Pernambuco, Northeast of Brazil. The method applied for endophyte isolation was the sonication. Seven isolated species were evaluated for antagonistic activities against the causal agent of cabbage black rot disease *Xanthomonas campestris* pv. *Campestris*. Two isolates, *Alcaligenes piechaudi* and *Kluyvera ascorbata* were effective in reducing the disease incidence under greenhouse and field conditions. There are several examples related on the control of plant diseases by endophytic microorganisms. More research on this subject using tropical hosts will probably produce results of agricultural importance.

Pereira (results not published) isolated endophytic bacteria from *Maytenus aquifolium*, a Celastraceae plant, which inhabits tropical and subtropical regions. The plant is claimed to have antitumoral and antiinflammatory properties. Twenty bacterial genera were isolated and the genera *Bacillus*, *Clavibacter* and *Streptomyces*, were the most frequently found, besides other actinomycetes. Preliminary tests are showing that some isolated endophytic bacteria can produce ansamacrolides, which is also produced by the host plant.

Actinomycetes are also bacteria, known to constitute a large part of the rhizosphere microbiota. They are important producers of bioactive compounds and are also found as endophytes. However, it is surprising that, excluding the coryneform bacteria, the only extensively studied species are from the genus *Frankia*, a nitrogen-fixing bacteria from non-leguminous plants (Benson and Silvester, 1993). Endophytic actinomycetes have been the object of few researches all over the world. Preliminary investigations resulted in the isolation of some genera from temperate hosts (Sardi et al., 1992; Matsukuma et al., 1995; Matsumoto et al., 1998). The results obtained so far from tropical hosts have revealed that they are very frequently found and may play an important role in plant development and health. Some recent work done in Brazil will be presented.

Stamford (1997) isolated endophytic and epiphytic fungi and bacteria from roots of yam bean (*Pachyrhizus erosus*), a leguminous plant cultivated in tropical areas of Brazil and Mexico. Endophytic actinomycetes from the genera *Streptomyces*, *Streptosporangium* and *Nocardiopsis* were studied for the production of enzymes related to the starch hydrolysis. Satisfactory production of a  $\alpha$ -amylase and amyloglucosidase was achieved by some strains of *Streptosporangium* and *Nocardiopsis* at low pH and high temperatures. Matsuura (1998) isolated 31 endophytic actinomycetes from caupi bean (*Vigna unguiculata*) growing in Manaus, State of Amazonas, Brazil.

The genera found were *Streptomyces*, *Nocardiopsis*, *Streptosporangium*, *Actinomadura* and *Nocardia*. About 20% of the isolates have shown antibiotic activity against other bacteria. Britto (1998) also isolated actinomycetes from *Phaseolus vulgaris*, the common bean, from Recife, State of Pernambuco, Northeast of Brazil. Thirty-two isolates were obtained from roots and leaves, the great majority being derived from roots (78 %) and 6 of them had antibiotic activity against *Staphylococcus aureus* and *Bacillus subtilis*. Araujo et al. (1999) isolated 53 actinomycetes from leaves and roots of maize growing in Northeast of Brazil.

About 43% of the isolates presented antimicrobial activity against some bacteria and one yeast strain. Maitan (1998) isolated seven actinomycetes from *Solanum lycocarpum*, an alleged medicinal plant growing in the State of Goiás, Central Brazil. The genera *Streptomyces*, *Rhodococcus*, *Microthricum* and *Luteococcus* were found. Probably two isolates are new species. Other two isolates were antibiotic producers. All these results obtained from actinomycetes isolates from tropical plants, when compared to the scarce results in the literature related to this type of bacteria, demonstrate again the richness and potentiality of these endophytes for biotechnological purposes as for pharmaceutical and biocontrol uses.

### Recent studies on Amazon endophytic fungi



Some data from Amazon endophytic bacteria and fungi were already presented in this review. More recently however, the Pereira's group in the University of Amazonas, Manaus, initiated a survey of endophytic microorganisms from Brazilian Amazon plants, most of them of economical importance. The great majority of the results has not yet been published or has only been presented as short communications in local meetings and as part of Ms. Theses. *Paullinia cupana* var. *sorbilis* (guaranazeiro), a Sarpindaceae collected in the Amazon Basin (Manaus and Maués), has been the first species studied. From *P. cupana* var. *sorbilis* is produced a soft drink, the guaraná, which has great acceptance in Brazil and several other countries. The four most common genera of endophytic fungi found in the host were *Guignardia*, *Phomopsis*, *Glomerella* (*Colletotrichum*) and *Xylaria*. Other genera less frequently found were *Fusarium*, *Dreschella*, *Pestalotia*, *Curvularia*, *Humicola* and *Nodulisporium*. Unidentified fungi and mycelia sterilia were also isolated.

