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par

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Titre de la thèse

Etude de la résistance du *Coffea arabica* au nématode *Meloidogyne exigua* conférée par le gène *Mex-1* et mise au point des outils pour son analyse fonctionnelle

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Al abuelo Norman y la abuela Sara…

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Résumé de la thèse

Pour les pays producteur de café latino-américains, les nématodes phyto-pathogènes, en particulier les nématodes à galles, sont fréquemment observés sur des racines de caféier. Ils causent des baisses spectaculaires des rendements et l'affaiblissement de la plante qui mène souvent à sa mort. En Amérique latine, les nématodes sont souvent contrôlés avec des nématicides, qui sont parmi les molécules les plus toxiques utilisées dans l'agriculture. D'ailleurs, l'efficacité de ces produits est limitée puisque les nématodes vivent une partie de leur cycle de vie dans le sol à l'abri des pesticides. Une alternative consiste à développer la gestion intégrée des parasites (IPM) et à employer des variétés résistantes de caféier. Ces deux approches constituent une alternative écologique et durable par rapport à l'utilisation souvent irrationnelle des produits phytosanitaires, qui représentent un danger pour la santé de l'homme et pour son environnement.

La plupart des pays producteurs de café n'ont jamais fait l'effort de créer les variétés d'arabica qui soient résistantes aux nématodes. Dans ce contexte, l'IRD et le CIRAD avaient développé depuis 1990 un programme destiné à créer puis à multiplier des variétés d'arabica sélectionnées entre autres pour la résistance aux nématodes de galles (*Meloidogyne* sp.). Ce programme a commencé en Amérique Centrale, puis dernièrement au Brésil avec le soutien des laboratoires à Montpellier. Il s'appuie de nombreuses équipes nationales. Ce programme de recherche international produit la principale connaissance scientifique à ce sujet et a conduit au développement et à la diffusion de variétés résistantes (Etienne et al. 2002).

Lors d'une première phase, les études se sont concentrées sur la diversité de nématode (taxonomie). En effet, il y a il existe une quinzaine d'espèces de nématodes de galles, pour lesquelles très peu de choses sont connues. Puis, des échantillons représentatifs d'espèces de caféier ont été testés pour leur résistance aux principales espèces de nématodes. Ce travail initial s'est concrétisé par la mise à disposition de la variété porte-greffe 'Nemaya' (Bertrand et al. 2002).

Les patho-systèmes *Meloidogyne exigua*/*C. arabica* et *M. paranaensis*/*C. arabica* ont alors été choisis par l'équipe IRD-CIRAD pour étudier plus étroitement les mécanismes de résistance, le déterminisme génétique, et l'expression des gènes de résistance. Un résultat majeur fut l'identification du gène de résistance à *M. exigua* dans les lignées de *C. arabica* en cours de sélection. Ce gène de résistance (*Mex-1*) était introgressé dans l'arabica suite à un croisement avec

l'espèce voisine *C. canephora* var. Robusta. Ce gène est en cours de clonage et la séquence putative sera bientôt disponible.

Le but de cette thèse était de développer les outils efficaces (transgénèses et bio-essais) pour l'analyse fonctionnelle des gènes de résistance de caféier, en utilisant un procédé de transformation basé sur *Agrobacterium rhizogenes*. A court terme cet outil devrait permettre de valider la fonctionnalité du gène *Mex-1*.

Pendant ce travail de thèse, une tentative a été faite d'accomplir les objectifs qui étaient fixes. Dans cette résume générale, les résultats principaux sont rappelés et discutés par rapport aux questions augmentées. Des perspectives sont indiquées pour l'usage des technologies développées, tandis qu'également essayant d'augmenter la connaissance des mécanismes de transformation.

Rappel des résultats de force et de leurs implications

Dans la première section, une revue bibliographique complète est développée sur la culture et la multiplication du café et plus spécifiquement sur la transformation génétique. Une délibération trait-sage au sujet des principaux accomplissements des sources de distributeur de gène, des méthodes utilisées, le choix du tissu transformé, la régénération des usines entières, et les futures perspectives de la transgénèse pour la validation fonctionnelle de *Mex-1* sont décrits.

Dans la deuxième section, on le confirme que l'expression dominante du gène *Mex-1* existe dans des génotypes heterozygous et provoque une résistance intermédiaire qui réduit le développement des juvéniles de nématode, et pas nécessairement la pénétration. Nous avons prouvé qu'une telle résistance intermédiaire était stable sous la pression élevée de parasite dans des conditions contrôlées et de champ. Ces résultats suggèrent un effet possible de dosage du gène *Mex-1*.

Dans la troisième section, est décrit l'établissement d'un protocole de transformation du caféier par *A. rhizogenes*, qui permet la production efficace et rapide des « plants composites » (racines transformées induites sur les pousses non-transformées). On lui a montré que les racines transformées dans des états de pépinière maintien le phénotype de résistance / susceptibilité à *M. exigua* des variétés desquelles elles ont été dérivées.

Dans la quatrième section, une description est donnée des conditions de culture pour la prolifération efficace des racines velues. La similitude des phénotypes entre racines transformé et non-a transformé a été indiquée. En utilisant un protocole d'analyse d'image, des phénotypes aberrant ont été efficacement et éliminés avant une analyse fonctionnelle des gènes de racine.

Dans la cinquième section, on démontre que protocole de transformation du caféier par *A. rhizogenes* développé dans la troisième section était fiable avec trois vecteurs binaires différents (pBin19-p35S-*uidA*, pBin-p35s-*gfp*, pCambia2300-p35s-*gfp*) montrant les niveaux élevés de l'efficacité de transformation. Par analyse histologique on a démontré que l'anatomie des racines transformées par *A. rhizogenes* était semblable aux racines non-transformées dans conditions *in vitro* et *ex vitro*. Nous avons prouvé que l'activité de glucuronidase était hétérogènes et montré profil de l'expression variable entre différentes racines transformées avec le gène *uidA*. De même, l'expression d'epifluorescence du gène *gfp* était hétérogène entre différentes racines transformées. L'analyse exécutée par microscopie confocal menée à la conclusion que le modèle de l'expression du promoteur CaMV35 était mosaïque et hétérogène entre différent racines transformées.

General introduction

In this introduction, we intend to describe the context into which my thesis work fits.

For Latin American coffee growing regions, phyto-pathogenic nematodes, particularly root-knot nematodes, are frequently found on coffee tree roots. They cause spectacular drops in yields, and decay that often leads to early death. In Latin America, nematodes are controlled with nematicides, which are among the most toxic molecules used in agriculture. Moreover, the effectiveness of those products is limited as nematodes live part of their life cycle in the soil and are sheltered from the pesticides. One alternative consists in developing integrated pest management (IPM) and using resistant coffee tree varieties. Those two approaches are an ecological and sustainable alternative to often irrational use of phytosanitary products, which are a danger to human health and his environment.

Most coffee producing countries have never made much of an effort to create Arabica varieties that are resistant to nematodes. In that context, IRD and CIRAD have been working since 1990 on developing a programme to breed Arabica varieties for resistance to root-knot nematodes. The programme began in Central America, and latter in Brazil with support from the laboratories in Montpellier. It relies on numerous national teams. This leading international programme is producing the main scientific knowledge on this subject and has led to the development and dissemination of resistant varieties (Etienne et al. 2002).

In an initial phase, the studies focused on nematode diversity (taxonomy). Indeed, there are around fifteen species of root-knot nematode, of which little is often known. Then, representative samples of coffee tree species were tested for their resistance to the main species of nematodes. That initial work led to the first new variety (example of the 'Nemaya' rootstock variety, Bertrand et al. 2002).

The *Meloidogyne exigua/Coffea arabica* and *M. paranaensis/C. arabica* patho-systems were then chosen by the IRD-CIRAD team to take a closer look at resistance mechanisms, genetic determinism, and the expression of resistance genes. One major result was identification of the gene of resistance to *M. exigua* in the Arabica lines undergoing selection at the time.

That resistance gene (*Mex-1*) was introgressed into Arabica by crossing from the close species *C. canephora* var. Robusta. It is now being cloned and putative sequence is available.

The work described in this thesis was mainly devoted to developing a functional analysis method to validate genes of resistance to root-knot nematodes, and more particularly the *Mex-1* gene. However, in section II, we describe characterization of the expression of that gene under controlled and natural conditions, in order to show that the expression of resistance is not as simple as it might have seemed previously and completes my MSc work. Sections III and IV are devoted to the development of a genetic transformation method. Section V deals with an analysis of the expression of reporter genes under the control of promoter 35S from the cauliflower mosaic virus in transformed roots. Lastly, the main results obtained are described and discussed in relation to the fixed hypotheses and objectives. Prospects for taking this work further are proposed with a view to using the techniques developed for a functional analysis of genes of interest, particularly resistance genes, paying particular attention to the *Mex-1* gene.

Chapter I

Bibliography introduction

1. Coffee (*Coffea arabica)*

Economical and social importance

Coffee industry currently worth roughly $ 10 billion per year, standing next to petroleum as the most valuable commodity exported from the tropics. For many developing countries coffee constitutes a major source of foreign exchange. The coffee production chain consists of producers (mostly smallholders who grow coffee on family plots and which number is estimated around 25 million in world), intermediates (like millers, traders, exporters and importers), coffee roasters and consumers. The intermediates provide a lot of coffee-related jobs in the countries of origin and five coffee roasters companies (Kraft, Nestle, Sara Lee, Procter&Gamble and Tchibo) buy almost half of the world's supply of green coffee beans (Oxfam, 2002).

Since the creation of the International Coffee Organization (ICO) in 1960, coffee international trade was regulated by an agreement of exportation quotas by country. The quotas system "ruled" during decades the coffee market and maintained minimal selling prices for producing countries and, during this time, farmers used to make a good profit from their crop. The rupture of the international coffee agreement in 1989 brought market liberalization and overproduction caused by the subsidized entry of new producers in South East Asia (principally Vietnam), as well as a substantial increase in production in traditional Latin American producing countries like Brazil. As a result, prices felt down and almost all coffee producing countries entered in economical crisis which has not yet finished. This situation generated the disintegration of national coffee agencies and consequently, agronomic research and extension activities disappeared. Rural development and livelihood began to degrade in many coffee regions and loans from banks for maintenance and renovation of plantations became rare or inexistent (Bates, 1999).

In addition, coffee growers had to face an additional problem: the global atmospheric disturbances. An example is "El Niño" phenomenon which periodically appearance provoke that some tropical regions in the world receive worth of rain in few months or weeks, causing extensive flooding and the destruction of roads and infrastructure, whereas other regions suffer droughts and disastrous forest fires (Solow et al. 1998; Noss, 2001).

Illustration of *Coffea arabica* var. Caturra

The price crisis especially affected the Central American countries, due to their higher production costs and subsequent lower competitiveness in relation to Brazil and Vietnam. Large numbers of coffee growers, in order to overcome the crisis, have been forced to differentiate their product and supply a higher-value product. This "differentiation" includes an increasing interest to plant varieties with comparable beverage quality to traditional varieties but with intrinsic resistance to principal pest and diseases in order to reduce dependency to agrochemicals and target the actual demand from consuming countries of sustainable coffees (Kilian et al. 2004).

Taxonomy and origin

Coffea arabica species is originated from the high plateau of Ethiopia and forms part of the genus *Coffea* that includes more than 103 different species (Davis & Stoffelen, 2006). With exception of *C. arabica*, which present 44 (2n=4x=44) all the rest of species, have 22 chromosomes (2n=2x=22). *C. arabica* is an allotetraploid plant originated from two different wild diploids species: *Coffea eugenioides* and *Coffea canephora* (Lashermes et al. 1999). The self-fertilization of the species is not strict. The rate of auto-fecundation was estimated at 90% in conditions of plantation (Carvalho, 1988a). The meiotic diploid behavior of the Arabica was confirmed by Lashermes et al. (2000) by the observation of the segregation of markers RFLP in F2 progenies.

Polyploidy is described as "the formation of a higher chromosome number by the addition of extra whole chromosome sets present in one or more ancestral organisms" (Grant, 1981). Hence, polyploid can result from doubling a single species' genome (autopolyploidy) or from joining together two or more different genomes (allopolyploidy). Fusion of unreduced gametes that contain a diploid, rather than haploid, chromosome complement is the most probable route to both types of polyploidy (Soltis & Soltis, 2000). Polyploid may have broader ecological amplitudes than their diploid progenitors because of their increased genetic and biochemical background. The association of the parental genomes can involve the evolution and/or a differential regulation (inactivation) or expression of certain genes. These regulations of genes could lead to the "functional" stabilization of polyploid species () and give rise to novel patterns of gene expression (Comai, 2000; Soltis et al. 2003). Thus, the polyploid character of *C. arabica* can be the origin of the ability of this species to colonize marginal environments. (low temperatures, full sun exposition and few rains). *C. arabica* grows ideally in localities between 17 to 25°C, from 600 to 2000 masl and with annual precipitations from 1200 to 2000 mm^3 (Wrigley, 1988).

Genetic diversity

In 1964-1965, the United Nations Food and Agriculture Organization (FAO), then in 1966 the ORSTOM (presently Institute de Recherche pour le Développement, IRD), organized the prospections of spontaneous and sub-spontaneous coffee-trees in Ethiopia (FAO, 1968). This material was distributed in several world collections.

An important rate of heterozygosity for *C. arabica* was believed to exist (Anthony & Lashermes, 2005). Progenies of some accessions showed a rate of cross-fertilization from at least 13%. However, the diversity of the wild coffee-trees doesn't appear very important when it was studied by molecular markers. Anthony et al. (2001) studied 88 spontaneous and sub-spontaneous Arabica accessions. The accessions were divided into four sub-groups, structured according to their geographical origins. The principal was located in the south-west of Ethiopia and contains the majority of polymorphism. The other three groups represented the coffee-trees from the south and south-east of Ethiopia and it was presumably derived from the first group.

Two other origins for *C. arabica* are also reported, one from the high plateau of Boma in Sudan and from the Marsabit Mount in Kenya (Anthony et al. 1987). Coffee-trees from Sudan (Rume Sudan) do not differ from the coffee-trees coming from Ethiopia and those from the Marsabit Mount are close to the cultivated forms according with the same author. The coffee-trees of Yemen could constitute a secondary center of diversity introduced into this country around the sixth century (Eskes, 1983).

In spite of its economic and social importance, genetic resources of *C. arabica* are extremely threatened. Although, wild populations still naturally exist in the highland forests of the south-west of Ethiopia, these forests are under strong pressure to disappearing due the combined action of agricultural activities and climatic changes (Gole et al. 2002; Harwell et al. 2002). The lack of prospecting missions endangers also the species donors of the actual genome of *C. arabica* in Uganda, where *C. canephora* and *C. eugenioides* reported to be sympatric (Anthony & Lashermes, 2005). Thus, all scientific research that succeed to increase the knowledge of the diversity and evolution of resistances genes to major diseases, will allow a more reasoned exploitation and conservation of the genetic diversity and the natural environment around (Harvell et al. 2002; Altizer et al. 2003).

2. Principal pest and diseases

While in Asia and Africa coffee breeders had to deal against leaf rust and berry disease since the establishment of first commercial plantations in the middle of the XIX century, the absence of those diseases in Latin America until the end of the XX century, caused that *C. arabica* breeding programs focused mostly on the creation of high-yield cultivars. Millions of hectares of Arabica cultivars susceptible to leaf rust were affected when this fungus arrived in 1970 to Brazil and quickly spread to rest of coffee-growing countries, causing a great economic impact to farmers' revenues and the beginning of the fungicide use dependency in detriment of the environment around coffee-growing areas.

Leaf rust

Coffee leaf rust caused by *Hemileia vastatrix*, cause serious defoliation in *C. arabica* trees in lower altitudes in almost all producing countries. Such defoliation adversely affects subsequent crops and repeated epidemics may weaken trees so much that eventually no worthwhile yield is produced (Van der Vossen, 2001). Resistance genes (*R*-genes) transfer from either wild *C. arabica* accessions or diploid related *Coffea* species has been a breeding priority in most coffee producing countries (Bettencourt & Rodrigues, 1988). Hypersensitive-like reaction was observed in leaves of resistant coffee varieties to leaf rust (*Hemileia vastatrix*) as a defense response (Silva et al. 2002). Coffee resistance to leaf rust appears to be conditioned by at least nine dominant *R*-genes (S_H1-S_H9), either singly or in combination. These S_H genes are dominant and condition total susceptibility to compatible races and specific resistance to incompatible races on the basis of the gene-for-gene concept (Rodrigues et al. 1975). The genes S_H1, S_H2, S_H4 and S_H5 have been found in *C. arabica* accession form Ethiopia, while S_H6, S_H7, S_H8 and S_H9 have been only found in Timor hybrid descendants (Bettencourt & Rodrigues, 1988). The S_H3 gene originates from *C. liberica* (Prakash et al. 2004) its genetic map was recently published (Prakash et al. 2006) and physic map is under work (Lashermes et al. 2005).

Coffee genotypes are classified in resistance groups according to their interaction with leaf rust physiological races. *C. arabica* cultivars descendent from Timor hybrid are namely *A*-group and are considered the most important source of resistance to leaf rust because expresses resistance to all races identified so far (Varzea et al. 2002). Until present, resistance conferred by *A*-group has been efficient in countries where Arabica coffee is grown in high altitude areas (i.e. Colombia,

Central America and East Africa). However, in countries as India, extremely favourable weather conditions for leaf rust development lead to an enormous pressure for the apparition of new races and therefore, failure of Timor hybrid resistance is always latent (Van der Vossen, 2005).

Coffee Berry Disease

Coffee berry disease (CBD) caused by the fungus *Colletotrichum kahawae* is the major constraint to coffee production in highland production regions of East Africa and is still limited to the African continent (van der Vossen, 2006). CBD was first detected in 1922 in Arabica plantations of Mt. Elgon in Western Kenya (van der Vossen & Walyaro, 1980). CBD under prolonged wet and cool weather conditions can destroy 50-80% of the berries [6-16 weeks after anthesis] (van der Vossen, 2006). Preventive control by frequent fungicide sprays is currently the main way of controlling the disease; treatments have to be carried out five to seven times per year on precise dates avoiding any delays, depending on the prevailing weather conditions (E. Gichuru, pers. com. 2006).

In most African coffee producing countries, the local varieties are susceptible to CBD. The creation of resistant varieties was initiated about 40 years ago in Kenya, Ethiopia and Tanzania (van der Vossen et al. 2001). However, the dissemination of such varieties remains low and limited to few thousand hectares mainly in Kenya and Ethiopia (van der Vossen, 2006). Resistance to CBD in *C. arabica*, is believed to be stable and controlled by major dominant-*R* and recessive-*K* genes from Rome Sudan variety and dominant-*T* from Timor Hybrid (van der Vossen & Walyaro, 1980). Recently, Gichuru et al. (2006), achieved identification and mapping of simple inherited major resistance gene, designated *Ck-1* (likely to be *T*-gene) in certain Timor Hybrid genotypes.

Coffee berry borer

The coffee berry borer (CBB), *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae) is the most serious pest of commercial coffee in almost all producing countries of the world (Dufour et al. 1999). It was first recorded in coffee seeds of unknown origin being traded in France in 1867 and first reports of the pest in Africa were from Gabon in 1901 and Zaire in 1903 (La Pelley, 1968). However, the true origin of this pest remains unclear (Damon, 2000). In Latin America it was accidentally introduced in 1923 to Brazil from Africa (Jaramillo et al. 2005).

The suggestion that the original host of *H. hampei* was *C. canephora* was initially strengthened by the lack of reports of CBB presence in Ethiopia (Damon, 2000). However, after Abebe (1998) reported CBB to be present almost all major coffee-growing areas of the south and south-west of the country, with relatively higher infestation at lower altitudes (< 1000 m), the situation changed and new hypothesis were set about either a recent introduction of the pest, or, a very effective control of CBB by natural enemies or plant resistance, which would then suggest that *H. hampei* has co-existed with *C. arabica* for a very long time in Ethiopia or possibly originated there (Damon, 2000). This last observation enriches discussion of that, natural mechanisms of resistance probably exist and could be exploited in future and that more detailed research studies need to be done in order to confirm or discard this hypothesis.

Predominantly, coffee growers try to fight against *H. harnpei* infestations through the use of broad-spectrum insecticides. However, growing environmental concerns and increasing problems with insecticide resistance (Brun and Suckling, 1995; Gongora et al. 2001) stimulated the search for more environmentally friendly control strategies against this pest (Bustillo et al. 2000). However, biological control has so far given disappointing results and only a few minority of environmental conscious coffee farmers have established an effective long-term integrated CCB management (Dufour et al. 1999; Damon & Valle, 2002; Pérez-Lachaud et al, 2004). An additional problem to control this pest is the wide range of host plants (over 300), which undoubtedly reduces the efficacy of sanitary crop practices as the carefully picking of remaining berries after harvest (Damon, 2000).

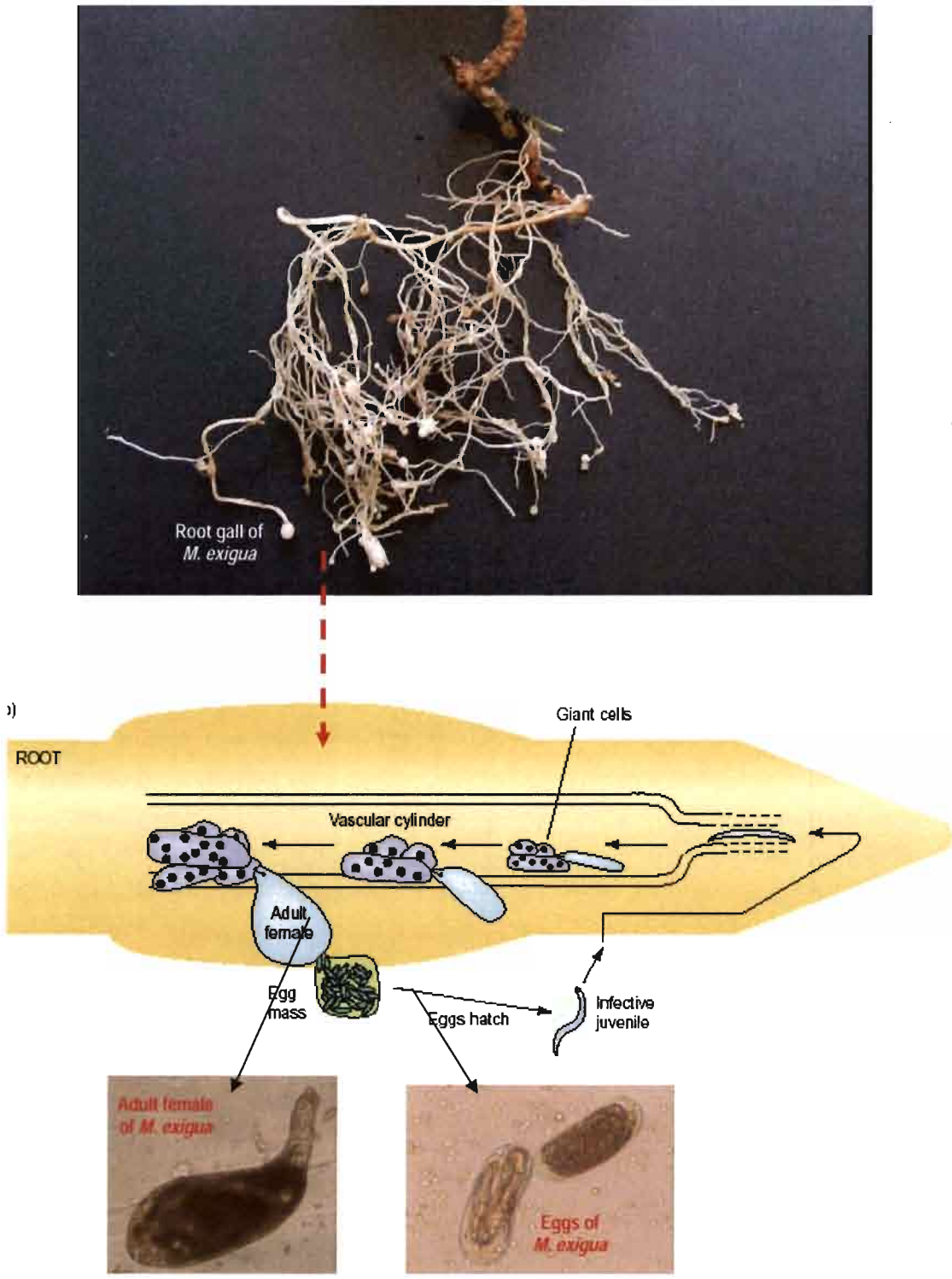

Figure 1. Illustration of the life cycle of root-knot nematodes. (Williamson & Gleason, 2003).

Nematodes

Generalities

Nematodes are found generally as species of free life that not cause parasitism. However a few species are well-known as plant parasites. The plant parasite nematodes are classified in several groups according to their nutritional habits. The genera *Meloidogyne* are sedentary endoparasites, because they remain in the same feeding site almost all their life cycle (Sijmons et al. 1994).

The life cycle of *Meloidogyne* involves four larvae stages and one adult stage (Fig. 1). The only stage they remain in soil is during the second juvenile stage (J2). In this stage, nematode is attracted to the root elongation zone, which it penetrates and then it moves at the intercellular level (Taylor & Sasser, 1983). Penetration is done by using mechanical force and by secreting products from the esophagus glands that are injected by the stylet (Jones, 1981; Smant et al. 1998). Cell wall-modifying enzymes are known to be secreted by nematodes during migration (Davis et al. 2000; Qin et al. 2004). Also, plant genes encoding cell wall-loosening proteins are induced or repressed during feeding site development (Vercauteren et al. 2001; Jammes et al. 2005). These cells become elaborated sites of feeding denominated giant cells, from which they obtain permanently nutrients from the root phloem (Wyss et al. 1992). These giant cells distorted root structure and block the transport of nutriments from the soil to the aerial part of the plant Generally after the development of the female, that it takes around 3-4 weeks, this one release a mass of eggs to the root surface starting a new cycle (Williamson & Hussey, 1996).

For all *Meloidogyne* sp. life cycle is essentially the same. Nevertheless, conditions of temperature, acidity and texture of the soil could induce variations. In conditions of drought or low temperatures, the egg appearance stops during limited periods, then rains reactivate the outbreak of a high number of free J2 individuals in the soil (Schwob et al. 1999). This explains why the greater incidence of attack of nematodes happens mainly after the beginning of the rain season. *Meloidogyne* sp. has one of the largest weed host range in tropical conditions (Quénéhervé et al. 2006) which reduces the efficacy of nematicide applications since these are generally applied near the trunk of the tree.

Nematodes attacking coffee

Root-knot nematodes

Root-knot nematodes, *Meloidogyne* sp., are among the most important pathogens for Arabica coffee plant, leading in some cases to let entire coffee plantations or pushing farmers to change agricultural activity i.e. sugar cane or orange in the state of Sao Paulo in Brazil or rubber tree in the Pacific coast of Guatemala (Campos & Villain, 2005). Root-knot nematode species of coffee can be separated in two categories depending on their dispersion and damage caused: (i) most damaging with widespread in Latin America are *M. exigua*, *M. incognita*, *M. paranaensis* whereas (ii) less widespread species are *M. arabicida*, *M. javanica*, *M. arenaria* and *M. hapla*.

Meloidogyne exigua

The french zoologist Jobert in 1878 made the discovery in Brazil of the first nematode disease reported in Latin America. He observed considerable injury to the roots of coffee trees in the state of Rio de Janeiro. In 1887, the National Museum of Brazil commissioned the Brazilian zoologist Goeldi to make a detailed study of this new disease. In his report, he agreed with Jobert's conclusions and described the genus and species: *Meloidogyne exigua* (Nolla & Fernandez, 1976). *M. exigua* was the first nematode described causing damage of economical importance to a cultivated plant.

As many other *Meloidogyne* sp. reproduction of *M. exigua* occurs through parthenogenesis and intra-species variability has been based on comparison of morphological characters (Eisenback and Triantaphyllou, 1991). Although *M. exigua* is able to reproduce in a wide range of crops as tomato, pepper, cacao (Oliveira et al. 2005) and rubber tree (Carneiro et al. 2000), this root-knot type nematode has been reported principally as a very common parasite present in roots of coffee trees in most Latin America producing countries (Campos & Villain, 2005). *M. exigua* has been found in all major coffee producing areas of Brazil, whereas in Central America, the main distribution on coffee seems to extend form southern Costa Rica to eastern Honduras (Hernandez, 1997; Hernandez et al. 2004; Carneiro et al. 2004). In Costa Rica, the presence of *M. exigua* in coffee was first described by Salas & Echandi (1961). Since then, many studies carried in that country confirm that this species is the most predominant in the coffee regions (Morera & Lopez, 1987; Avendaño & Morera 1987; Flores & López, 1989; Alpizar & Alvarado, 1999). This fact

could be caused by the transfer of vegetal materials propagated in inadequate nurseries, tools and workers between the different coffee areas. Yield losses caused by this species in full sun exposed plantations with appropriated agronomical management have been estimated between 10 to 15% in Costa Rica (Bertrand et al. 1997) and 45% in Brazil (Barbosa et al. 2004). In Brazil also, a *M. exigua* infested area treated during five consecutive years with nematicide did not eradicated the nematode (Lordello et al. 1990).

M. exigua causes rounded galls mostly on new roots formed after first rains in coffee regions with period of marked dry season. This *Meloidogyne* species produces eggs masses in the cortex under the root epidermis, and these galls which are initially white to yellowish brown, turn to dark brown as the root becomes older. In deep rich organic soils *M. exigua* can cause serious defoliation of coffee plant (Campos & Villain, 2005). On other hand, the perennial nature of coffee (a breeding cycle of 4-5 years) and the high variations of nematode infection in field, due to macro- and micro-climatical conditions (Herve et al. 2005) represent serious limiting factors for developing *C. arabica* varieties with stable resistance to *M. exigua*.

Root-lesion nematodes

Another important nematode genus attacking coffee is the root-lesion *Pratylenchus coffeae*. Initially described on coffee by Zimmermann (1898) in Java Island, is considered to be responsible for substantial damages in plantations of *Coffea arabica* worldwide. In Central America and particularly in Guatemala, for ungrafted plants cultivated in heavily infested plots with *P. coffeae*, the plant mortality rate reached 75% by the third harvest (Villain et al. 2000a). Similarly, in severe infested fields in the north-east of Brazil, the same species caused 70% *C. arabica* plant mortality (Moura et al. 2003). In such conditions, the economic consequences are considerably high, considering the costs of replanting a perennial tree such as coffee. In Brazil, *P. brachyurus* seems to be the most widely distributed root lesion nematode (Campos & Villain, 2005). *P. coffeae* and closely related species could be involved in a pathogenic complex in coffee roots in Central America (Anzueto, 1993; Villain, 2000b). Results of routine analysis performed by the Guatemalan Association of Coffee (ANACAFE) over the last fifteen years indicated that root lesion nematodes are abundant in many of the *C. arabica* producing regions of Guatemala (Villain et al. 1999). In Costa Rica, high frequency of *Pratylecnchus* sp. has been also reported in most coffee regions Araya (1994).

3. Genetic improvement of *C. arabica*

Low genetic diversity in major coffee-growing areas

Through human history, world major areas of coffee production have shifted over time. During the sixth century the Arabian Peninsula was the principal region of commercial coffee production. What is now Yemen played a major role in supplying the Arabic world with coffee, along with Abyssinia (now Ethiopia). In the sixteenth century Dutch's colonial entrepreneurs expanded coffee plantations to Ceylan, Timor, Sumatra, and other Southeast Asian regions. French colonial administration introduced coffee, first into Bourbon island (now Reunion island) then exported the variety "Bourbon" into their Caribbean territories in the eighteenth century (Pendergrast, 1999). This single source, together with Dutch introduction (from Amsterdam Botanical Garden) of variety "Typica" in South America, explains the very low genetic diversity and extremely vulnerability of the current commercial cultivars to the majority of diseases that attack the coffee-tree (Anthony et al. 2002).

Coffee green revolution in Latin America

In 1935, with the discovery in Brazil of a small and compact mutant genotype called "Caturra" within a Bourbon population (Krug, 1949), provoked that breeders adopted rapidly this characteristic on their varietal creation programs because the small size of the coffee-trees allowed high density planting (Carvalho, 1988b). Historically, the 'green revolution' on coffee was developed in mainly in Colombia, Brazil and Costa Rica in the decades of 60 and 70's. The production system based on high input of fertilizers, high plant density (i.e., 5000 tree/ha) with dwarf varieties. The 'green revolution' strategy increased considerably the production per unit area mostly due to a spread use of synthetic fertilizers (Varangis et al. 2003). Unfortunately, this increase in productivity was incompatible with maintaining the product quality.

Timor Hybrid as new source of disease resistance

During the second half of the last century, the imminent arrival to Latin America of the orange leaf rust (*Hemileia vastatrix*) imposed to coffee breeders the goal to create new dwarf cultivars with resistance to this fungus. Efforts turned to the creation of the Coffee Rust Research Center (CIFC) in Portugal, from where coffee breeding received a decisive impulse with the utilization of the Timor Hybrid (a natural hybrid between *C. canephora* and *C. arabica* that received from the later the *R*-genes to all leaf rust races actually known) as a resistant parent in the production of

hybrids distributed in most coffee-growing countries in F3 or F4 generations for local selection.

In Latin America, three origins of Timor Hybrid were used (CIFC 832/1, CIFC 832/2 and CIFC 1343) since 1960. Main coffee research centers as IAC, IAPAR and University of Vicosa (Brazil) and PROMECAFE (Central America) used CIFC 832/1 and 832/2. In both regions, the methodology used was the crossover between the Timor Hybrid origin with the dwarf commercial variety (Caturra) followed by several back-crossings with the cv. Caturra after a genealogical selection (Bertrand, 1999). At CENICAFE (Colombia), CIFC 1343 was exclusively used and there were no back-crossings with commercial varieties. Descendants from this origin were directly used by genealogical selection (Castillo & Moreno, 1986).

Lashermes et al. (2000) estimated by AFLP markers that introgression of *C. canephora* in descendants of Timor Hybrid (namely 'catimors' and 'sarchimors' varieties) varies between 9 – 29% of the genome in the lines derived from this spontaneous hybrid. This introgression was often accompanied with favorable characters of resistance to leaf rust, and sometimes to some species of *Meloidogyne* and to *Colletotrichum kahawae* (causal agent of the Coffee Berry Disease). However, it is highly likely that genealogical selection preserved also several fragments of DNA associated to a substantial drop in cup quality (Bertrand et al. 2003). For this reason, the majority of the national coffee research institutions temporarily suspended the diffusion of Catimors.

Current breeding priorities for *C. arabica*

In the actual context of overproduction and fluctuant prices of the coffee market, along with the increasing demand from consumers of food produced following sustainable agronomical practices, a common target became generally adopted by most breeding programs around the world: the maintain of the Arabica quality level in genetic material introgressed with alien material presenting resistance to principal local pathogens or diseases (Fazuoli et al. 2000; Leroy et al. 2006). Notably, after the failure of the Catimors program, the project initiated by the French cooperation (CIRAD/IRD) and the Central America Coffee Research Network (PROMECAFE) aiming the development and commercial production of F1 hybrids obtained by crossing of *C. arabica* wild Sudan-Ethiopian accessions with Timor hybrid descendants seems the most and faster efficient way to achieved the objective mentioned above.

Heterosis defined as the genetic expression of the superiority of a hybrid in relation to its parents, has been known since ancient civilizations and remains as the most important plant breeding scheme for commercial production of hybrid seeds (Miranda Filho, 1999). In *C. arabica*, proposals for developing hybrid lines for increasing genetic diversity and exploiting heterosis were first presented by Charrier (1978) and Van der Vossen (1985). Today significant results have already been obtained from these F1 hybrids. Bertrand et al. (2005a) demonstrated an increased yield (30-70%) of the hybrids in comparison with inbreed lines. These hybrids also showed a good performance in beverage quality, showing no difference when compared with traditional varieties used as reference (Bertrand et al. 2006).

Similarly in India, Arabica breeding shifted also on the development of synthetic hybrids. The outcome of hybridization program between a natural *C. arabica* x *C. canephora* hybrid with wild Arabica accessions involved some lines that shown good yield and resistance to leaf rust under different agro-climatic regions (Srinivasan et al. 2000).

Recently, the Tanzania Coffee Research Institute (TACRI) has embarked on an ambitious initiative with the aim of replant about five million coffee trees with new selected *C. arabica* hybrids that are resistant to leaf rust and CBD and that exhibit good beverage quality. Being able to avoid the use of fungicides would reduce farmers' costs by 60-80 per cent.

With regard to diseases, some Arabica wild coffee-trees used in above described breeding programs constitute an additional source of *R*-genes. Concerning coffee rust, it seems that certain accessions have a good level of partial resistance (Eskes, 1983; Gil et al. 1990; Holguin, 1993). Other Arabica origins have shown resistance to various species of *Meloidogyne* nematode (Anzueto et al. 2001; Bertrand et al. 2001). More recently a group of phytopathologist from CIRAD found 21 Arabica accessions exhibiting levels of partial resistance to CBD (Bieysse & Berry, unpublished data).

4. Plant resistance

Resistance mechanisms

Resistance of plants towards pathogens is based on a combined effect of preformed structural barriers and induced mechanisms. The induced resistance is based on the recognition of the pathogen at the time of contact with the plant which leads to a subsequent activation of defense mechanisms, which the pathogen must confront (Hammond-Kosack & Parker, 2003).

R-genes have been shown frequently to be single loci that confer resistance against pathogens that express matching avirulence genes (*Avr* genes) in a "gene-for-gene" manner (Flor, 1971). Therefore when *Avr*-genes products modify a protein conformation, the *R*-gene recognizes this modification and releases a defense reaction response. This type of specific resistance is often associated with a localized hypersensitive response, a form of programmed cell death in the plant cells proximal to the site of infection triggered by the pathogen (Dangl & Jones, 2001). The plant resistance response also includes generation of reactive oxygen species, production or release of salicylic acid, ion fluxes, etc. (Heath, 2000).

Resistance is durable when it remains effective in a cultivar that is widely grown for a long period of time in a environment favorable to the disease (Johnson, 1981). The previous, is generally achieved by accumulating as many genes as possible from different sources of resistance into a same cultivar (Parlevliet, 2002). The use of molecular markers can facilitate the pyramiding of resistance genes through marker-facilitated selection (Mohan et al. 1997; Hammond-Kosack & Parker, 2003).

R-genes

A significant proportion of the plant genome encodes proteins potentially involved in defense against diseases (Meyers et al. 1999). Currently, ~40 *R*-genes have been cloned and characterized among Arabidopsis and other crop species (reviewed by Martin et al. 2003; also by Trognitz & Trognitz, 2005). Five classes of gene-for-gene disease resistance (R) genes have been defined according to the structural characteristics of their predicted protein products (Martin et al. 2003). Interestingly, the majority of those *R*-genes cloned so far, encode proteins with similarity to the nucleotide binding site-leucine rich repeat (NBS-LRR) domain (Meyers et al. 2003). The fact,

that NBS domain is highly conserved among the pathways that different plants use to trigger defense responses suggests its essential role on *R*-protein activity (Huang et al. 2005).

Among plant species, NBS-LRR proteins are divided into two subgroups: (i) those encoded N-terminal domain with Toll/Interleukin-1 receptor homology (TIR-NBS-LRR); (ii) those encoded an N-terminal coiled-coil motif (CC-NBS-LRR) (Pan et al. 2000a; Meyers et al. 2003). CC-NBS-LRR (non-TIR) *R*-genes are located in the cell cytoplasm (Boyes et al. 1998) and are involved in protein–protein interaction during pathogen recognition process in both dicots and monocots species (Meyers et al. 1999) whereas TIR-NBS-LRR have not been detected in monocots (Pan et al. 2000a; Monosi et al. 2004). Later functional studies indicated that the highly variable LRR domains determine recognition of the pathogen *Avr* product (Van der Hoorn et al. 2005), whereas the more conserved TIR-NBS or CC-NBS regions are believed to propagate the perceived signal (Tao et al. 2000; Tameling et al. 2002).

Nevertheless in gene-for-gene hypothesis, *R*-protein and *Avr*-protein interact directly to activate disease resistance. Increasing reports suggest that one *R*-gene can use multiple signaling pathways to target various pathogens, each probably with different *Avr*-genes (Martin et al. 2003). i.e. *Gpa2* and *Rx* genes conferring resistance to nematode and virus in potato (Van der Vossen et al. 2000), or *Mi-1* gene that confer resistance to nematode and insect in tomato (Vos et al. 1998) and *Pto* gene to different *Avr*-proteins from the same bacteria in tomato (Kim et al. 2002).

R-*genes clusters*

Mapping studies of model plant species as *Arabidopsis* (Meyers et al. 2003), rice (Zhou et al. 2004), tomato (Zhang et al. 2002), soybean (Tian et al. 2004) and potato (Huang et al. 2005) have shown that, in many cases, different families of NBS-LRR genes tend to cluster and are often genetically linked to known disease resistance loci. *R*-genes clusters often comprise tandem arrays of genes that determine resistance to multiple pathogens as well as to multiple variants of a single pathogen, but the functional and evolutionary significance of this clustered arrangement still to be unclear (Kuang et al. 2004).

Sequences presenting similarities with *R*-genes cloned within the genomes of plants are denominated Resistance Gene Analogs (RGA). These RGA are generally obtained and

characterized following in vitro amplifications (PCR) using degenerated oligonucleotidic starters correspondent with preserved fields (i.e. NBS) of *R*-genes. The majority of these RGA are organized in clusters and co-localized with known genes of resistance (Pan et al. 2000a).

To date, RGAs, sequences with a close evolutionary relationship to *R*-genes as determined by a high percentage of sequence similarity, have been identified in different fruit trees species, including peach (Lalli et al. 2005), apricot (Soriano et al. 2005) and apple (Baldi et al. 2004). In coffee, Noir et al. (2001) achieved to isolated 27 RGAs from genomic DNA of two and four accessions of *C. arabica* and *C. canephora* respectively of known *R*-genes of several plant species (e.g. *Arabidopsis*, tomato, pepper, potato, lettuce). Bhat et al (2004) did a similar analysis including one variety of *C. arabica*, one of *C. canephora* and four additional wild coffee species. In both works the majority of RGAs were related to *R*-genes of the CC-NBS-LRR sub-group. The similarity between particular coffee RGAs with other angiosperm species (mentioned above) suggest an ancestral relationship and the existence of common ancestors. On other hand, the identification in both coffee species of closely related RGAs confirms the postulated recent speciation of *C. arabica* from *C. canephora* as one of the progenitors and indicates a slow evolution NBS RGAs within coffee plant (Noir et al. 2001).