Molecular analysis via RAPD have shown a high variability among isolates of *Glomerella cingulata* obtained from *P. cupana* var. *sorbilis* and the *Guignardia* isolates from the Maués region were shown to be highly polymorphic (Guimarães, 1998; Pereira, not published). Other plant host under investigation is *Theobroma grandiflorum*, a Sterculiaceae (cupuaçuzeiro), from the Amazon Basin and widespread from the States of Amazonas to Maranhão, in the North of Brazil. The fruits of *T. grandiflorum* are largely employed in the production of juices, sweets, ice cream, liquors and a kind of chocolate called cupolata. It is severely attacked by pathogenic fungi and insect-pests.

This host plant was chosen in an attempt to find some possible endophytic microorganisms, which could be useful in biological control. Among 182 isolates, *Guignardia*, *Phomopsis*, *Colletotrichum* and *Pestalotia* were the most common endophytic fungi found. Two other hosts were surveyed for endophytes, *Pueraria phaseoloides*, a leguminous plant originated from Asia and well adapted in Amazon and used as pasture and to increase nitrogen fertilization and *Scleria pterota*, an invading Cyperaceae common in Tropical and Subtropical America. Similarly to *T. grandifolium*, among 145 isolates from *P. phaseoloides* and 382 isolates from *S. pterota*, the most frequently endophytes isolated were *Colletotrichum*, *Guignardia*, *Phomopsis* and *Pestalotia* besides *Xylaria* and *Curvularia*.

From all these Amazon hosts, a number of unidentified fungi and micelia sterilia were found. RAPD analysis of *G. cingulata* isolates was also carried out, showing the high variability presented in isolates derived from the same and different hosts (Silva, 1998; Pereira, not published). Other host which is being analysed is *Dipteryx odorata*, an arboreal leguminous found in the low Amazon Basin and used in folk medicine, whose seeds are used for production of coumarin. Besides the endophytic genera commonly found in the other Amazon hosts studied (*Guignardia*,

*Colletotrichum*, *Phomopsis*, *Pestalotia* and *Xylaria*), other four unidentified fungi were common in *D. odorata* (Pereira, unpublished results). Other Amazon host studied was *Copaifera multijuga* (copaiba), a leguminous plant which produces a terpenoid resin mainly from its stem and leaves.

Copaiba-oil is extracted from its seeds, with several industrial uses as for instance, the production of dyes and varnishes. *Phomopsis* sp., which is a pathogenic fungi for this species, was the most common endophytic isolated from, apparently, healthy plants. *Guignardia*, *Colletotrichum*, *Xylaria* and *Fusarium* were also frequently isolated, besides several species not yet identified including some yeast. A preliminary study of endophytic bacteria from this host is being also carried out. From an Apocynaceae used in folk medicine of the species *Himatanthus sucuuba*, the same pattern of common Amazon endophytes was found, with predominance of *Phomopsis* sp. followed by species of the genera *Guignardia*, *Colletotrichum*, *Glomerella*, *Pestalotia* and *Xylaria*. The endophytic mycobiota from *Victoria amazonica*, an aquatic plant, and *Paulicourea marcgravii*, a Rubiaceae toxic for livestock, is now starting to be investigated (Pereira, not published).

## Final considerations

Although first described in the XIX Century, endophytic microorganisms were studied in more details only from the 80's onwards. Soon after that, they started to be recognized as being of great importance for the hosts, protecting the plants against pests, including among others, insects, nematodes and plant pathogenic fungi and bacteria. They also cause physiological modifications in their hosts, such as making them more resistant to hydric stress. Some endophytic microorganisms are able to produce compounds of biotechnological value as antibiotics and antitumor drugs.

The role of endophytes protecting plants against insects and diseases has been quite well studied. However, the research is based mainly on endophytes from temperate hosts and, besides, the work has been concentrated in some genera of grasses. Apart from isolated studies, only more recently, efforts are being directed to endophytes from tropical plant hosts.

This is a broad field of investigation that is almost entirely open to new findings. The results presented in this review show that, as expected, great diversity has been found among endophytes isolated from tropical hosts. They must, of course, play important roles as, for instance, protecting plants against pests and environmental stresses, as already found in temperate isolates of endophytes. What occurs is that, these factors, as incidence of pests, are much more drastic in tropical regions when compared to temperate ones. Thus, it is expected that new ways of interactions between endophytes and their hosts will be found for

certain. It is also expected that new drugs of biotechnological importance, produced by endophytes, will be discovered with the increase of studies focusing on these tropical microorganisms.

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