_R_-genes to nematodes

Resistance patterns in most crops damage by *Meloidogyne* sp. reflect the co-evolutionary forces between host and parasite. These highly specialized relationships resulted in specific *R*-genes. Some of these *R*-genes to various plant pathogenic nematodes have been identified and cloned in few crop plants or their wild relatives (Martin et al. 2003). The best-studied of these genes is the tomato *Mi* gene (Milligan et al. 1998), which confers resistance against three species of root-knot nematodes and one potato aphid (Vos et al. 1998). Other cloned nematode *R*-genes are *Hero* from tomato that confers resistance to all pathotypes of *Globodera rostochienses* (Ernst et al. 2002), *Gro1-4* also from potato confers resistance only to *Ro1* pathotype of the same species (Paal et al. 2004). *Gpa2* from potato confers resistance to Pa2/3 of *Globodera pallida* (van der Vossen, et al. 2000). *Hs1pro-1* from sugar beet confers resistance to *Heterodera schachtii* (Cai et al. 1997). Sequence analysis of all these genes indicates that they are all members of the non-TIR-type *R*-gene sub-group (with exception of *Gro1-4* gene). Moreover, phylogenetic analysis revealed an ancestral relationship among nematode *R*-genes from tomato and potato (Huang & Williamson, 2003; Tian et al. 2004).

Although nematodes are extra-cellular pathogens, they feed on the cytoplasm of living plant cells and are thought to inject secretions into plant cytoplasm to initiate the development of feeding cells (Williamson & Hussey, 1996). In the case of *Mi* gene, LRR region (located within cell cytoplasm) initiates transduction of the signal(s) leading to cell death after nematode recognition (Huang et al. 2000). The cellular hypersensitive response (HR) occurs near the head of the nematode 12 hr after inoculation, which corresponds roughly to the time when the nematode would be expected to inject saliva into the cytoplasm of developing vascular tissue cells to initiate giant cell development. This timing is consistent with the hypothesis that *Mi* recognizes nematode secretions into the plant cell (Milligan et al. 1998). Interestingly, cell death during aphid resistance, were not observed for *Mi* gene, leading to suggest that different defense pathways are triggered by aphids in leaves and by nematodes in roots (Huang et al. 2000).

In some cultivars of tomato (Williamson, 1999) that have in common resistance to *Meloidogyne* sp., it was observed that nematodes penetrate into the root and migrate to the feeding site like it happens to the susceptible plants but without displaying formation of giant cells; in their place, a necrosis in the cells around the head of nematode was observed suggesting an answer of induced resistance after the infection. However, Pegard et al. (2005) who also observed penetration of *Meloidogyne* sp. in resistant pepper described the occurrence of post-penetration biochemical defense mechanisms, which leads to block nematode reproduction. Hendy et al. (1985) observed that between the genes responsible for the resistance to *M. incognita* in pepper, some presented a resistance reaction more progressive than others; thus, the *Me* 1 & *Me* 4 genes were related to the imperfect formation of trophic cells after the penetration of nematode, whereas *Me* 2 & *Me* 3 to the HR (necrosis) immediately after nematode penetration. The previous demonstrates how unrealistic would be to determine the physiological expression of *R*-genes based only in histological preparations and how complicated would be also to classify the resistance reaction, when two or more genes are present in the same genotype.

Coffee resistance to *M. exigua*

Traditionally, the use of agro-chemicals has been the most used method for nematode control in coffee plantations with high technical management (Lordello, 1990); nevertheless, this practice has been widely questioned because treatments are expansive, punctually efficient and suffers accelerate degradation that allows soil re-colonization by nematodes in the short term (Campos &

Villain, 2005). Therefore, efforts on the development of nematode-resistant cultivars for rootstock usage or direct planting constitute the most promising option so far for controlling the pest.

Grafting

In Guatemala, hypocotyledonary grafting of *C. arabica* commercial varieties onto unselected *C. canephora* which is resistant to *Meloidogyne* sp. has been used during decades with successful results in order to control nematode populations (Villain et al. 2000a). In Brazil, an introduction of *C. canephora* LC2258 derived from clone T3561 from CATIE germplasm collection, showed resistance and/or tolerance to several *Meloidogyne* spp. including *M. exigua*. This variety was named Apoatã and has been used with successful results as rootstock in areas infested by both *Meloidogyne* sp. and *Pratylenchus brachyurus* (Gonçalvez et al. 1996; de Oliveira et al. 1999). In Central America, by crossing the same clone T3561 with another resistant clone, T3751 gave origin to a multi-resistant *C. canephora* hybrid which exhibits high tolerance to *Pratylenchus* sp. and resistance to most *Meloidogyne* sp. found in the region (Bertrand et al. 2000). This variety was named "Nemaya" and is currently commercialized in Guatemala, however attempts to implement this practice in other Central America coffee producing countries have until now failed, probably because of lack of capacitated personal and high cost of hand labor. In addition to nematode resistance, these rootstocks varieties produce abundant roots systems that offer resistance to drought and better tolerance to foliar pathogens by increasing aerial vigor.

Resistance in C. arabica *to* M. exigua

In *C. arabica*, although resistance to *M. incognita* and *M. arabicida* has been found (Anzueto et al. 2001), none resistance to *M. exigua* has been found in this coffee species until now. By contrast, resistance to *M. exigua* in *Coffea canephora* exists and was first documented by Curi (1970). Since then however, most Latin America breeding programs achieved introgression of resistance genes to this nematode species from *C. canephora* via the Timor hybrid (Gonçalvez & Pereira, 1998; Bertrand et al. 1997; 2001; Fazuoli et al. 2006). Bertrand et al. (2001) remarked the fact that selection of *C. arabica* lines with resistance to *M. exigua* was until recently done without any rational breeding strategy and conservation of introgressed resistance genes was due more to the randomness and the number of generation during the pedigree selection process than to a rational choice made by breeders. Conversely, Bertrand et al. (2003) found two Timor hybrids highly introgressed lines (T17934 and T17931) that revealed no difference for beverage quality

from non-introgressed *C. arabica* controls and that also showed genetic resistance to coffee leaf rust and *M. exigua*. With this work, the authors demonstrated that selection in coffee breeding programs can avoid accompanying systematically the introgression of resistance genes with a drop in beverage quality.

Characterization of resistance to M. exigua

Presence of necrotic cells around the feeding site (associated with HR) following inoculation of *M. exigua* in resistant introgressed *C. arabica* varieties was recently described (Anthony et al. 2005). The authors suggested that resistance response was based on gene-for-gene interaction. Noir et al. (2003) identified molecular markers in *C. arabica* derived from the interspecific Timor hybrid associated with resistance to *M. exigua*. Segregation data analysis of F2 progeny derived plants from the cross between the resistant introgression line T-5296 and the susceptible accession ET-6 had showed that such resistance is controlled by a simply inherited major gene, designated as *Mex-1* locus. However this major gene could have an incomplete dominant expression because most of the F2 population showed a gall index higher than the mean value of the resistant parent T-5296 (Noir et al. 2003). More studies should be done in order to determine the level of expression (complete or incomplete) of the *Mex-1* gene within homozygous or heterozygous *C. arabica* genotypes.

Anzueto et al. (1995) mentioned that although specific resistance to some species of *Meloidogyne* exist in some *C. arabica* hybrids, the lack of resistance to other sorts of nematodes, entails the danger that in fields infested with two or more nematode genera, the decrease of one population would promote the increase of other. This hypothesis of nematode competition between *M. exigua* and *Pratylenchus* sp. nematode populations has been demonstrated by Bertrand et al. (1998) and Herve et al. (2005). As demonstrated by Castagnone-Sereno (2002) on *M incognita,* virulent nematode populations are able to break down the plant resistance. More studies on coffee resistance stability are necessary. On other hand, Alpizar (2003) described significant differences in the virulence assessment between isolates of *M. exigua* from different coffee regions of Costa Rica. In Brazil, Carneiro et al. (2000) differentiated two phenotypes of *M. exigua* by comparative esterase profiles and by their distinct efficiencies to reproduce on tomato. More recently, Oliveira et al. (2005) found another original isolate, increasing to four the number of 'esterase phenotypes' known for *M. exigua*. The relation between those 'esterase phenotypes' and the pathogenicity should be done.

Prospects for enhanced identification and analysis of coffee resistance genes to biotic constraints

Despite the importance of coffee as an important international commodity and the indiscriminate use of pesticides to control main biotic constraints (e.g. coffee berry borer, nematodes, leaf rust, coffee berry disease) in detriment of environment and human health, scarcely attention has been given to the characterization and sequencing of genes involved in insect/pathogen resistance compare with efforts on research of genes involved on fruit development and chemical composition with the aim of improving the quality of the final commercial product. Conversely, most coffee breeding programs in producing countries aiming the introduction of resistance traits from wild relatives still laborious and highly expensive because the need of establishment of long term field trials in order to discriminate and select plants showing a more durable resistance to specific pathogens.

On other hand, in most cereals, solanaceous and brassicaceae species molecular biology advances have enabled to generate novel insights into the complexity of plant defence and host–pathogen co-evolution and to employ this knowledge on highly effective breeding strategies as marker-assisted selection or even "within-the-gene" molecular markers (Hadmmond-Kosack and Parker, 2003). The recently evidence that coffee and solanaceous species share similar chromosome architecture and genome sequence, offers an interesting opportunity to expedite biotechnological research in coffee breeding programs (Lin et al. 2005). The previous open the possibility to identify orthologous NBS-LRR sequences between coffee and tomato plants by comparing genome maps from large-scale EST sequencing projects.

Gene-transformation technology complements the genomic mapping and is crucial to validate *R*-gene function which will serve later to variety improvement. The recent construction and characterization of a BAC library of *C. arabica* (Noir et al. 2004) would enable in near future to the isolation of *R*-genes by PCR amplification of NBS-LRR encoding genes from tomato or other solanaceous species. Such progress need to be accompanied by an efficient coffee transformation protocol that could allow the transfer of several DNA segments susceptible to contain a putative *R*-gene into the genome of a genotype lacking that gene in order to elucidate their function. However the few reports of successful transformation of coffee plants still hampered by the low rate of transformation events obtained until present and the lack of suitable conditions to carry studies that permit to develop *R*-gene functional validation analysis.

5. Plant genetic transformation

Generalities

Plant genetic transformation consists in introducing one or various genes with particular proprieties into a cell where they were naturally absent in order to express their proprieties. Through human history, gene transfer has been done spontaneously in natural environments between related plant species. However, recent biotechnological advances allow to achieve this gene transfer accurately and briefly in virtually any living cell.

Two fundamental differences between classical selection and plant improvement by the transgenic way exist: i) plant transformation breaks sexual barriers ties to the vegetal species notion, because it allows the integration of one gene from any type of living organism to other completely different, ii) the transgenic approach limits the genetic modification to one character, in contrast with classical methods of varietal selection, where the transmission of other hereditary characters occurs in a mixed and aleatory way (Franche & Duhoux, 2001). In coffee breeding, the difficulty to introduce resistance genes without introgression of other genes from *C. canephora* without lead a decrease in cup quality is well known (Bertrand et al. 2003; 2005b).

Production of transgenic plants is recent. The first significant works come out almost three decades ago (Zambryski et al. 1983) and historically, plant genetic transformation was possible thanks to major discoveries of plant pathologists working on a gall disease provoked in many fruit trees by two types of naturally occurring soil bacteria: *Agrobacterium tumefaciens* and *A. rhizogenes*. This soil bacterium possesses the natural ability to transform its host by delivering a well-defined DNA fragment, called "transferred" (T) DNA into the host cell (Chung et al. 2006). Since then, the delivery and stable integration of foreign genes into the genome of extensive range of plants has made *Agrobacterium* the most favored tool today for plant genetic engineering.

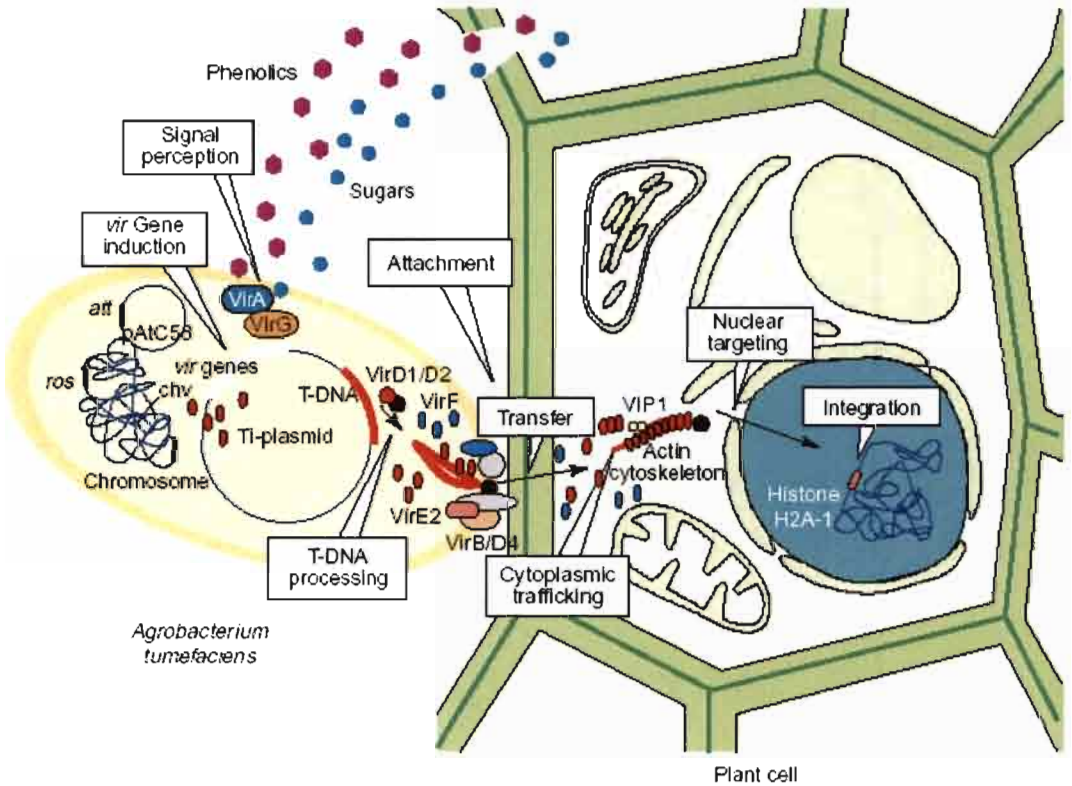

Figure 2. Model for the process of Agrobacterium-mediated transformation. This model depicts the various steps in the transformation process, including those occurring within the bacterium (perception of phenolic and sugar signals from wounded plant cells, virulence gene induction, T-strand processing from the Ti-plasmid and the subsequent export of the processed T-strand and Virulence proteins) and those involving the plant cell (bacterial attachment, T-strand and Virulence protein transfer, cytoplasmic trafficking and nuclear targeting of the T-complex, and T-DNA integration into the plant genome). VIP1 is hypothesized to influence events in the plant cytoplasm, whereas histone H2A-1 influences events within the nucleus (From Gelvim, 2003).

Transformation methods of plants

Non-Agrobacterium-*mediated genetic transformation*

Plant transformation can be achieved by an important number of unrelated techniques collectively referred to as 'direct-DNA transfer' (reviewed by Twyman et al. 2002). These include DNA delivery into the host cell by microinjection (Crossway et al., 1986), particle bombardment (Christou, 1992), electroporation (Fromm et al. 1985) and sonoporation (Liu et al. 2006). The only similarity among these "direct-gene-transfer" techniques is that external physical or chemical factors mediate DNA delivery into the plant cell (Kohli et al. 2003).

Agrobacterium-*mediated genetic transformation*

Agrobacterium is a gram-negative, non-sporing, motile, rod-shaped, soil-borne bacteria; belonging to the genus Agrobacterium, family Rhizobiaceae. Along with *A. tumefaciens* (a naturally pathogenic bacteria that causes crown gall disease on the stem of trees of economical importance as roses, apple, pear, peach and cherry), *A. rhizogenes* causes the formation of cell tumors and formation of hairy roots in infected inoculum site mainly in dicots plants.

Genetic transformation mediated by *Agrobacterium* involves the transfer of DNA (T-DNA) from the bacteria to the eukaryotic host cell, and its integration in the host genome (Stachel & Zambrysky, 1989; Zupan et al. 2000). Although used mainly for plant genetic engineering *Agrobacterium* can transform almost any living cell (Gelvim, 2003), i.e. other prokaryotes (Kelly & Kado, 2002), yeast (Piers, 1996) and fungi (de Groot et al. 1998).

Another technology referred as "agro-infiltration" which was first develop for study compatible plant-pathogen interactions (de Witt et al. 1982), consist of the intercellular injection of *Agrobacterium* fluids on leaf. It presents the advantage over complete transformed plants that it does not require stable integration of the T-DNA into the host genome, neither the development of a long morphogenetic pathway, due that protein expression can be achieved in only few days after infection (Yang et al. 2000).

The "transferred DNA" named T-DNA comes from two bacterial plasmids: the tumor-inducing (pTi) or the root-inducing (pRi) plasmids from *A. tumefaciens* and *A. rhizogenes* respectively.

Numerous strategies have been developed to introduce foreign DNA into the Ti plasmid used later as a vector to introduce this DNA into plant cells. Within these strategies T-DNA binary vectors revolutionized the use of *Agrobacterium*-mediated procedure for plant transformation. T-DNA binary vectors were created after Hoekema et al. (1983) and Frammonf et al. (1983) have determined that pTi carries two separated but necessary components for genetic transformation: the virulence (*vir*) and T-DNA regions. The *vir* region encodes most of the bacterial proteins necessary for processing, transport and integration of the T-DNA into the host cell and is induced by phenolics exudates from wounded plants. The T-DNA consist of 25 bp direct repeats delimited by two borders (left and right) and does not encode any gene important for the transfer process, but it is possible to place any DNA between the T-DNA borders, which once within the plant cell, is processed and transferred by the *vir* proteins to the nucleus, where it becomes stably and integrates into a plant chromosome (Zupan & Zambrisky, 1995; Zupan et al. 2000; Gelvin, 2000).

In pRi-type plasmids of *A. rhizogenes* agropine-manopine strains, the T-DNA region is divided in two parts: left (T_L) and right (T_R). In the T_L-part, are located the genes responsible for agropine synthesis (*rol*), whereas the T_R-part harbors the auxin synthesis genes (*aux*). The T_R-part is the only one that exhibits homology with pTi T-DNA. The protein sequences deduced from the *A. rhizogenes* auxin synthesis genes are very similar to those of the corresponding genes of *A. tumefaciens*, but in contrast, the promoter regions of these genes are substantially different in pTi and pRi plasmids, which can be due to different forms of regulation (Camilleri & Jouanin, 1991).

The transformation process occurs as following (Fig. 1): in the presence of phenolic exudates from wounded plants, *Agrobacterium* initiates gene expression from the *vir* region of the pTi or pRi plasmid with specific proteins that recognize the border repeats of the T-DNA and produce the trans-acting factors for the transfer of linear single-stranded copy of the T-DNA (Zupan et al. 1996). Once the T-DNA from the agrobacteria has been transferred to the cell, the generation of genetically transformed tissues can be achieved by *in vitro* growth culture conditions.

The understanding of the molecular pathways by which T-DNA from *Agrobacterium* is transferred to plant cell is still incomplete (Kohli et al. 2003; Tzfira et al. 2004). While the bacterial factors necessary for the infection are relatively well characterized and the complete sequences of the nopaline-type T-DNA region and the entire Ti plasmid have recently been determined (Suzuki et al. 2000), much less is known about the host cell factors involved in this process.

Clean DNA transformation

Initially, *Agrobacterium*-mediated transformation was considered as "a clean" transformation system in which only sequences between the T-DNA borders were introgressed into the plant genome (Kooter et al. 1999). However, in the both *A. tumefaciens* and *A. rhizogenes* a co-transfer of the prokaryotic backbone vector (defined as Ti or Ri-plasmid sequences outside the conventional T-DNA borders that are also incorporated into the plant DNA) has been almost systematically reported at high frequency in many studies (independently of the agrobacteria strain used). This phenomenon generally occurs because T-DNA transfer starting from the right border skips the left border and part of the binary plasmid is transferred to coffee genome. The problem worsens if the vector backbone with bacterial antibiotic resistance markers is transferred into the plant genome. The excessive presence of binary vector DNA adjacent to transgene sequences could lead to poor expression or methylation of the transgene loci (Iglesias et al. 1997). Also, limiting amounts of *vir* gene products could result in inefficient cleavage of the right or left border sequences of the T-DNA and co-transfer of non-T-DNA sequences (Matzke et al. 1994; Ramanathan & Veluthambi, 1995).

A similar problem was found in traditional particle bombardment methodology, where plant transformation carried out with whole plasmid vectors, caused also introduction of the vector backbone into the plant genome along with the transgenes of interest (Kohli et al. 2003). This superfluous DNA was undesirable because often leads in transgene silencing, since vector backbone sequences were prokaryotic origin, then triggered *de novo* DNA methylation and also promoted transgene rearrangement through the production of multi-copy transgenic loci (Kohli et al. 2003)

Reporter genes and selection markers

Most protocols for development of transformed plants required that plasmid vectors possessed selection markers genes in order to restrict the development to transformed cells or tissues. Common examples of selection markers are genes encoding resistance to antibiotics as kanamycin (*nptII*) and/or to herbicides as hygromycin (*aphIV*), nevertheless, those selection systems of transformed plants based upon antibiotics or herbicide selection allowed frequent escapes of non-transformed cells (Peña & Séguim, 2001). This system also presented the difficulty of removing such genes selectively and cleanly from transgenic lines after regeneration

(Agrawal et al. 2005). Also, the growing of transformed cells or tissues in media containing these compounds affected negatively their regeneration efficacy, making the protocol even laborious and arising the timing necessary to produce a whole transformed plant.

Due to the previous disadvantages of selection marker genes, reporter *uidA* (GUS) gene started to be used widely to detect transformed cells. However, the use of this gene became undesirable for monitoring the time course of transgene expression in living cells or for rapid screening of primary transformants, mainly because assays for measure GUS activity required histochemical staining with exogenous substrate and was destructive (tissue analyzed needs to be excised from the plant). Moreover, differences in cell size and metabolism as well as variations of penetration and diffusion of the staining substrate can contribute to differences in staining intensity that could lead to make wrong conclusions about promoter efficacy (Jefferson et al. 1987). Therefore, Southern or Northern blotting analyses are necessary to be performed at the target organ level to obtain accurate conclusion that plants did not express the *uidA* RNA.

Therefore, *uidA* gene became replaced by the *gfp* gene, a most efficient marker that expresses the jellyfish green fluorescent protein (GFP) from *Aequorea victoria* algae, and serves as an *in vivo* reporter, since expression can be detected in living cells (Hasselof and Amos, 1995). Since the first report that GFP from a wild-type *Aequorea* could be visualized in *Citrus sinensis* cells (Niedz et al. 1995), constant genetic engineering improvements have been done in order to increase GFP protein expression and stability (Chiu et al. 1996; Pang et al. 1996; Davis and Viestra, 1998). The use of GFP as marker selection of transformed embryos or explants, had replaced partially or completely antibiotic or herbicide selection in sugarcane, tobacco, maize and lettuce (Elliot et al. 1999), rice (Vain et al. 2000), citrus (Ghorbel et al. 1999) and woody plant species transformation (Tian et al. 1999) mainly because GFP transformed tissue can be identify, then isolated easily and subcultured in the absence of the selection agent, achieving therefore, faster regeneration of transformed plants. This "real time" GFP expression allowed for example to monitory simultaneously the expression of a target *cry1Ac* gene in tobacco (Harper et al. 1999)

Another gene commonly used as morphological marker to study the variability of transgene expression in plants is the *rolC* gene (Flandung et al. 1997). This type of gene offers as advantages that major morphological alterations can be observed in greenhouse or field conditions, also serve to study complete or incomplete transgene suppression (Fladung, 1999; Kumar & Fladung, 2001).

Transgene integration and stability

Plant transformation does not always result in efficient transgene expression (Gelvim, 2003). Although, the mechanism of T-DNA integration is suggested to be similar in perennial and annual plants (Kumar & Flandung, 2002), little is known about analysis of transgenes expression in trees (Hawkins et al. 2006). Of particular interest is the role of transgene integration in silencing phenomena (Kooter et al. 1999; Kohli et al. 2003). Stably expression of foreign DNA occurs at telomeric regions (near the ends of chromosomes), which are typically gene rich in the plant genome, whereas unstably transgene expression have been localized in heterochromatic (centromeric) regions (Iglesias et al. 1997). Both in the case of *Agrobacterium* mediated transformation and direct DNA transfer, the position of transgene integration in the chromosome can vary considerably among independent transformants, and may repress in some cases transgene expression (Meyer & Saedler, 1996; Kumar & Fladung, 2001). The packaging of transgenes into a chromosome is regulated via sophisticated chromatin remodeling mechanisms that define whether and when a gene becomes accessible to the transcription machinery, thus chromatin plays an important role in plant epigenetics (Meyer, 2000)

A second class of silencing phenomena can arise, not by the alteration of the T-DNA transgene sequence, but when multiple inserts of the T-DNA are present in the transgenic plant genome (Muskens et al. 2000). Two different mechanisms can be distinguished: transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) (Stam et al. 1997). TGS is classified as an abolished transcription of the transgene, associated with methylation of promoter sequences of the transgene (Vaucheret et al. 1998). As opposed, in PTGS even thought high transcription of the transgene occurs in the nucleus, the mRNA does not accumulate in the cytoplasm (Kooter et al. 1999). PTGS has been hypothesized to derivate from a natural post-transcriptional defense mechanism against pathogens like virus and transposable elements (Waterhouse et al. 2001). Also it has been suggested that PTGS is enhanced under the control of the strong promoters as 35S (Elmayan & Vaucheret, 1996; Porsch et al. 1998) because an over-production of transgene RNA above putative threshold level could trigger an irreversible degradation of RNA by the cellular machinery involved in post-transcriptional antiviral defense (Vaucheret et al. 1998).

In order to obtain more predictable transgene expression levels, single-copy transformants are often preferred for phenotypic analyses, although they are not guaranteed to produce predictable transgene expression levels. However, there is now considerable evidence that single-copy and single-locus transformants also undergo silencing i.e in rice (Kohli et al. 1999), *Arabidopsis* (De Buck et al. 2004) potato (Craig et al. 2005). Hence, transgene copy numbers may be highly variable irrespective of the transformation method and are probably dependent on numerous factors which have not been identified (Butaye et al. 2005).

In tomato, Goggin et al. (2004) found that *Mi R*-gene mRNA levels in all evaluated transgenic plants were comparable to levels observed in the resistant control to *Meloidogyne incognita*; however resistance efficacy was reduced in comparison with the later. The authors suggested that transgene expression could be hampered during foreign protein translation or post-translational processing and/or that transgene integration into the plant genome may disrupt the function of other important genes critical to nematode resistance, resulting in an aberrant phenotype.

Influence of transformation method in transgene stability

Agrobacterium-mediated transformation methods exhibited less transgene rearrangement when compared with direct transformation methods in *Arabidopsis* (Lechtenberg et al. 2003) and barley (Travella et al. 2005). The lower prevalence of transgene rearrangement during T-DNA integration must be linked to its association with *Agrobacterium* vir proteins that protects it from degradation during the integration process (Kohli et al. 2003). Interactions between species or ecotype and type of explant transformed with *Agrobacterium* strains may underlie differential infectivity responses (Heeres et al. 2002). In *Arabidopsis* Karimi et al. (1999) reported more complex T-DNA arrays when *A. rhizogenes* was used instead of *A. tumefaciens*.

At the plant level, differences in susceptibility of various trees cultivars to different *Agrobacterium* strains have been observed and factors such as endogenous phytohormone levels of the explants have been proposed to influence successful transformation (Flandung et al. 1997).

Influence of promoter in transgene expression

Stability of transgene expression is an important character of genetically modified plants. Transgene instability that leads to silencing of the transgene and subsequent loss of the expected phenotype can result from various factors above mentioned, but another possible cause might be the promoter of the T-DNA binary plasmid itself. The 35S promoter, derived from the cauliflower mosaic virus, is the most common component of transgenic constructs and is used in more than 80% of genetically modified plants (Hull & Dale, 2000). In general, 35S is considered to be constitutive, which means that it is expressed in all of the plant cells, however, several studies have revealed that expression of most common reporter genes like *uidA* and *gfp* driven by 35S promoter are tissue-specific and define expression through development: tobacco (Benfey et al. 1990); tomillo (Assad-Garcia et al. 1992); *Casuarina* tree (Smouni et al. 2002; Obertello et al. 2005).

Conversely, GUS and GFP expression in tobacco plants growing in field conditions was reported to be similar when both genes were controlled by 35S promoter (Harper & Stewart, 2000). Halfhill et al. (2001) in soybean and El Shemy et al. (2004) in canola demonstrated that plants expressing 35S-GFP protein grew and reproduced normally, and GFP expression was inherited to the progeny. On other hand, homology between transgene construct and viral genes can occur in the promoter region, leading to the suppression of the inserted genes due to transcriptional down-regulation of the 35S promoter in oilseed (Al-Kaff et al. 2000). As mentioned before, RNA interference [RNAi] can naturally occurs as a post-transcriptional gene silencing [PTGS] in plants as a mechanism of defence against foreign genes like virus in which double-stranded RNAs [dsRNAs] in cells caused degradation of RNAs that share the same sequence with the dsRNAs (Voinnet, 2001).

Agrobacterium-mediated transformation with large DNA fragments and gene isolation

Gene isolation by positioning cloning frequently requires several rounds of transformation with traditional binary vectors. Even in well-studied crops for which multiple DNA markers are available, still difficult to narrow down the chromosomal region of interest to a manageable size for gene identification (Tao et al. 2002). To minimize the resources invested and to accelerate the process, recent research advances allow the use large DNA fragments in transformation experiments followed by analysis of transgenic plants to assess functional complementation.

Commonly, the size of T-DNA to be transferred to the plant by *Agrobacterium* mediated-transformation is generally large (> 40 kb) and cloning can be time-consuming and laborious. Moreover, traditional binary vectors generally do not allow transfer of DNA sequences larger than 25 kb. Recently, larger binary vectors i.e. BIBAC (Hamilton et al. 1996), TAC (Liu et al. 1999) have provided a fast and reliable alternative to the cloning of large genomic-DNA fragments (< 150 kb) for standard *Agrobacterium*-mediated transformation protocols in several plant species. Plant-transformation-competent BIBAC and TAC libraries representing the entire genome were constructed in tomato (Hamilton et al. 1999), *Arabidopsis* (Chang et al. 2003) and rice (Tao et al. 2002; Qu et al. 2003) to facilitate genome-related research and determination of gene function. This technology can be also useful in the transfer of clusters of genes (naturally occurring or synthesized) into plant genome. Consequently, several disease resistance genes or genes belonging to a specific biochemical pathway can be inserted through one transformation event and subsequently inherited as a single locus.

Functional analysis of *R*-genes to nematodes

Genetic engineering in most important agricultural commodities had increased considerably the knowledge about *R*-gene function during the last decade, mostly due to the accuracy of protocols for introgression of desired resistance genetic factors and the establishment of reliable pathogen tests for each plant species. Among the most studied *R*-genes for any plant-pathogen studied, functional complementation of *R*-gene to cyst (*Heterodera* and *Globodera* sp.) and root-knot (*Meloidogyne* sp.) nematodes had received a worldwide important attention, since significant damages are caused by these root endoparasites in human or animal alimentary security crops i.e. tomato, potato and soybean (see page 22).

Until date, functional validation of cloned plant genes has been done preferably using *A. tumefaciens*-mediated transformation. Principal reasons for this are: i) disposition of reliable protocols for efficient obtention of transformation events, ii) higher availability of vectors and iii) the fact that larger size of T-DNA can be transfer into the plant genome compared with direct transformation methods. However, an important inconvenient of transformation protocols using *A. tumefaciens*, is that regeneration of whole plants for nematode tests from initially transformed callus still constitutes a time consuming and tedious process, e.g. with actual regeneration procedures approximately 1 year is required before the plant with the introduced sequences can be evaluated.

On other hand, *R*-genes to nematodes can be rapidly and effectively validated by producing *in vitro* transformed roots expressing candidate resistance genes obtained after infection by *A. rhizogenes* (Remeeus et al. 1998). Such roots, when cultured *in vitro* are commonly called "hairy roots" because they frequently exhibited a particular phenotype (rapid growth, high branching and plagiotropic development). Hairy roots have been successfully used to study *Mi* and *Hs1pro-1* *R*-genes function in tomato and sugar beet, respectively (Cai et al. 1997; Kifle et al. 1999; Hwang et al. 2000). Moreover, further advances in *A. rhizogenes*-mediated transformation will contribute to elucidate some uncertainties associated with this transformation method as the number of copies, site of localization and stability of the expression of transgenes following insertion in the plant genome.

6. Genetic transformation of coffee

Coffee genetic engineering emerged during the last decade as a potential tool to achieve objectives from two different research strategies: i) study the introgression impact (functional *in vivo* validation) of agronomically interesting genes that would be introduce later by traditional breeding programs, ii) serve as a tool to introduce desirable traits into commercial genotypes (i.e. protection against insects to which no source of resistance has been identified naturally in *Coffea* sp.).

However, the previous was only available after the establishment of protocols for regeneration of coffee plantlets for both principal commercial species (*C. arabica* and *C. canephora*). In that order, two types of somatic embryogenesis (both using leaf sections as explants) have been preferably used since enables efficient regeneration of plantlets from different tissues with numerous technical simplifications: i) *direct somatic embryogenesis*: somatic embryos are obtained quickly (approximately 70 days) on only one medium with the production of limited callusing. This procedure is particularly suited to *C. canephora* and ii) *indirect somatic embryogenesis* based on the use of two media: an induction medium for primary callogenesis, and a secondary regeneration medium to produce friable embryogenic callus regenerating several hundred thousand somatic embryos per gram of callus on both *Coffea* sp. (Etienne et al. 2005).

Gene source

The recent development of high through-put methods for analyzing the structure and function of genes represents a new paradigm with broad implications for agricultural production. With the end of the sequencing of the first model organisms and the development of centers of excellence in genomics, the knowledge of genomes and their expression increases exponentially, inclusive for woody plants like coffee. During the last decade, important efforts were made in coffee to develop EST data bases. The Brazilian government funded an ambitious Coffee Genome Program with the objective to establish a data bank of more than 200 000 expressed sequence tags (EST) which could lead to the identification of more than 30 000 genes. Recently, a mixed Nestlé-Cornell University team published an EST database of around 47 000 cDNA clones, corresponding to 13 175 unigenes (Lin et al. 2005). In parallel, BAC libraries of both coffee species, *C. arabica* and *C. canephora*, were established (Noir et al. 2004; Leroy et al. 2005). Such maps are of central strategic importance for marker assisted breeding, for straight-forward positional cloning of agronomical important genes, and analysis of gene structure and function.

As a consequence of the recent efforts on coffee genomics, a lot of coffee gene candidates had been identified and some of them are currently under cloning. Among them, pathogen resistance genes as: *Mex-1* gene to *Meloidogyne exigua* root-knot nematode (Noir et al. 2003), *Sh-3* gene to race 3 of coffee leaf rust (*Hemileia vaxtratix*) (Prakash et al. 2004) and *Ck-1* gene to coffee berry disease (*Colletotrichum kahawae*) (Gichuru et al. 2006) and others involved in physiological functions such as sucrose synthase (Leroy et al. 2005), caffeine synthase (Ogita et al. 2004; Satyanarayana et al. 2005), seed's oil content (Simkin et al. 2006) and osmotic stress response (Hinniger et al. 2006). For the mentioned intrinsic genes and for the ones to be discovery, the availability of efficient genetic transformation procedures becomes necessary in order to validate their function and to analyze their structure. On attendance, the majority of transformation events done until present in coffee corresponds principally to *uidA* and/or *gfp* reporter genes inserted between T-DNA borders in pBIN or pCambia type binary backbone (see Table 1). Exceptions to the previous was done by Leroy et al. (2000), who achieved stable transfer of *cry1Ac* gene (synthesized from *Bacillus thurigiensis* and that encodes an endotoxin active against coffee leaf miner *Perileucoptera coffeela*) and of *csr1-1* gene (conferring resistance to chlorsulfuron herbicide).

In addition, Ogita et al. (2003) and Kumar et al. (2004) reported the production of transformed plants with suppression of the expression of the genes that encode the enzymes threobromine synthase (*CaMXMT1*) and N-methyl transferase (*NMT*) respectively, both involved in caffeine biosynthesis. In particular, Ogita el al. (2003), achieved production of doubled strained RNA interference (RNAi) method. RNAi sequences homologous to the *CaMXMT1* mRNA given as a result that leaves of 1-yr-old transformed trees exhibited reduced theobromine and caffeine content (30 to 50% compared with the control).

Markers and reporter genes

For successful recovery of transgenic plants, the choices of a suitable selection marker, its concentration and timing of application are critical. During the period following transformation, the selecting agent must permit the proliferation of transformed cells and further development of transformed embryos along limiting the negative toxicity effects related to the polyphenolic oxidation from the surrounding tissues. Although, in most coffee transformation reports T-DNA transfer has been done through the use of binary vectors containing both the gene of interest and one or various selection marker(s) genes, few research teams have compared different concentrations and timings of antibiotic (*hpt* hygromycin-R, *nptII* kanamycin-R) or herbicide (*csr1-1* chlorsulfuron-R, *bar* ammonium glufosinate-R, *ppt* phosphinothricine-R) resistance markers genes in order to find the parameters that allow best rates of transformed plant regeneration.

First works in coffee transformation were done using kanamycin. However, this antibiotic has exhibited contradictory results as potential agent for transformed coffee embryos. Barton et al. (1991) and Spiral et al. (1993) concluded that kanamycin possess poor selective capacity since non-transformed somatic embryos could regenerate even at high concentration doses (400 mg/L), whereas Giménez et al. found that secondary somatic embryogenesis was inhibited at 50 mg/L. Giménez et al. (1996) and Van Boxtel et al. (1997) agreed that embryogenic suspension cultures showed tolerance to 400 mg/L of kanamycin. Despite the previous warnings, kanamycin was recently used at 400 mg/L by Cuhna et al. (2004) and at 100 mg/L by Canche-Moo et al (2006), both achieving acceptable regeneration of transformed embryos.

Hatanaka et al. (1999), Naveen et al (2002) and Ogita et al. (2004) observed that hygromycin at 50-100 mg/L allowed an acceptable regeneration frequency of *A. tumefaciens*-mediated transformed somatic embryos. Kumar et al. (2006) using *A. rhizogenes*-mediated transformation reported that hygromycin 20 mg/L interfered with secondary embryos growth and therefore so sub-optimal levels of selection pressure (5 mg/L) were employed during early embryogenesis development in order to retain nature of the transformed cell. However, because of the biosafety concerns about transferring of bacterial antibiotic resistance markers to plants and from these to animals or humans, plant transformation strategy turned into the utilization of other types of selection markers as herbicide selection or positive selection in order to avoid this potential biohazard.

Van Boxtel et al. (1997) proposed a "hybrid" protocol for selection of coffee transformed tissues, where kanamycin should be used only during early phase of callus development (100 to 200 mg/L) with subsequent use of glufosinate ammonium (3 mg/L) on later developmental stages of somatic embryogenesis (Glufosinate ammonium or phosphynotricin PPT, is an herbicide that inhibits glutamine synthetase, an enzyme able to detoxify the ammonium produced by the reduction of nitrate, the degradation of aminoacids and the photorespiration); the gene *bar* codes the phosphynotricin acetil transferase -PAT- that acetylates the free NH_2 from PPT, producing resistance to the herbicide). Van Boxtel et al. (1997) and Fernandez Da Silva & Menéndez-Yuffá (2004) showed that low concentration of glufosinate (6 mg / L) were enough to inhibit non-transformed callus growth. Consequently, ulterior works carried by Ribas et al. 2005a and Ribas et al. 2006a) confirmed the reliability of *bar* gene (showing resistance to this agent) as a selection marker for regenerate only transformed somatic embryos. Leroy et al. (2000), reported efficient selection of somatic embryos from necrotic callus using chlorsulfuron at 80 µg / L. Later works succeeded to regenerate also transformed plantlets containing *ppt* gene conferring resistance to phosphinothricine on selective medium containing 10 µM of the herbicide (Cruz et al. 2004).

Others marker genes as phospho-mannose isomerase (*pmi*) (Joersbo et al. 1998) and xylose isomerase (*xylA*) (Haldrup et al. 1998) started to gain attention for their use in coffee genetic transformation. They differ from conventional (antibiotic or herbicides) selection markers as they are based on supplementing the transgenic cells with a recovery metabolic advantage rather than killing transgenic shoots after transformation (Joersbo, 2000). In coffee, Samson et al. (2004) tested the regeneration of somatic embryos in presence of mannose and xylose in media devoid of glucose, and demonstrated that they were able to regenerate into explants in the presence of

mannose as carbohydrate source, but not in different concentrations of xylose, indicating thus the potential use of *XylA* gene as a positive selection marker in coffee transformation.

Recently, following environmental concerns about transferring herbicide resistance through natural pollinization to non-transformed related plant species growing near to transgenic crop fields, along with the aim of simplify transformation procedures, visually markers started to replace those based in herbicide selection. In that order Ogita et al. (2004) and Chance-Moo et al. (2006) used *gfp* and *DsRFP* reporter genes respectively for visual selection of somatic embryos of *C. canephora* following *A. tumefaciens*-mediated transformation.

Promoter

With few exceptions, the 35S promoter, derived from the cauliflower mosaic virus, has been the most common component of transgenic constructs used with either direct or indirect *Agrobacterium*-mediated coffee transformation (see Table 1). Surprinsingly, apart of Van Boxtel et al. (1995) and Rosillo et al. (2003) who tested different promoters controlling *uidA* gene and compared its expression in endosperm, somatic embryos and leaf explants of *C. arabica*; none other research group had done this type of research in coffee. Van Boxtel et al. (1995) founded that EF-1α promoter (from *A. thaliana* EF-1α translation elongation factor p35S drive) revealed the most efficient GUS gene transient expression; however the use of this promoter has been restricted to this research group (Leroy et al. 2000; Perthuis et al. 2005). Rosillo et al. (2003) compared the efficacy of transfer and expression of GUS gene driven by 35S with respect to two coffee promoters (α-tubulin and α-arabicin); under their protocol conditions, all promoter constructs resulted in similar transient expression of GUS gene, opens the possibility that pCaMV35S could be replaced with an equivalent coffee promoter.

Not less important is the lack of studies about the characterization and specific localization of p35S drived-GUS activity in transformed tissues of coffee. Sreenath and Naveen (2004b) carried an histochemical survey of p35S.GUS activity comparing various tissues in untransformed and transformed embryogenic calli and somatic embryos, and demonstrated that coffee possess an endogenous GUS activity which is expressed to different levels in different tissues, but differs from introduced bacterial GUS activity in optimal pH requirement and sensitivity to methanol. Hatanaka et al. (1999) demonstrated that leaves and roots and of p35S.*uidA* transformed plants exhibited a deep blue color on reaction with X-gluc, while non-transformed plantlets did not react

with X-Gluc. Leroy et al. (2000) found that 50% of the embriogenic callus transformed with pEF-1α.GUS drived-promoter and growing in culture media containing a high dose of chlorsulfuron (80 µg / L) did not exhibit a blue staining. The previous clearly demonstrated that increasing the selective marker concentration did not alleviate the problem of escapades and even decreased the rate of transformation events. Therefore, this research team used GUS histochemical test as a secondary screening of whole plantlets.

Ogita et al. (2004) demonstrated that epifluorescence of p35S.*gfp* gene was constitutively detected throughout the entire somatic embryo. Similarly results were observed by Chance-Moo et al. (2006) in somatic embryos using p35S.*DsRFP*. Satyanarayana et al. (2005) recently achieved cloning of the first promoter for the N-methyl transferase gene involved in caffeine biosynthesis, which will be very useful in near future for studying the regulation of caffeine biosynthesis genes.

Methods employed

Direct gene transfer

After first report from Barton et al. (1991) of transformation of coffee embryos by ***electroporation*** using the *nptII* (kanamycin resistance) gene, this method remained unmentioned until Da Silva-Hernandez and Yuffá-Menéndez (2003) described some improved conditions to regenerate transformed *C. arabica* somatic embryos expressing the GUS and *bar* genes. Their experiments showed that the electroporation of somatic embryos in torpedo stage can be promissory as a method for coffee transformation since expression of GUS and *bar* genes was detected by PCR in tissue from the regenerated plants several months after transformation (De Guglielmo y Menéndez-Yuffá, unpublished results).

Biolistic delivery method (Klein et al. 1987) has been improved considerably since the first report of GUS transient expression in coffee using a powder driven gun by Van Boxel et al. (1995a). Rosillo et al. (2003), studied diverse interactions between osmotic preconditioning of *C. arabica* suspension cells and physical parameters using helium gun device. They determinate that a short period of endosperm pretreatment with two non-metabolixed agents (mannitol and sorbitol) increased the number of cells expressing GUS gene without causing cell necrosis after bombardment. The authors discuss the advantage of using these types of osmotic agents instead

of sucrose in order to reduce the turgor pressure of cells and increase cell survival by avoiding the leakage after the shock wave created during bombardment. Ribas et al. (2005a) described a protocol for transformation of embryogenic callus and somatic embryos of *C. canephora* using a helium gun, following by cell transferring into medium containing mannitol before and after bombardment. Their protocol allowed apparition 12.5% of transformed callus expressing GUS-positive reaction to histochemical activity assay.

De Guglielmo & Menéndez-Yuffá (unpublished results), using a pistol at low helium pressure evaluated the efficacy of this technique in transformation efficacy of torpedo and globular somatic embryos, embryogenic callus and vitroplant leaves of *C. arabica*. Based on the GUS transient expression, survivorship and regeneration of the tissues, the authors determinate that torpedo embryos were the best material for biolistic gene delivery. The same authors applied the improved conditions to transform also *C. arabica* with the complete pUBC plasmid and the genetic cassette Ubi-cry-Nos (promoter-codingsequence-terminator). Such modification of the transformation procedure avoids or eliminates the negative effects attributed to the backbone of the vector of transformation that could be responsible for transgenic silencing, besides the number of foreign sequences introduced in the plant genome, including genes of resistance to antibiotics, which is controversial from the point of view of biosafety.

Indirect gene transfer

Hatanaka et al. (1999) achieved the first successful *A. tumefaciens*-mediated transformation of *C. canephora* plants exhibiting strong GUS stable expression. Leroy et al. (2000) reported also transformation and efficient regeneration of coffee plants of both *Coffea* sp. containing *uidA* and *cryIAc* genes. Ribas et al. (2006a) also transformed *C. canephora* explants submitted to sonification during immersion on *A. tumefaciens* suspension strain encoding *uidA* and *bar* genes and regenerated transformed plants. Chance-Moo et al. (2004) transformed leaf explants through *A. tumefaciens*-mediated transformation involving a vacuum infiltration protocol in a bacterial suspension, followed by a step of somatic embryogenesis induction. *A. tumefaciens*-mediated transformation had also served to induce stable gene silencing through RNAi technology of genes encoding theobromine synthase (Ogita et al. 2004) and N-methyl transferase (Kumar et al. 2004) [both involved in caffeine biosynthesis] in both *C. arabica* and *C. canephora* species. Ribas et al. (2005b) achieved inhibition of ethylene burst of *C. arabica* by means of introducing the transgene in antisense orientation.

Agrobacterium rhizogenes-mediated transformation in both *C. canephora* and *C. arabica* species was first reported by Spiral et al. (1993) and Sugiyama et al. (1995) respectively. Leroy et al. (1997) also achieved stable transformation of both *Coffea* sp. somatic embryos following the protocol developed by Spiral et al. (1993). Kumar et al. (2006) described an adapted method for *A. rhizogenes* sonification-assisted embryos transformation. Their technique allowed the transformation and direct regeneration of transformants through secondary embryos bypassing an intervening hairy root stage. Interestingly, *C. arabica* resembles less amenable to *Agrobacterium* infection that *C. canephora* and, although transient and stable expression has been reported in both species (Kumar et al. 2006).

Regeneration of whole transformed plants

The long life cycle of coffee tree calls for stability of the transgenes over several years and, until present, regeneration of stable transgenic coffee has been scarcely studied and seems still far from suitable to be use in routine regeneration of different *Coffea* species.

First regeneration of transformed *C. canephora* was achieved by Spiral et al. (1993) and of *C. arabica* by Sugiyama et al. (1995). In both studies, transformed plantlets were regenerated from *A. rhizogenes*-mediated transformed somatic embryos and roots respectively; however in both studies regeneration protocol was laborious and plants showed a "hairy" phenotype with short internodes and stunted growth. Since van Boxtel and Berthouly (1996), developed an optimized protocol that allowed high frequency somatic embryogenesis from coffee leaves, homogeneous and efficient regeneration of vigorous coffee plantlets from embryogenic suspensions cultures become the most common technique to regenerated transformed plants following either direct or indirect cell transformation (See Table 1). Such technique was later used by Leroy et al. (2000), however embryogenic callus was induced only in 1 and 0.1% from total *A. tumefaciens*-mediated transformation events for *C. canephora* and *C. arabica* respectively. From this callus, only 30 and 10% developed into secondary embryos, and from these, only 50% regenerated into plantlets. The previous results clearly demonstrated the genotype effect on coffee regeneration (higher in *C. canephora* with respect to *C. arabica*).

Ogita et al. (2004) developed an advantageous method which consisted in the production of direct somatic embryos directly formed from the epidermal tissues of the initial embryos (without callus formation and where somatic seedlings stage is reach within 3-6 months). Kumar et al. (2006)

reported that regeneration of coffee plantlets following *A. rhizogenes*-mediated transformation of somatic embryos was barely efficient (only 3% of total transformation events). They also described some plantlets exhibited a "hairy root" phenotype with abnormal elongation and brittle and wrinkled leaves phenotype. However, the percentage of plantlets in the previous study aberrant phenotype was significantly low compared with the results described by Sugiyama et al. (1995), who described that all regenerated coffee plantlets showed a "hairy root" phenotype. Such abnormal phenotype is stable in time as demonstrated Perthuis et al. (2005) who demonstrated that four out of the nine independently transformed *C. canephora* clones obtained with *A. rhizogenes* were still displaying this phenotype in field conditions, however all these plants died rapidly after planting.

Although a considerable number of reports have done regeneration of transformed coffee plants, all plants from such experiments were grown in transgenic green-houses. Perthuis et al. (2005) reported the sole work of successful regeneration of transformed coffee plants up to establishment a lot in field conditions.

Testing

Herbicide resistance

Leroy et al. (2000) regenerated transformed explants expressing resistance to chlorsulfuron. However, not all exhibit amplification of *csr1-1* gene, demonstrating the important occurrence of escapes during herbicide selection. Apart from chlorsulfuron, selection of transgenic coffee plants has also been conducted by means of regenerating somatic embryos encoding *bar* gene on selective medium containing ammonium glufosinate (Ribas et al. 2005). Regenerated plants supported up to eight times the herbicide recommended field application doses (Ribas et al. 2006a).

Pathogen resistance

Reports about coffee genetic engineering approach to insect resistance had increased considerable during the last years, since to date, none resistance to coffee berry borer (CBB) or coffee leaf miner (CLM) have been reported in *Coffea* sp. In the case of CLM, research has focused primarily on the use of *cry1Ac* gene from *Bacillus thuringiensis* (Bt), which posses the most

efficient ß-endotoxin against leaf miner (*Perileucoptera coffeella*) (Guerreiro et al. 1998). Leroy et al (2000) described that *C. canephora* transformed plants with this gene, achieved to synthesis and express resistance to the insect in green-house conditions. Perthuis et al. (2006) reported that such resistance was stable and operationally effective resistance after six releases of a natural population of *P. coffeella* during four years of field assessments.

In other hand, production of transformed coffee plants with resistance to CBB is actually conducted by Cruz et al. (2004) with the *α-All* gene from common bean. The authors achieved transformation of *C. canephora* plants with this gene and bioassays with the insect are under way to confirm functional validation of its proteins in coffee.

Following recent achievements of other crops, gene pyramiding could be envisage in coffee in order to introduce a large number of resistance genes to diverse races of one pathogen or combined different pathogens.

Physiological traits

Ribas et al. (2005b) achieved inhibition of the ethylene burst by introducing the *ACC oxidase* gene in antisense orientation; this technique would permit the understanding of genes involved on fruit maturation and ethylene production. Satyanarayana et al. (2005) reported the cloning of the promoter for the gene N-methyl transferase (NMT) involved in caffeine biosynthesis pathway. The authors mentioned that efforts are on to use this promoter sequence for down regulation of NMT gene through transcriptional gene silencing.

The recent cloning of the first promoter for the gene involved in caffeine biosynthesis (Satyanarayana et al. 2005) together with the proximity identification of genes involved in sucrose and drought tolerance metabolism (Pot et al. 2006) opens up the possibility for coffee plant transformation to validate and study the molecular mechanisms that regulate the production of these important targets for *Coffea* sp. cultivation.

Thesis research objectives

The **principal research objective** of this work was to develop efficient tools (transgenesis + bioassays) for the functional analysis of resistance genes using the *Agrobacterium rhizogenes*-mediated transformation procedure.

This thesis research was part of a scientific project seeking to gain a clearer understanding of biodiversity, with a view to optimized exploitation of genetic resources. Better knowledge of the genetic diversity of R-*genes* and how they evolve is essential for improving the conservation and exploitation of genetic resources. The transfer of R-*genes* into cultivars needs to be rational, not only in terms of sustainability, but also controlled in order to limit undesirable effects on agro-morphological traits and on the quality of agricultural production. By mastering the *A. rhizogenes* transformation process along with bio-assays for nematode resistance evaluation, it should be possible to validate the functionality of the *Mex-1* gene.

Following that study, it will be possible to analyse, in functionality terms, the diversity of orthologue sequences derived from a genetic resources analysis. It will then be possible to determine the molecular bases of resistance specificity associated with that locus.

Various specific objectives were pursued during this thesis work:

i. It was investigated whether the *Mex-1* gene could confer intermediate resistance associated with incomplete expression and whether that expression is stable.

In coffee trees, several *C. arabica* lines derived from the interspecific Timor hybrid (wild *C. arabica* x *C. canephora*) have displayed resistance to the *M. exigua* nematode and the IRD-CIRAD team confirmed that resistance to that nematode came from the *C. canephora* parent (Bertrand et al. 2001). Recently, Noir et al. (2003) identified molecular markers associated with the resistance to *M. exigua*. A simply inherited major gene, called the *Mex-1* locus, was identified. That major gene may have incomplete dominant expression because most of an F2 population showed a gall index that was higher than the mean value of the resistant parent. Intermediate resistance to plant pathogens has frequently been reported for numerous host/parasite interactions.

Tzortzakakis et al. (1998) suggested that the number of copies of the *Mi* gene inserted in tomato hybrids could modify the expression of *R* genes. The availability of the complete sequence of the *Mex-1* gene in the near future will make it possible to carry out a functional analysis of *Mex-1* and maybe provide an opportunity for studying how the number of copies of the *Mex-1* gene in the *C. arabica* tree genome influences the expression of resistance to *M. exigua*.

ii. The development of an *A. rhizogenes*-mediated transformation protocol enabling a rapid regeneration of transformed roots and the subsequent production of composite plants.

In coffee, protocols for *A. rhizogenes* mediated-transformation and plant regeneration have been reported (Spiral et al. 1993; Kumar et al. 2006), but the transformation efficiency achieved to date has been very limited and unsuitable for use in the functional analysis of genes. One of the main objectives of this thesis work was to establish reliable culture conditions for rapid regeneration of *A. rhizogenes*-transformed roots.

Since *A. rhizogenes*-transformed roots are often characterized by a 'hairy root' phenotype (rapid growth, high branching and plagiotropic development), the potential for composite plant production (transformed roots on untransformed shoots) was studied and it was determined whether that system offered major advantages over the 'hairy roots' approach for functional genomics studies of genes conferring resistance to nematodes. Consequently, a procedure for the infection of transformed composite plants was also developed.

iii. The establishment of suitable conditions for effective long-term proliferation of hairy root axenic cultures.

In coffee, culture conditions for long-term maintenance of axenic root cultures have not yet been described. However, a command of the hairy root (*A. rhizogenes*-transformed roots) proliferation tool is important at different stages of the procedure:

- to maintain a library of transformation events,
- to have large quantities of biomass, hence of DNA, for molecular studies associated with functional genomics (PCR and Southern Blot analyses),
- to regenerate whole transformed plants and be able to study any expression of a promoter or of transgene in the whole plant.

The development of such a technique would also provide a clearer understanding of the morphological changes in hairy-roots and define i) whether morphological variables can be used to distinguish between the different phenotypes ii) identify the responsible genes from RiT-DNA for the altered phenotypes.

iv. Finally the expression of the CaMV35S-GUS and CaMV35S-GFP constructs within transformed roots was analysed.

For perennial plants, except maybe *Populus* and *Casuarina*, there is still little information available on the expression pattern of the cauliflower mosaic virus promoter in transgenic trees. It is increasingly being reported in the bibliography that such expression may not be constitutive. In the coffee tree, that type of information is particularly lacking and there is no information available about the way it is expressed (level and localization) in roots. That approach was taken using the *gus* and *gfp* reporter genes. The stability of CaMV35S-GUS and CaMV35S-GFP was also studied. It was important to assess the reliability of that promoter for future functional validation studies.

Chapter II

Study of *Coffea arabica* resistance to *Meloidogyne exigua*

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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots

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Abstract The possibility of rapid validation and functional analysis of nematode resistance genes is a common objective for numerous species and particularly for woody species. In this aim, we developed an *Agrobacterium rhizogenes*-mediated transformation protocol for *Coffea arabica* enabling efficient and rapid regeneration of transformed roots from the hypocotyls of germinated zygotic embryos, and the subsequent production of composite plants. The *A. rhizogenes* strain A4RS proved to be the most virulent. High transformation efficiencies (70%) were obtained using a 2-week co-cultivation period at a temperature of 15–18°C. Using a p35S-*gusA-int* construct inserted in the pBIN19 binary plasmid, we could estimate that 35% of transformed roots were GUS positive (co-transformed). Using the GUS assay as visual marker, 40% composite plants bearing a branched co-transformed rootstock could be obtained after only 12 weeks without selection with herbicides or antibiotics. Transgenic coffee roots obtained with *A. rhizogenes* did not exhibit the 'hairy' disturbed phenotype and were morphologically similar to normal roots. PCR analyses demonstrated that all co-transformed roots were positive for the expected *rolB* and *gusA* genes. Transformed and non-transformed root systems from both susceptible and resistant varieties were inoculated with *Meloidogyne exigua* nematode individuals. Inoculation of composite plants from the Caturra susceptible variety resulted in the normal development of nematode larvae. Numbers of extracted nematodes demonstrated that transformed roots retain the resistance/sensibility phenotype of varieties from which they are derived. These results suggest that composite plants constitute a powerful tool for studying nematode resistance genes.

Keywords *Agrobacterium rhizogenes* · Coffee · Composite plant · Genetic transformation · Nematode

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Introduction

Plant parasitic nematodes are obligate parasites that represent a major threat for crop production throughout the world. Recently, the cost of nematode parasitism to world agriculture was estimated to be US $125 billion annually (Chitwood 2003). The most severe damages are caused by sedentary root endoparasites, like cyst (e.g. *Heterodera* and *Globodera*) and root-knot nematodes (e.g. *Meloidogyne*). Breeding for nematode resistance has been a major objective for many important crops like tomato, potato, sugar beet and soybean. Consequently, a number of resistance genes to various pathogenic nematodes have been identified and cloned in these crops. The best studied is *Mi* gene from tomato (Milligan et al. 1998), which confers resistance to three species of *Meloidogyne*. Other cloned genes are *Hs1pro-1* gene from sugar beet that confers resistance to *Heterodera schachtii* (Cai et al. 1997) and *Gpa2* and *Hero* genes from potato (van der Vossen et al. 2000; Ernst et al. 2002) conferring resistance to *Globodera pallida* and *G. rostochiensex*, respectively.

Coffee is the most widely cultivated tropical tree crop and represents the leading agricultural export product. The main *Coffea arabica* cultivated varieties were shown to be susceptible to numerous pathogens, notably sedentary

endoparasitic root-knot nematodes (*Meloidogyne* spp.) (Campos et al. 1990). In many production regions, *Meloidogyne* is a major agricultural constraint and can result in significant drops of production. So far, more than 15 species have been reported as parasites of coffee. Sources of resistance specific to root-knot nematodes have been identified in coffee trees (Bertrand et al. 2001) and the *Mex-1* gene conferring resistance to *M. exigua* in *C. arabica* is in the process of being isolated (Noir et al. 2003).

Genetically improving plants for resistance to nematodes requires increased knowledge of resistance genes and associated mechanisms. Molecular dissection of resistance genes and better understanding of resistance specificity basis and evolution would contribute to an enhanced durability and utility of the identified resistance genes. For this purpose, the development of a genetic transformation procedure and additional tools for functional analysis that enable rapid validation and study of resistance genes to nematodes would be extremely useful. Using *Agrobacterium tumefaciens*-mediated genetic transformation is one way envisaged for conducting such studies. This technology exists for the coffee tree (Hatanaka et al. 1999; Leroy et al. 2000; Ribas et al. 2005) but, as with other woody species, it is lengthy, labour consuming and not efficient enough to be used in functional analysis studies.

It has been shown that resistance of genes to nematodes can be rapidly and effectively validated by producing in vitr transformed roots expressing candidate resistance genes (Remeeus et al. 1998). In many dicots, such roots can be produced after infection by *Agrobacterium rhizogenes*, a soil-borne pathogenic bacterium responsible for the development of the hairy root disease, that induces at the inoculation site the development of adventitious roots that are genetically transformed, following transfer of the T-DNA of the Ri (root-inducing) plasmid (Chilton et al. 1982; Tepfer 1990; Christey 2001). *A. rhizogenes* is also able to transfer the T-DNA of binary vectors, thereby enabling the production of transformed roots bearing other foreign genes on a second plasmid (co-transformation). When cultured in vitro, *A. rhizogenes*-transformed roots were often characterized by rapid growth, high branching and plagiotropic development ('hairy root' phenotype).

Hairy root cultures have been used to study nodulation and nitrogen fixation, for the production of plant secondary metabolites and for studying interaction with other organisms, such as mycorrhizal fungi or nematodes. Cho et al. (2000) showed that the soybean cyst nematode could complete its entire life cycle on soybean hairy root cultures. Hairy roots were proposed as an easy system for testing nematodes resistance in crop plants and were successfully used to study *Mi* and *Hs1pro-1* gene function in tomato and sugar beet, respectively (Cai et al. 1997; Remeeus et al. 1998; Kifle et al. 1999; Hwang et al. 2000).

Nevertheless, the use of hairy roots to study nematode resistance presents several limits associated with their phenotype variability and the subsequent requirement to work with many lines that must be transferred regularly to fresh medium plates (Plovie et al. 2003). Moreover, it was reported the difficulty in maintaining axenic culture conditions in a system with three organisms, i.e. plant/nematode/*A. rhizogenes* (Narayanan et al. 1999) and the problematic difference between natural root environment and the use of sterilized nematodes and plant material devoid of aerial system when studying plant–nematodes interactions. In coffee, although the regeneration of *A. rhizogenes*-transformed roots has been reported (Spiral et al. 1993; Kumar et al. 2005), culture conditions for long-term maintenance of axenic root cultures have not yet been described.

An alternative strategy for studying the function of genes in roots is to use 'composite' plants that can be efficiently and rapidly generated by inducing transformed roots on non-transformed shoots after inoculation with *A. rhizogenes* (Hansen et al. 1989; Akasaka et al. 1998; Boisson-Dernier et al. 2001). Composite plants offer the major advantage over axenic hairy root cultures of generating information at the whole plant level and give the possibility to realise functional analysis studies in non-axenic conditions. To our knowledge, composite plants have never been used to study plant–nematode interaction. In this paper, we describe (i) the development of a convenient *A. rhizogenes*-mediated transformation protocol for *C. arabica* enabling efficient and rapid regeneration of transformed roots from the hypocotyls of germinated zygotic embryos, and the subsequent production of composite plants without selection with antibiotics or herbicides; and (ii) the successful infection of composite plant transformed roots expressing the *gus* gene with the *M. exigua* root-knot nematode.

Materials and methods

Plant material and culture conditions

Two varieties of *C. arabica*, Caturra and IAPAR-59 were used in this study. The Caturra variety is susceptible to *M. exigua* whereas IAPAR-59 is resistant to it. The germinated embryos used for transformation were obtained as follows: seeds of these cultivars were surface-sterilized after hand removing the parchment. Sterilization was carried out by immersing the seeds in 8% HClO (w/v) bleach solution. The seeds were stirred for 5 min, then submitted to a vacuum for 20 min and stirred again for 5 min. They were finally rinsed three times in sterile water and soaked in Petri dishes (2 cm deep) containing sterile water and placed in the dark at 27°C. Under these conditions, the seeds were totally imbibed after 48–72 h. Embryos were extracted after removing the endosperm. Germination was obtained by culturing the zygotic embryos in 5.5-cm diameter Petri dishes (three embryos/dish and 12.5 ml of medium) on semi-solid GER medium (Etienne 2005) in the dark at 27°C for 8 weeks.

Bacterial strains and binary plasmid

Five strains of *A. rhizogenes* were compared for their transformation efficiency (Fig. 1): A4RS, an agropine

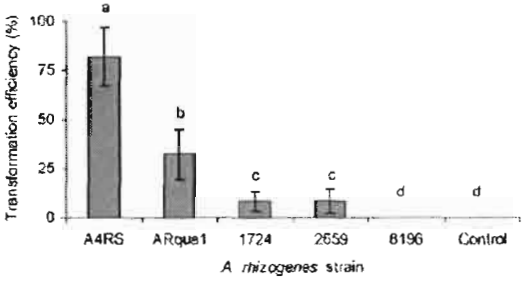

Fig. 1 Comparison of the transformation efficiency of five wild *A. rhizogenes* strains: A4RS, ARqua1, 1724, 2659 and 8196, on *C. arabica* var. Caturra. Transformation efficiency was assessed 10 weeks after infection with *A. rhizogenes*. Control was done on embryos wounded with a sterile scalpel. Each value represents the mean of three replicates on 20 embryos each. Values with different letters are significantly different at $P \leq 0.05$ (Neuman–Keuls test)

mannopine type strain (Jouanin et al. 1986), ARqua1, an agropine mannopine type strain (Quandt et al. 1993), 1724, a mikimopine type strain (Shiomi et al. 1987), 2659, a cucumopine type strain (Daimon et al. 1990), and 8196, a mannopine type strain (Hansen et al. 1991). The A4RS strain was derived from the wild strain A4 modified for resistance to rifampycin and spectinomycin antibiotics. The binary vector pBIN19 was introduced into strain A4RS by the electroporation method (Sambrook 1989). The *uidA* (*gusA*) bacterial gene isolated from *E. coli* coding for β-glucuronidase was introduced in the T-DNA of the pBIN19 plasmid, with an additional intron for specific expression in plants (Vancanneyt et al. 1990). The gene was controlled by the cauliflower mosaic virus (CaMV) 35S promoter and terminator. The armed A4RS-p35S-*gusA-int* strain was used to estimate the transfer efficiency of the binary plasmid (co-transformation frequency) using the GUS assay. The *A. rhizogenes* strains were grown on LB semi-solid medium. The wild strains were cultured without any antibiotics and the A4RS-p35S-*gusA-int* with appropriate antibiotics: 50 mg l^{-1} rifampycin, 500 mg l^{-1} spectinomycin, 50 mg l^{-1} kanamycin at 28°C for 48 h prior to use for genetic transformation.

Transformation procedure

Embryos germinated for 2 months were infected with *A. rhizogenes* by wounding different organs (root hypocotyls, cotyledons) with a scalpel dipped in the 48 h *A. rhizogenes* culture. Control embryos were wounded in the same way using a sterile scalpel. Co-cultivation was carried out during 14 days at 20°C by placing the infected embryos on MS medium (Murashige and Skoog 1962) supplemented with sucrose (40 g l^{-1}) and solidified by adding 2.5 g l^{-1} phytagel (Sigma), in 50 mm diameter Petri dishes. Cultures were placed in dark. To optimise the co-cultivation conditions, different temperatures (15, 18, 20, 22, 24 and 28°C) and co-cultivation durations (0.4, 1, 3, 7, 10 and 14 days) were compared by assessing subsequent transformation efficiency.

Selection of co-transformed roots and production of composite plants

Co-cultured germinated embryos were decontaminated by immersion in MS medium with cefotaxime (500 µg ml^{-1}) for 2 h and rinsed twice with water. The non-transformed radicals were sectioned approximately 4 mm above the collar. Embryos were then subcultured every 4 weeks on MS germination medium containing decreasing cefotaxime concentrations (500, 200, 100 µg ml^{-1}). Transformed roots appeared at the wounding site after 4–8 weeks. Transformation efficiency was calculated as the percentage of inoculated embryos that regenerated at least one transformed root at the wound site. The percentage of GUS-positive (co-transformed) transformed roots was evaluated after 12 weeks by dipping one root tip of well-ramified roots *in staining solution*. The GUS-negative roots were eliminated and only one branched GUS-positive transformed root per embryo was kept on the non-transformed stem to generate co-transformed composite plants. Transformed embryos and composite plants were subcultured every 4 weeks on MS medium and maintained under 14 h photoperiod (20 µmol m^{-2} s^{-1} light intensity) at 26°C until acclimatization.

Histochemical GUS assays

To assay ß-glucuronidase activity, sectioned transformed roots were drenched with a *staining solution* containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) in microwell plates and incubated overnight at 37°C, as indicated by Jefferson (1987). To confine the localization of blue staining, 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)$ were added as catalysts.

Polymerase chain reaction (PCR)

DNA from transformed roots that displayed a positive reaction to the GUS histochemical test was extracted using the Dneasy® Plant Mini Kit No. 69104 (Qiagen®). The following primers were used for amplification of a 584-bp fragment of the *gus* (*uidA*) gene: 5′-GAATGGTGATTACCGACGAAA-3′ and 5′-GCTGAAGAGATGCTCGACTGG-3′; 438-bp fragment of the *virD* gene: 5′-ATGTCGCAAGGACGTAAGCCGA-3′ and 5′-GGAGTCTTTCAGCATGGAGCAA-3′ and 423-bp fragment of the *rolB* gene: 5′-GCTCTTGCA GTGCTAGATTT-3′ and 5′-GAAGGTGCAAGCTACCT CTC-3′. The PCR mixture consisted of 5 ng of plant DNA, 2.5 µl of 10 × Taq buffer, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 5 mM dNTP, 1.25 units of Taq DNA polymerase, 1 µl from each 10 pmol primer in a final volume of 25 µl. PCR analysis was performed with a PTC-100TM thermocycler

Fig. 2 Regeneration of coffee (*C. arabica*) transformed roots using *A. rhizogenes* (A4RS strain)-mediated transformation. **A** Growth of transformed root at the wound site on hypocotyl 4 weeks after *A. rhizogenes* infection. **B** Aspect of branched transgenic roots 12 weeks after infection. **C** Morphological aspect of normal (*right*) and transformed root from composite plants (*left*) in nursery 5 months after *A. rhizogenes* infection. **D** *C. arabica* composite plants in soil substrate ready for nematode inoculation in resistance tests. **E** & **F** Histochemical localization of β-glucuronidase (GUS) gene expression in transgenic roots of *C. arabica* transformed with the p35S-*gus*A-*int* gene construct. The different tissues actively expressing the GUS gene are stained in blue. Transgenic root with strong staining in the (**E**) meristematic region and central cylinder (Bar = 5 mm) and (**F**) staining restricted to the meristematic regions (Bar = 5 mm)

(MJ Research Inc., San Francisco, CA, USA). For DNA amplification, samples were heated to 94°C for 5 min, followed by 29 cycles at 94°C for 30 s, at 56°C for 30 s, and at 72°C for 1 min and then 56°C for 10 min. The amplified products were separated by electrophoresis on 1.5% agarose gels stained with 0.5 mg l^{-1} ethidium bromide in 0.5 × TAE (Tris-acetate/EDTA electrophoresis buffer) and visualised by fluorescence under UV light.

Nematode infection on composite plants

Composite plants were transferred into 200 cm^3 plastic pots filled with N_2-sterilized substrate (Neuhaus®) and sand (3:1 ratio) in a growth chamber under 14 h photoperiod (50 µmol m^{-2} s^{-1} light intensity) at 26°C. Once the composite plants reached the three pair of leaves stage, i.e. 5 months after transformation (see Fig. 2C and D), they were inoculated with *Meloidogyne exigua* individuals (J2 larvae + eggs) collected from inoculated susceptible Caturra cv. plants grown in a greenhouse. Seven composite plants or normal plants were inoculated for each cultivar, with three replicates. The inoculum dose was 300 individuals (eggs + J2) applied to the collar of each plant. Four months after inoculation, nematodes were extracted following the protocol of Hussey and Baker (1973) by root maceration and sieve extraction with NaOCl. Number of nematode individuals per gram of fresh root for each composite plant was determined under stereo-microscope (G × 4) in three replicates.

Statistical analysis

The data were compared by ANOVA followed by a comparison of the means using a Neuman–Keuls multiple range test. Values followed by different letters are significantly different at $P \leq 0.05$.

Results and discussion

Reactivity to different *A. rhizogenes* strains

Four of the five *A. rhizogenes* strains tested led to regeneration of transformed roots on the Caturra variety (Fig. 1). Only strain A4RS, similar to the A4 strain successfully used by Spiral et al. (1993) on coffee, proved to be very virulent (80% transformation efficiency). Transformed roots were obtained with almost all the

Table 1 Response of different organs from germinated zygotic embryos of two *C. arabica* varieties inoculated with *Agrobacterium rhizogenes* strain A4RS-p35S-*gusA-int*

Type of inoculated organs	Transformation efficiency* (%)		Number of transformed roots at inoculation site	
	Caturra	IAPAR-59	Caturra	IAPAR-59
Cotyledon	$14 \pm 12^{c**}$	14 ± 13^{b}	3.5 ± 2.1^{a}	1.0 ± 0^{a}
Hypocotyl	82 ± 17^{a}	51 ± 9^{a}	1.5 ± 1.0^{b}	1.5 ± 0.7^{a}
Root	20 ± 12^{b}	11 ± 3^{c}	1.0 ± 0^{a}	1 ± 0^{a}

Transformation efficiency and number of transgenic roots at the inoculation site were assessed 12 weeks after infection with *A. rhizogenes* for both varieties. Each value represents the mean of three replicates on 30 embryos each

*Frequency of inoculated organs that gave rise to at least one transformed root

**Values in the same column followed by different letters are significantly different at $P \leq 0.05$ (Neuman–Keuls test)

strains except for the 8196 strain. The embryos wounded with a sterile scalpel were used as the control. Root formation was never seen in merely wounded hypocotyls, confirming that formation of transformed roots resulted from a morphogenetic response attributable to the presence of *Agrobacterium* and not to physiological stresses.

Table 1 shows that very different reactivity values were obtained depending on the type of organ inoculated within the germinated zygotic embryo. The hypocotyl proved to be the most reactive organ. Hypocotyl wounding with *A. rhizogenes* led to transformed root regeneration with transformation efficiencies ranging from 50 to 80% depending on the varieties. Much lower transformation efficiencies were obtained by wounding cotyledons and roots. High hypocotyl reactivity provided an opportunity for composite plant production. No significant variability in the number of transformed roots regenerated at the wound site was found for the organs tested. Similarly, it has been reported that the reactivity of different plant organs after transformation by *A. rhizogenes* strongly depends on the plant species and on the different bacterial strains used for a same host (Phelep et al. 1991; Chaudhuri et al. 2005).

Root formation and characterization

Two weeks after *A. rhizogenes* infection, hypocotyl swelling accompanied by curvature was seen at the wound site in embryos that subsequently regenerated a transformed root. Roots appeared 6 weeks after infection (Fig. 2A). They were well-branched 12 weeks after infection (Fig. 2B), white in colour, with few phenotype variations between roots. Unlike reported for other species (Tepfer 1990; Franche et al. 1999; Królica et al. 2001), transformed coffee roots did not display *in vitro* all the distinctive characteristics associated with the 'hairy roots' phenotype. These morphological alterations (fast growth, high branching, plagiotropic root development, absorbent hairs) arose from the integration and expression in the plant cell of oncogenes such as the *rol* genes, *aux* genes involved in auxin synthesis or genes synthesizing opines, borne by the T-DNA of the Ri plasmid (Grant et al. 1991). Coffee transformed roots could not be morphologically distinguished from non-transformed roots (Fig. 2C). Their diameter and branching were similar. A histological analysis confirmed the apparent similarity of tissue organization in transformed and non-transformed roots, in the vicinity of the apex, corresponding to a differentiating zone, as well as in the vicinity of the collar, where secondary tissues were in place (data not shown). Lastly, we never found any transformed root developing in the air above the medium, indicating that the geotropism of the transformed roots was not disturbed. A comparison of transformed root and normal root growth rates also indicated that hairy roots grew twice as quickly (4.3 ± 1.0 cm per week) as non-transformed taproots (2.1 ± 0.7 cm per week). Nevertheless, that difference remained slight when compared to other species (Bonhomme et al. 2000). In previous works targeting the regeneration of coffee plants that were totally transformed by *A. rhizogenes*, information has not been provided about the phenotype of transformed roots (Spiral et al. 1993; Kumar et al. 2005). The verification that *A. rhizogenes*-transformed roots exhibit a similar phenotype than non-transformed normal roots was required before using this transformation procedure for posterior functional analysis studies.

Optimisation of co-cultivation conditions

For the A4RS strain, transformation frequencies increased in line with co-cultivation duration, stabilizing after 14 days (Fig. 3). This optimum co-cultivation duration was applied for subsequent experiments. The temperature used during the co-cultivation period greatly affected the efficiency of transformation by *A. rhizogenes* for the two coffee varieties (Fig. 4). The optimum temperature for transformed root regeneration was around 15–18°C. Beyond those

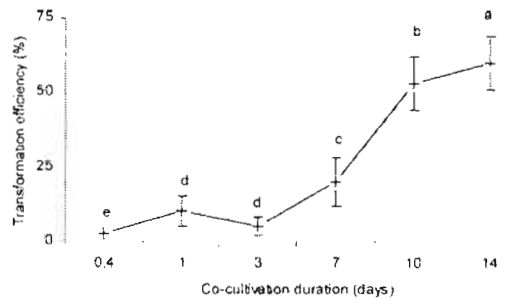

Fig. 3 Influence of co-cultivation duration on transformation efficiency of zygotic embryos of *C. arabica* var. Caturra mediated by *A. rhizogenes* strain A4RS. The transformation efficiency (i.e. frequency of infected embryos that gave rise to at least one transgenic root) was assessed 8 weeks after infection with *A. rhizogenes*. Each value represents the mean of three replicates of 30 zygotic embryos each. Values with different letters are significantly different at $P \leq 0.05$ (Neuman–Keuls test)

temperatures, the efficiency of the transformation process dropped considerably. It is interesting to notice that the optimum temperature was substantially lower than those used in earlier works on coffee, either with *A. rhizogenes* (Spiral et al. 1993) or *A. tumefaciens* (Hatanaka et al. 1999; Leroy et al. 2000) where a temperature of 25–26°C was used during co-cultivation. For several plant species, the marked effect of temperature on transformation efficiency was previously described for both types of agrobacteria. It has never been reported for coffee. Similar to our results on coffee, Boisson-Dernier et al. (2001) showed in *Medicago truncatula* that the percentage of embryos transformed by *A. rhizogenes* increased from 3.3% at 27°C to 63% at 20°C. With *A. tumefaciens*, 19°C was shown to be the optimum for plasmid transfer (Fullner and Nester 1996). The optimised co-cultivation conditions (i.e. low temperature and long duration) are probably responsible for the high transformation efficiencies obtained in the present study ranging up to 70%. Leroy et al. (2000) using an *A. tumefaciens* system reported that 0.4% of the somatic embryos infected regenerated transformed plants. Recently, Kumar et al. (2005) reported a 3% transformation efficiency using *A. rhizogenes*-mediated transformation system.

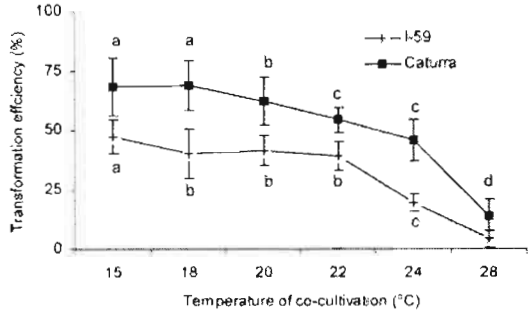

Fig. 4 Effect of temperature during co-cultivation on transformation efficiency mediated by *A. rhizogenes* strain A4RS-p35S-*gusA-int*. The transformation efficiency was assessed 12 weeks after infection with *A. rhizogenes* by evaluating the frequency of infected embryos from two *C. arabica* varieties (Caturra and IAPAR-59) that gave rise to at least one transformed root. Each value represents the mean of four replicates from 20 independent transformation events. For each cultivar, values with different letters are significantly different at $P \leq 0.05$ (Neuman–Keuls test)

Regeneration of GUS-positive transformed roots and selection of co-transformed composite plants

By using the armed A4RS-p35S-*gusA-int* strain, it was possible to measure the co-transfer frequency of the Ri T-DNA and of the T-DNA of the binary vector containing the *gusA* reporter gene following 14 days of co-cultivation at 20°C. The two varieties responded in a similar way to agroinfection (Table 2). Around 70% of infected embryos regenerated at least one root at the wound site and 40% were co-transformed as they generated at least one GUS-positive root. The average number of co-transformed roots per inoculation site was higher in Caturra. This 40% frequency also represented the frequency of co-transformed composite plants obtained by transformation of germinated embryos. In fact, as the GUS test was performed on a root tip of an already branched root system, it was possible to preserve just that GUS-positive root on the stem to obtain co-transformed composite plants. Other roots were eliminated. These results demonstrate that *A. rhizogenes*-mediated transformation is a potent tool to produce transformed roots in coffee. Moreover, the visual selection with the *gusA* reporter gene efficiently allowed avoiding the selection of co-transformed roots with antibiotics or herbicides. The fact that transformation and co-transformation frequencies were significantly different (approximately 72% vs. 36%) for the two varieties is not surprising. *A. rhizogenes* can transfer the T-DNA of binary vectors in trans, but it has been reported that the integration of *rol* genes from the Ri-plasmid T-DNA and T-DNA from the binary vector are independent (Shahin et al. 1986). Recently, Kumar et al. (2005) have shown in *C. canephora* that during transformation process, co-transfer of T-DNA from both Ri plasmid and binary plasmid is not obligatory.

We found variability in the distribution of GUS expression among transformed roots (Fig. 2E and F). The strongest GUS expression was always seen in the root tip and in the central cylinder (Fig. 2F). This staining pattern has often been observed in transformed plants when the CaMv 35S promoter was used (Terada and Shimamoto 1990; Diouf et al. 1995). However, in some roots, all the tissues displayed GUS activity (Fig. 2E). This wide

Table 2 Comparison of transformation efficiency and co-transformation frequency of *A. rhizogenes* strain A4RS-p35S-*gusA-int* between two *C. arabica* varieties: IAPAR-59 and Caturra

Genotype	Total infected embryos	Transformation efficiency (%)*	GUS (+) transformed roots (%)	GUS (+) transformed roots per co-transformed embryo	Co-transformed composite plants (%)**
Caturra	216	$74.4 \pm 16.0^{a***}$	35.5 ± 8.3^{b}	2.1 ± 0.4^{a}	40.8 ± 19.1^{a}
IAPAR-59	216	71.8 ± 8.3^{b}	38.0 ± 7.3^{a}	1.5 ± 0.3^{b}	38.6 ± 3.8^{a}

Each value represents the mean of three replicates on 72 embryos each

*Frequency of inoculated embryos that gave rise to at least one transformed root

**Corresponds to the frequency of inoculated embryos that gave rise to at least one GUS-positive transformed root

***Values in the same column followed by different letters are significantly different at $P \leq 0.05$ (Neuman-Keuls test)

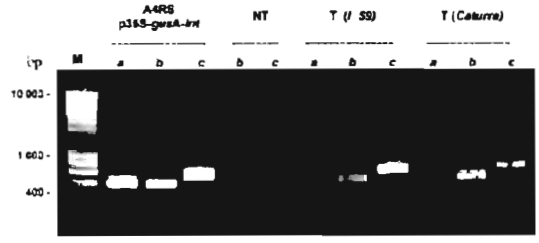

Fig. 5 PCR amplified DNA fragments of *virD* (a), *rolB* (b), and *gusA* (c) genes in transformed *C. arabica* roots. Total DNA from *A. rhizogenes* A4RS-p35S-*gusA-int*, DNA from non-transformed *C. arabica* var. Caturra roots (NT), and DNA from two GUS-positive transformed roots of the two *C. arabica* varieties IAPAR-59 and Caturra (T). DNAs were primed with oligonucleotides specific to the *virD*, *rolB* and *gusA* gene sequences. *Lane M* is a 10-kbp ladder (Smartladder, Eurogentec)

variability in the spatial distribution of GUS expression has often been reported in hairy roots. It might result from differences in the number of inserted *gusA* gene copies, in T-DNA distribution or rearrangements (Kohli et al. 2003). In coffee, such variation in GUS expression patterns has only been found between transformed roots, whereas the expression pattern was uniform for the same transformed root (Fig. 2E and F). Each composite plant was therefore characterized by a uniform GUS expression pattern.

PCR analysis of transformed roots

To demonstrate the presence of both Ri and binary plasmids in the transformed root genome, a PCR analysis of plant genomic DNA was performed. PCR analyses performed on all putative co-transformed roots lead to the amplification of the expected *rolB* and *gusA* fragments (Fig. 5). The PCR products were of the expected size (423 and 584 bp, respectively) and identical to those of the positive control (A4-p35S-*gusA-int* strain). These amplifications demonstrated the presence of the *rolB* and *gusA* genes and consequently, the integration of the Ri T-DNA and of the binary plasmid T-DNA in the plant. No product was obtained from either the non-transformed roots DNA (negative control) or from all the tested transformed roots when using the *virD* primers. This result confirmed that the detection of *rolB* and *gus* genes in transformed roots was not due to contamination by the *A. rhizogenes* strain.

Use of composite plants to study plant–nematode interaction

Infection of transformed roots of the susceptible cultivar with nematode individuals of *M. exigua* resulted in the development of numerous gall symptoms 4 months after inoculation (Fig. 6A and B). Thus, the nematode could complete its entire life cycle and proliferate in transformed roots. Numbers of extracted nematodes obtained from composite plant transformed roots of resistant and susceptible varieties inoculated with *M. exigua* were comparable with those from their non-transformed counterparts (Fig. 7). This data demonstrate that transformed roots of coffee composite plants retain the resistance/sensibility phenotype of varieties from which they are derived. Similar information was previously reported with hairy root cultures for sugar beet (Kifle et al. 1999), soybean (Narayanan et al. 1999) and tomato (Hwang et al. 2000). Compared to hairy root cultures, the present composite plant system developed in coffee permits to avoid the critical step of nematode sterilisation and the subsequent difficulty in maintaining axenic culture conditions in a system with three organisms, i.e. root cultures/nematode/*A. rhizogenes* (Narayanan et al. 1999). Another advantage over hairy root cultures is the possibility to realize functional analysis studies on root genes at the whole plant level.

Composite plants have been extensively used in legumes, as they represent an ideal system for analyses of gene expression involving infection of rhizobia and nitrogen fixation (Christey 2001). We report for the first time the possibility to use composite plants in functional analysis studies on genes involved in the plant–nematode interaction. We established a convenient transformation protocol that enables the production of transformed coffee roots at the infection site expressing a co-transferred transgene cloned in a binary vector, with high efficiency in the two

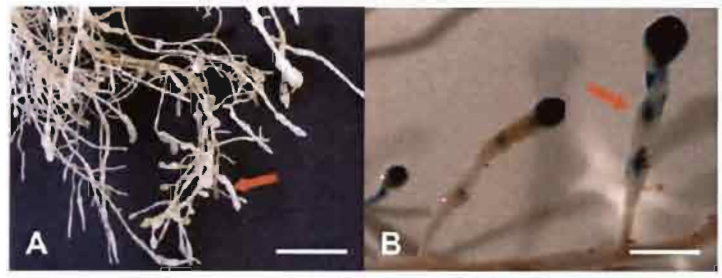

Fig. 6 Proliferation of the *M. exigua* root-knot nematode on *C. arabica* composite plants obtained after transformation with *A. rhizogenes*. **A** Gall symptoms caused by *M. exigua* on transformed roots of susceptible *C. arabica* var. Caturra four months after nematode inoculation. Bar = 20 mm. **B** GUS expression in feeding sites induced by *M. exigua* in transformed roots (var. Caturra). Bar = 5 mm

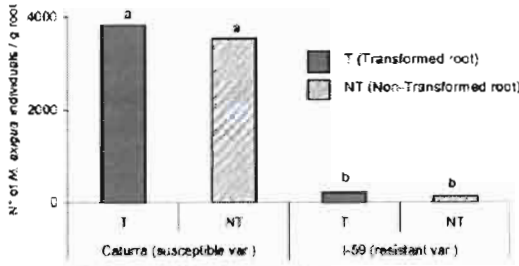

Fig. 7 Numbers of *M. exigua* individuals extracted from on *A. rhizogenes*-transformed vs. normal roots in susceptible (Caturra) and resistant (IAPAR-59) varieties. Numbers of nematode individuals per gram of fresh root were determined 4 months after *M. exigua* inoculation. Each value represents the mean of three replicates from 10 composite or normal plants for each variety. For each variety, values with different letters are significantly different at $P \leq 0.05$ (Neuman–Keuls test)

varieties tested. This method based on infecting germinated embryo hypocotyls with *A. rhizogenes* is very rapid and not particularly laborious as compared to *A. tumefaciens*-mediated methods. It resulted in the production of acclimatized composite plants bearing well-developed transformed rootstocks in 5 months and directly usable for rapid validation and functional study of resistance genes to nematodes. In comparison, using an *A. tumefaciens*-mediated transformation with selective pressure, it usually takes 14 months to produce similar well-developed transformed plantlets starting from primary explants, i.e. leaf pieces (Hatanaka et al. 1999; Leroy et al. 2000).

This composite plant strategy and the nematode resistance test could be used to study resistance genes to nematodes in other plant species, particularly in woody species for which *A. tumefaciens*-mediated transformation protocols are not efficient. In coffee, a localized genetic map of the chromosome carrying the major dominant *Mex*-1 gene conferring resistance to *M. exigua* in *C. arabica* was constructed (Noir et al. 2003) and the physical mapping of the *Mex*-1 region is currently realized. We will soon apply this plant composite technique to validate in vivo the *Mex*-1 gene by functional complementation. This technique could also be applicable to functional analysis studies of coffee genes involved in the resistance to other agronomically important nematode species (*M. paranaeinsis*, *M. arabicida*, *M. incognita*), in root development or in mycorrhizal symbiotic associations.

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Chapter III

Development of efficient regeneration and proliferation conditions of *A. rhizogenes* in coffee transformed roots

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Intermediate resistance to *Meloidogyne exigua* root-knot nematode in *Coffea arabica*

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Abstract

The root-knot nematode *Meloidogyne exigua* is a huge threat in major Arabica coffee growing areas in Latin America. The development of nematode-resistant coffee trees constitutes the most promising option for controlling the pest. *Coffea arabica* resistance to *M. exigua* is controlled by a simply inherited major gene, called the Mex-1 gene. The objective of this study was to determine the level of expression (complete or incomplete) of the Mex-1 gene within homozygous or heterozygous *C. arabica* genotypes, and the stability of that expression under field conditions. Resistant and susceptible pure line cultivars were compared with clones of hybrid cultivars derived from crosses between resistant and susceptible lines. The results under controlled conditions in a greenhouse revealed that reproduction of the nematode was significantly higher on hybrid cultivars than on resistant pure line cultivars, but much lower than on a susceptible pure line cultivar. The same result was confirmed under field conditions. There were fewer galls in the hybrid cultivar than in the susceptible pure line cultivar. A significant number of the galls in the susceptible cultivar were large, whilst in the hybrid cultivar, only small galls were found. A histological analysis of galls did not reveal any differences in patterns of giant syncytial cells between susceptible and homozygous or heterozygous resistant genotypes. After being monitored for 4 years, a *M. exigua* field population was multiplied by a factor 14 on a susceptible cultivar and by a factor 1.9 on the hybrid cultivar. We concluded that Mex-1 could have incomplete dominant expression that allowed nematode penetration, but inhibited the durable reproduction of the nematode. Finally we introduce the "gall diameter" as a new parameter that could be used to characterize the intermediate resistant phenotype for breeding purposes in coffee.

Keywords: Gene incomplete dominant expression; *Meloidogyne exigua*; Root-knot nematode; Mex-1; *Coffea arabica*

1. Introduction

The root-knot nematode *Meloidogyne exigua* is a very common parasite in the roots of coffee trees in Latin America (Campos and Villain, 2005). Yield losses arising from parasitism by this root-knot nematode in full-sun-exposed plantations with appropriated agronomical management have been estimated eliminate between 10% and 15% in Costa Rica (Bertrand et al., 1997) and 45% in Brazil (Barbosa et al., 2004). Nematicide treatments are expensive and hazardous for the environment and human health. Genetic resistance seems the best way of controlling root-knot nematodes for many important crops like tomato, potato, sugar beet and soybean (Luc and Reversat, 1985). In coffee trees, we previously identified several *Coffea arabica* lines derived from the interspecific Timor hybrid (wild *C. arabica* × *C. canephora*) that displayed resistance to the *M. exigua* nematode and we confirmed that resistance to this nematode came from the *C. canephora* progenitor (Bertrand et al., 2001). Recently, Noir et al. (2003) identified molecular markers associated with the resistance to *M. exigua*. A segregation data analysis of F2 progenies derived from a cross between the resistant introgressed cultivar T-5296 and the susceptible Ethiopian accession ET-6 showed that resistance to *M. exigua* was controlled by a simply inherited major gene, called the Mex-1 locus. However, we hypothesized

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June 2001. The study was carried out on two plots of 150 1-year-old trees, at "La Hilda" coffee farm, located at Poas, in Alajuela Province (CR), at 1300 m above sea level, on an andosol soil with 2500 mm of annual rainfall and an annual average temperature of 20 °C. The cultivars studied were the susceptible cv. Catuai and the intermediate resistant Xw hybrid cultivar. The coffee trees were spaced 0.90 m apart along the row, with 1.90 m between rows (5880 trees/ha). The *M. exigua* population on the farm was characterized beforehand by electrophoresis patterns (Carneiro, unpublished data). The population of *M. exigua* (J2) was recorded each year from December 2002 to December 2005 on roots, following the same extraction protocol as for Trial 1. In 2005, the observation of the number of galls and their diameter was carried out on root units of around 20 cm in length, and fresh weight comprised between 1.8 and 4.3 g. The diameter of the galls was measured. The galls were classed in three categories depending of their diameter: [0.2–1 mm], [1–3 mm] and [> 3 mm]. Results were expressed as the number of galls for 10 g of fresh weight and for each of the categories.

2.4. Histological examinations

Histological sections of infested roots from Trial 1 plants were studied to observe plant reactions to the parasite. The observations were restricted to small galls measuring 1 mm in diameter or less, in order to compare the reactions occurring after *M. exigua* infection in roots from the cv. CR-95, cv. IAPAR-59 and the Xw hybrid cultivars. The root samples collected from three trees per cultivar were fixed in FAA (formaldehyde alcohol, acetic acid and distilled water) for 48 h. They were then gradually dehydrated in an ethanol series (70–100%), 1 h in each bath, embedded in historesin 7100 (LKB), at 4 °C overnight and then moulded. Tangential or longitudinal 3 µm sections were cut and stained with PAS (periodic acid Schiff), which stained polysaccharides red (walls and starch), and NBB (naphthol blue black) which revealed soluble and insoluble proteins in blue (Fisher, 1968).

2.5. Statistical analysis

For both greenhouse and field trials, values were [log(x + 1)] transformed before analysis to standardize the variances. Data from the resistance evaluation trials under controlled conditions and from Trial 2 (durability) were analysed by ANOVA. Mean values were compared with the Duncan test at $P = 0.05$. Data from Trial 1 (population dynamics) were analysed by comparison of the area under the nematode infestation progress curve (AUNIPC) for each treatment, according to Shanner and Finney (1977), using the trapezoidal integration method between two months for each replication. The AUNIPC was estimated as $\text{AUNIPC} = \sum_{i}^{n-1} [(r_i + r_{i+1})/2](t_{i+1} - t_i)$, where n was the number of assessment times and r_i expressed in absolute values, was the disease intensity measured at time t_i. An ANOVA for AUNIPC was used to compare the treatments. All analyses were carried out using the PROC GLM procedures of the SAS statistical package (SAS Institute Inc., 2003).

3. Results

3.1. Resistance under controlled conditions

Three months after inoculation, the number of nematodes per gram of root (nem. g^{-1} root) reached more than 4290 ± 1104 for cv. CR-95, whereas it was only 231 ± 614 for cv. IAPAR-59 (Fig. 1). As expected, these two cultivars used as controls confirmed their susceptibility and high level of resistance, respectively. For the two hybrid cultivars, Xw and Xy, the number of nem. g^{-1} root was 786 ± 1608 and 410 ± 611, respectively. These two hybrid cultivars revealed a highly significantly smaller number of nematodes than the susceptible control ($P < 0.001$), and proved to be significantly higher ($P < 0.03$) than in the resistant control.

3.2. Resistance under field conditions

3.2.1. Nematode population dynamics in resistant and susceptible cultivars

Large numbers of *M. exigua* J2 larvae were present in the roots of the susceptible cv. CR-95 (Table 1). In contrast, very few J2 larvae were observed in the completely resistant cv. IAPAR-59. A low level of nematode populations was recorded in the Xw hybrid cultivar. However, these results need to be interpreted carefully when examining nematode dynamics in the field (Fig. 2A). The nematode population present in the roots of the susceptible cv. CR-95 varied throughout the year. It rose from under 1500 nem. g^{-1} root in November and December to over 4000 nem. g^{-1} root in January–April. It then decreased from May onwards.

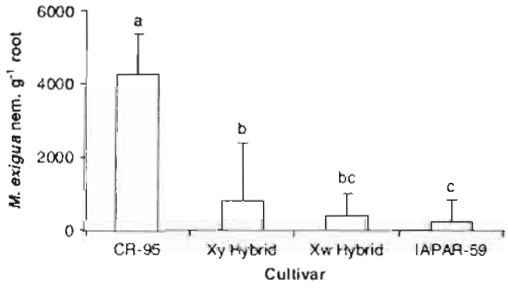

Fig. 1. Evaluation of resistance to *M. exigua* under controlled conditions. Average number of nematodes per gram of root in four *C. arabica* cultivars [CR-95, IAPAR-59, Xw and Xy hybrid] was assessed three months after infection with 200 *M. exigua* individuals per plant. For each cultivar, 10 plants were used with three replicates. Bars with different letters were different at $P < 0.05$.

that, this major gene could have incomplete dominant expression because most of the F2 population showed a gall index higher than the mean value of the resistant parent T-5296. Intermediate resistance to plant pathogens has frequently been reported for numerous host-parasites interactions. Concibido et al. (1996) reported diverse degrees of resistance to cyst nematodes (*Heterodera glycines*) in soybean breeding lines. In the common bean, Geffroy et al. (2000) reported that by crossing two different resistant parental lines, it was possible to obtain hybrids with partial resistance to *Colletotrichum lindemuthianum*. In rice, the Xa21D gene family is known to confer intermediate resistance to *Xanthomonas oryzae* (Wang et al., 1998). The objective of this study was to investigate whether the Mex-1 gene can confer intermediate resistance associated with incomplete expression and whether that expression is stable.

2. Material and methods

2.1. Plant material

Six *C. arabica* genotypes were used to evaluate the resistance to *M. exigua*. The IAPAR-59 and T-5296 cv. are pure line cultivars derived from the same accession called C1669 (from Instituto Agronomico de Campinas, Brazil) which originated from the cross between 'Timor Hybrid CIFC 832/2 × cv. Villa-Sarchi'. Those two cultivars are well known for being highly resistant to the *M. exigua* nematode (Bertrand et al., 2001). The cultivars cv. Catuai and cv. CR-95, are pure line cultivars that are known to being highly susceptible to *M. exigua*. New clones called Xw and Xy, derived from the hybrid cultivars T-5296 × ET-6 and T-5296 × ET-25, respectively, were compared with the pure line cultivars used as controls. The parents ET-6 and ET-25 which are Ethiopian accessions are characterized as susceptible to *M. exigua* nematode (Bertrand et al., 2001). The clones were vegetatively propagated *in vitro* using the somatic embryogenesis method described by Etienne (2006). In the remainder of this article, we shall refer to these two clones as 'hybrid cultivars'.

2.2. Resistance evaluation under controlled conditions

Resistance to *M. exigua* was evaluated in a greenhouse at a mean temperature of 24 °C at CICAFE (Costa Rican Coffee Institute Research Station). The original nematode population used for inoculations came from a severely infested plot at Naranjo, Alajuela province, Costa Rica (CR). A pure population was isolated using a single egg mass. That *M. exigua* population was reproduced in the greenhouse on coffee plantlets of the susceptible cv. Catuai. After 4 months' reproduction, the population was used to assess the level of resistance in cv. CR-95, cv. IAPAR-59 and the hybrid cultivars. Ten coffee plantlets of each cultivar were used per treatment with three replicates. The coffee plantlets were cultivated in plastic pots (200 cm^3) containing 3:1 sterilized coffee pulp compost and fine sand. Once the coffee plantlets reached the two pair of leaves stage, they were inoculated. The inoculum dose was 200 nematode individuals (eggs + J2-juvenile larvae stage) applied to the collar of each plantlet using a micropipette. Three months after inoculation, the plantlet roots were washed until all the soil was completely removed. Nematodes at the J2 stage were extracted in a mist chamber from the roots of each plantlet, following the protocol recently described by Anthony et al. (2005). All the roots from each plant were chopped into 3, 4 mm long pieces and placed on a paper tissue over a coarse sieve (Hooper, 1986). The sieve was placed inside a funnel; this was placed inside a 1 l-bottle. The mist cycle used was as described by Seinhorst (1962), 90 s mist cycle every 10 min at 27 °C. Overflow escaped through a hole made in the upper section of the bottle. Nematodes were pipetted from the bottom of the bottle and counted twice, after 7 (R1) and 14 (R2) d in the mist chamber. For each plantlet, the numbers of individuals nematodes per gram of root (nem. g^{-1} root) were analysed using the sum of R1 + R2.

2.3. Evaluation of resistance under field conditions

Two studies were conducted in CR in plots severely infested by *M. exigua*:

Trial 1: Population dynamics in resistant and susceptible cultivars

In this plot, periodic measurements were taken over a year on roots from 10-years-old coffee trees, which had been infested by a nematode population since their planting. The study was carried out at the CICAFE research station, at Barva, Heredia province (CR) on an andosol soil at 1100 m above sea level, with an annual average temperature of 21 °C and 2200 mm of annual rainfall. The cultivars studied were the susceptible cv. CR-95, the completely resistant cv. IAPAR-59 and the putative intermediate resistant Xw hybrid cultivar. All cultivars were located in plots of 250 trees. The coffee trees were spaced 0.84 m apart along the row, with 1.68 m between rows (7090 trees/ha). The *M. exigua* population at the station was characterized beforehand by electrophoresis patterns (Carneiro et al., 2004). Root samples were collected each month at a distance of 30 cm from the base of the trunk. A minimum of 20 g of coffee roots were taken from each tree. For each cultivar, 10 trees were randomly chosen with three replicates. Nematode extraction was performed using a mist chamber as described earlier (Seinhorst, 1962), and the nematodes were recovered on 7 (R1) and 14 (R2) days. The results were analysed using the sum of the two counts. Moreover, the number and the size of the galls were evaluated for the three cultivars studied.

Trial 2: Stability of the intermediate resistance phenotype

This trial involved several measurements taken over 4 years from 2002 to 2005 in order to monitor any changes in the nematode population in young coffee trees planted in

Rainfall increased substantially in May, remaining at more than 200 mm of rain per month up to October, then decreasing in November (Fig. 2B). During what is conventionally called the rainy season, the nematode population decreased. At the end of the rainy season, i.e. November, the nematode population continued to decrease, reaching the lowest level in November–December.

Table 1
Resistance of *C. arabica* to *M. exigua* under field conditions (Trial 1)

Cultivar	*M. exigua* (nem. g^{-1} root)	SD
CR-95	2677	1380
Xw hybrid	264	153
IAPAR-59	78	102

Annual average of nematodes per gram of root (nem. g^{-1} root) was analysed by comparing the area under the nematode infestation progress curve (AUNIPC) for each cultivar. $AUNIPC = \sum_{i}^{n-1}[(y_i + y_{i+1})/2](t_{i+1} - t_i)$, where n is the number of assessment times and y_i expressed as an absolute value is disease intensity measured at time t. Disease intensity corresponds to the number of nematode per gram of root (nem. g^{-1} root) on three *C. arabica* cultivars [cv. CR-95, Xw hybrid and cv. IAPAR-59], recorded monthly at CICAFE, Heredia (Costa Rica), from August 2001 to August 2002. For each cultivar, 10 plants were used with three replicates.

At the same time, it was seen that the coffee production cycle could be divided into three phases: a vegetative first phase with no-fruit present (from the end of December to the end of March), a second phase of fruit development after anthesis which was followed by a third phase including fruit starch storage and pulp ripening (from July to the end of November (Fig. 2B). The nematode population present in the roots of the resistant cv. IAPAR-59, remained virtually the same throughout the year, although a very slight increase was seen from November to February. In the roots of the hybrid cultivar Xw, the nematode population dynamics varied with two peaks (October–November then January–February) which could amount to populations that were 4–10 times larger than on the resistant control cv. IAPAR-59. Keeping track over the months, it was found that the total population of nematodes present on the roots of hybrid Xw compared to the total population of nematodes on the roots of the susceptible cv. CR-95, varied from $\frac{1}{5}$ to $\frac{1}{10}$ and fell to $\frac{1}{100}$ in April, when there was an outbreak peak for the susceptible cultivar. An examination of the number and types of galls observed *in situ* revealed that the susceptible cv. CR-95 exhibited many large galls (Fig. 4) while the resistant cultivar only had a few small galls (not shown). Moreover,

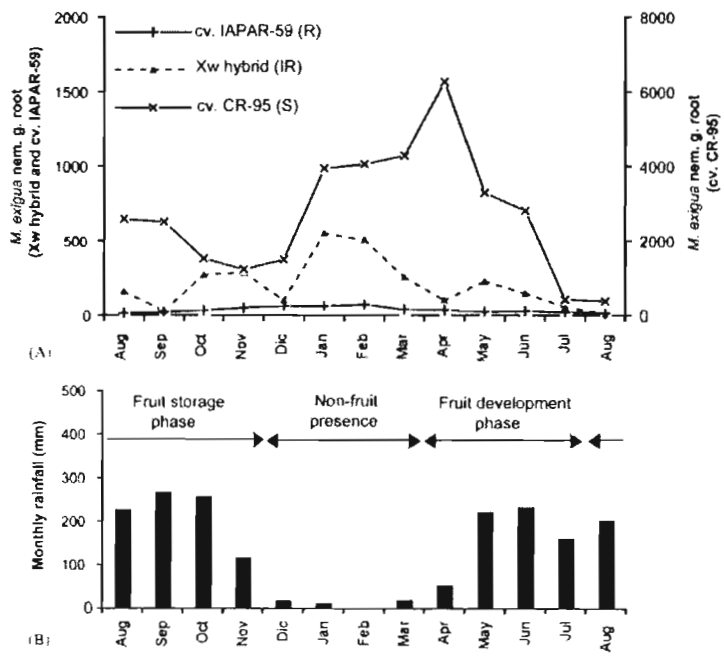

Fig. 2. (A) Dynamic of *M. exigua* population in seven-year-old trees from two *C. arabica* resistant cultivars (Xw hybrid and IAPAR-59) and one susceptible (CR-95). Average number of nematodes per gram of root was monthly assessed at CICAFE, Heredia (Costa Rica), from August 2001 to August 2002. For each nematode sample, 20 g of fresh root mix from 10 trees were used with three replicates. (B) Monthly rainfall (mm) and fruit development during the period of the study.

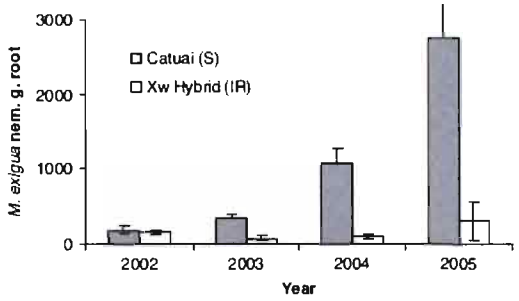

Fig. 3. Evolution of *M. exigua* population under field conditions in susceptible and intermediate resistant cultivars (Trial 2). Average number of nematodes per gram of root on young trees (planted in June 2001) from two *C. arabica* cultivars: susceptible homozygous cv. Catuai and intermediate resistant Xw hybrid at Poas de Alajuela, Costa Rica (sampling was assessed in December of years 2002–2005).

hybrid Xw had only small galls, but in a larger proportion than in the resistant cv. IAPAR-59 (Figs. 3,4).

3.2.2. Field stability of the intermediate resistance phenotype

The infestation of young plants of the susceptible cv. Catuai (196 ± 50 nem. g^{-1} root) and the hybrid cultivar Xw (164 ± 32) were similar in the first year after planting (Fig. 3). In the second year (2003), significant differences in infestation levels appeared. The susceptible cultivar exhibited 336 ± 70 nem. g^{-1} root, whereas the hybrid cultivar Xw decreased slightly compared to the previous year (81 ± 28 nem. g^{-1} root). In the third year (2004), marked differences between the susceptible and the hybrid cultivar were observed, when the nematode population in the roots of the cv. Catuai (1076 ± 200 nem. g^{-1} root) trebled, but it was still stable at a low level in the hybrid Xw (104 ± 35 nem. g^{-1} root). Lastly, in the fourth year (2005), the nematode population continued to increase considerably in the susceptible cultivar (2700 ± 400 nem. g^{-1} root), whereas in the hybrid Xw there were 314 ± 400 nem. g^{-1} root. Over the 4-year period, the nematode population had multiplied by a factor 14 on cv. Catuai and by a factor 1.9 on the hybrid cultivar. In 2005, we also observed the size and number of galls. The galls were classified in three categories according to their diameter (Fig. 5). For both cultivars, we found that small galls (with a diameter comprised between 0.2 and 1 mm) were the most numerous. However, there was a large quantitative difference between the two cultivars. For the susceptible cultivar, 47% of the galls were classified in this category, as opposed to 64% for the hybrid cultivar displaying intermediate resistance. We found an equivalent distribution between the two genotypes (36% and 35%, respectively, for the hybrid and cv. Catuai) in the medium sized-gall category (diameter between 1 and 3 mm). More important was the fact that 17% of the galls in the cv. Catuai were large gall

Fig. 4. Morphological aspects of *C. arabica* roots infested by *M. exigua*. (A) Aspect of the numerous and big sized-galls found in the infected roots of the susceptible CR-95 cultivar (gall diam. > 3 mm). (B) Aspect of the few and small sized-galls found in the infected roots of the intermediate resistant Xw hybrid (gall diam. < 3 mm).

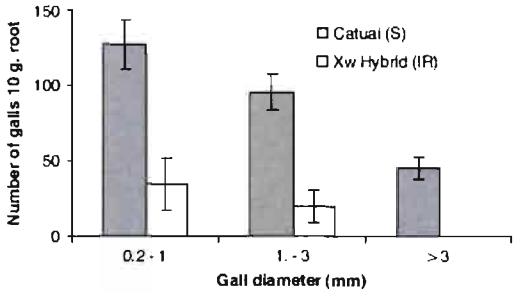

Fig. 5. Distribution of *M. exigua* root galls according to their diameter for two *C. arabica* cultivars (Trial 2): susceptible homozygous cv. Catuai (S) and intermediate resistant Xw hybrid (IR) at Poas de Alajuela, Costa Rica (sampling was assessed in December 2005).

with a diameter over 3 mm, whereas this type of gall was never observed in the hybrid cultivar. The number of galls, was significantly larger in cv. Catuai than in Xw for the first two categories (Fig. 5) with approximately four times more galls. Overall, trees from the cv. Catuai exhibited 193 galls per 10 g of root as opposed to 38 galls for the hybrid cultivar.

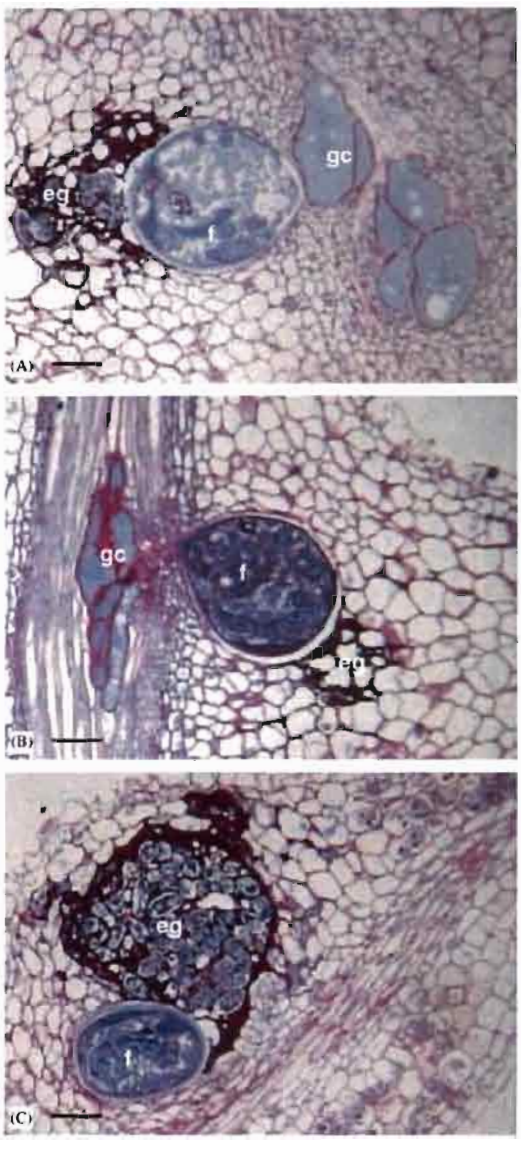

Fig. 6. Transversal sections stained with toluidine blue of root galls from (A) cv. CR-95 (susceptible), (B) cv. IAPAR-59 (resistant) and (C) Xw hybrid (intermediate resistant) inoculated with *Meloidogyne exigua*. (gc) giant cells, (f) female nematode, (eg) egg mass. Scale bar = 20 μm.

3.3. Histological observations

Only small galls with a diameter of 1 mm or less were observed to compare the three cultivars. The sectioned galls on roots of the susceptible cv. CR-95, revealed several females deeply buried in the vascular bundles and the presence of giant cells and egg masses (Fig. 6A). These results agreed with similar anatomical changes in coffee roots caused by *M. exigua* described in *C. arabica* susceptible materials by Di Vito et al. (2000), Rodrigues et al. (2000) and more recently by Anthony et al. (2005). Histological sections from galls did not reveal any difference between the susceptible and resistant cultivars (Figs. 6B and C).

4. Discussion

According to the hypothesis formulated by Noir et al. (2003), intermediate resistance to *M. exigua* should exist in *C. arabica*. In our study, we demonstrated for the first time, under controlled conditions and two sets of field conditions, that such a phenomenon does exist. The Xw and Xy hybrid cultivars grown under greenhouse conditions with high nematode pressure expressed an intermediate level of resistance to the parasite compared to the susceptible and resistant cultivars.

Under controlled conditions, the control susceptible and resistant cultivars used (cv. CR-95 and cv. IAPAR-59, respectively) expressed symptoms in relation to *M. exigua*, that tallied with what was previously described by Bertrand et al. (2001) and Anthony et al. (2005). Compared to these two well-characterized controls, the two hybrid cultivars displayed intermediate symptoms, with an infestation that was significantly larger than for the resistant control, but significantly smaller than for the susceptible control. In the study of Anthony et al. (2005), the maximum number of nematodes extracted per gram of roots was from 5–20 times greater in the susceptible compared with the resistant cultivar. Similar results were found in our study, with 18 times more nematodes per gram of roots in the susceptible than in the resistant cultivar. Under these conditions, hybrids Xw and Xy displayed intermediate resistance with 1.7–3.4 times the level of the resistant cultivar, respectively.

Under field conditions, the population dynamics monitored over 1 year characterize more precisely the intermediate resistance phenotype of the hybrid cv. Xw in comparison with the susceptible and resistant phenotypes. By monitoring the rainfall curve and coffee fruit development periods at the same time, we found that the nematode population in the roots of the susceptible control varied more in relation to coffee fruit development than with rainfall. The nematode population was the smallest during the storage phase. Our hypothesis is that fruits are priority metabolic sinks that compete very strongly with root

growth. Moreover, it is well known that *M. exigua* prefers to develop in very actively growing young roots. The nematode population found in the hybrid cultivar roots varied in proportions close to zero (same number of nematodes) to five times more, whereas in the susceptible control the population evolved from 5 100 times during the period of study. Following the previous hypothesis, there was a first outbreak peak in the hybrid cultivar that corresponded to the months preceding and following anthesis. The population then decreased and increased again during the fruit storage phase. This observation, which seems to contradict the previous model, might be explained by the considerable vigour of hybrid cultivars in *C. arabica*, which are capable of ensuring bean filling and vegetative growth at the same time (pers. obs.). The nematode population in the roots of the resistant control was always limited and stable over the year. Results from field conditions confirmed the observations under controlled conditions which placed the phenotype of the hybrid cultivar in an intermediate resistance position, but much closer to the resistant phenotype than to the susceptible phenotype. In a third approach, we estimated the durability of the intermediate resistance phenotype over 4 successive years. During that time the nematode population did not increase in the roots of the hybrid cultivar, whereas there was a large and continual increase in the susceptible control. We conclude that the intermediate resistance phenotype is stable. However, it is not possible to conclude on the durability of the resistance, which has to be observed over more years.

Gall observations revealed that the intermediate resistance phenotype was characterized by the existence of galls, but in much smaller quantities than for the susceptible cultivar. The number of galls is doubtless not a sufficient criterion for characterizing intermediate resistance. On the other hand, gall diameter seems to be a good characterization criterion, since the intermediate resistance phenotype only displayed small to very small galls, similarly to the resistant pure line cultivar. As shown by Anthony et al. (2005), small galls found in resistant or susceptible cultivars sheltered fertile females. According to those authors, resistance conferred by the Mex-1 gene is strongly associated with a hypersensitive reaction (HR) phenotype. This HR prevents gall formation. However, as that type of reaction does not systematically occur, a certain number of galls are likely to form. In fact, the rare juveniles that escaped the plant defence response might eventually reach the globose stage and maturity, and could complete their reproductive cycle in the resistant cultivar. Pegard et al. (2005), who also observed penetration of *Meloidogyne* spp. in resistant pepper, suggested that active post-penetration biochemical defence mechanisms may occur, which blocks nematode reproduction. In the present study, the histological analysis of the intermediate resistance hybrid cultivar was not precise enough to conclude on the existence of a hypersensitive reaction. Further investigations are required to gain a clearer understanding of the underlying mechanisms of resistance involved at both cellular and molecular levels. Jaquet et al. (2005) showed on tomato plants carrying the Mi resistance gene, that reproduction of *M. incognita* was greater on heterozygous genotypes than on homozygous resistant genotypes, suggesting a possible Mi gene dosage effect. They observed that intermediate resistance was associated with at least two heterozygous tomato genotypes. However, their experimental designs were not adequate for concluding whether or not that relation was consistent. Tzortzakakis et al. (1998) suggested that further studies were needed on the influence of the number of copies of the Mi gene inserted in tomato hybrids after controlled hybridization. In common bean, the resistance to root-knot nematodes conferred by the gene Me2 (Omwega and Roberts, 1992) was found to be completely dominant at 26 °C but showed an allelic dosage response of incomplete dominance at 28 °C.

Resistance genes to other nematode species, i.e. Mi in tomato (Hwang et al., 2000), $Hs1^{pro-1}$ in sugar beet (Kifle et al., 1999) and Gpa2 in potato (van der Vossen et al., 2000), have been validated by *Agrobacterium rhizogenes* genetic transformation procedures. The recent application of this technology in coffee (Alpizar et al., 2006), and the availability of the complete sequence of the Mex-1 gene will make it possible to carry out a functional analysis of Mex-1 (transcription level, kineties, tissue localization) but also to embark upon a structural study of the gene to understand the basis of its specificity. Transgenesis will also provide an opportunity for studying how the number of copies of the Mex-1 gene in the *C. arabica* tree genome influences the expression of resistance to *M. exigua*, and then verify whether the intermediate resistance phenotype might be linked to a Mex-1 dosage effect.

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Coffee hairy roots: development of reliable proliferation conditions and characterization of morphological variability

Introduction

Axenic cultures of transformed roots regenerated after *Agrobacterium rhizogenes*-mediated genetic transformation have been widely used for various horticultural crops (Mugnier, 1987; Tepfer, 1990). *A. rhizogenes* is a pathogenic soil bacterium that induces 'hairy root' disease on dicotyledonous plants, characterized by root proliferation at the infection site (Gaudin et al. 1994; Meyer et al. 2000). The term 'hairy root' is commonly used for *A. rhizogenes*-transformed roots and refers to the particular phenotype of those roots, often characterized by a highly branching root pattern and plagiotropic development attributed to increased endogenous auxin content (Nilsson & Olsson, 1997). Hairy roots have the capacity to grow even when removed from the plant and offer the interesting property of easily regenerating whole plants, avoiding callus formation and thus circumventing problems of somaclonal variation in a range of plant species (Tepfer, 1990). The *Agrobacterium* genes involved in rhizogenesis through the modification of plant cell growth and developmental regulation are commonly called *rol* and *aux* genes. Those genes are located in the T_R-DNA and T_L-DNA regions respectively of the Ri (root-inducing) plasmid of *A. rhizogenes* agropine strains. Some of the genes are involved in auxin biosynthesis which causes differences in hairy root growth and morphology when compared to non-transformed roots (Meyer et al. 2000; Christey, 2001).

Hairy roots have been extensively used to produce secondary metabolites for commercial use (review by Hamill & Lidgett, 1997), in root nodule research (Diouf et al. 1995; Akasaka et al. 1998; Boisson-Dernier et al. 2001), to study genes specifically involved in plant morphology and development (Mikami et al. 1999) and as a system to validate and study genes of resistance to root-specific pathogens, such as nematodes. For example, hairy roots have been used successfully to study *Mi*, $Hs1^{pro-1}$ and *Gpa2* gene function in tomato, sugar beet and potato, respectively (Kifle et al. 1999; Hwang et al. 2000; van der Vossen et al. 2000). One of the main constraints with hairy roots for functional analysis studies is their frequent phenotype variability. For example, was shown to be responsible for large variations in nematode multiplication rates and thereby complicated the interpretation of results, thus making it necessary subsequently to work with many clones (Plovie et al. 2003).

Noteworthy phenotype and growth variations (i.e. branching intensity, root diameter and growth rate) have often been observed among hairy root clones derived from independent transformation events, most of the time; this variability was only visually described. Phenotype variations were attributed to differences in the integration of genes from the T_L-DNA and T_R-DNA regions of *A. rhizogenes* in the host genome (Jouanin et al. 1987; Ambros et al. 1986; Mano et al. 1989). In *Chataranthus*, Batra et al. (2004) demonstrated for example that an absence of *aux* genes did not affect either hairy root morphology or growth, whereas an absence of *rol*A&B genes induced callusing and slow-growing morphology.

In coffee, protocols for *A. rhizogenes* mediated-transformation and plant regeneration were first described by Spiral et al. (1993) then by Kumar et al. (2006). Although an efficient protocol for routine regeneration of hairy roots has been recently established for coffee (Alpizar et al. 2006), the transformed roots were unable to proliferate on semi-solid medium and died after two or three subcultures. Similar observations were made by Kumar et al. (2006). To our knowledge, suitable conditions for the effective maintenance of hairy root axenic cultures of coffee have yet to be described. The purpose of this study was: i) to establish the culture conditions for efficient proliferation of hairy roots, ii), to characterize several clones and determine if morphological differences exist between them, iii) to assess the possibility of eliminating non true-to-type phenotypes before using the hairy root methodology as a tool for functional analysis.

Material and Methods

Regeneration of hairy roots

Hairy roots of *Coffea arabica* var. "Caturra" were regenerated by inoculating germinated zygotic embryos with *A. rhizogenes* (strain A4) according to procedures previously described by Alpizar et al. (2006). Briefly, zygotic embryos were infected by wounding the hypocotyl with a contaminated scalpel previously soaked in an agrobacterium culture for 48 h. Co-cultivation with *Agrobacterium* was carried out by placing the inoculated embryos on a MS medium (Murashige & Skoog, 1962) supplemented with sucrose (30 g/l) and solidified by adding 2.8 g/l of phytagel. Cultures were placed in 50-mm diameter Petri dishes in the dark for 12 days at 20°C. Co-cultivated embryos were decontaminated by immersion in MS liquid medium with cefotaxime (500 µg/ml) for 2 hours and washed twice. They were then subcultured every 4 weeks onto MS

germination medium containing decreasing cefotaxime concentrations (500, 200, 100 µg/ml). Hairy roots appeared at the wound site after 8-10 weeks.

Development of culture conditions for hairy root maintenance

Root fragments of approximately 40 mm in length were excised from hairy roots derived from independent transformation events and were cultured on a medium containing full-strength MS salts and the following vitamins: 10 mg/l L-cystein, 10 mg/l thiamine-HCl, 1 mg/l pyridoxine-HCl, 2 mg/l glycin, 1 mg/l nicotinic acid. The medium was supplemented with 30 g/l sucrose and solidified by 2.8 g/l of phytagel. Hairy roots were subcultured every 4 weeks by transferring 40-mm root tips onto fresh medium in 9-cm diameter Petri dishes. Cultures were kept at 26C° in the dark and 55-60% relative humidity.

The previous culture conditions were modified in the aim of establishing efficient conditions for hairy root proliferation:

i) Auxin addition. Different auxins and concentrations were tested: IBA (indole 3-butyric acid) and NAA (napthelene acetic acid) were added to the proliferation medium prior to autoclaving, whereas IAA (indole 3-acetic acid) was filter sterilized. Auxins were added to the medium at concentrations of 0.125, 0.25, 0.5 and 5 µM.

ii) Light conditions. Three different light conditions were compared: darkness, 50 µmol m^{-2} s^{-1} (full light conditions) and 20 µmol m^{-2} s^{-1} (indirect light) with a 16-h photoperiod.

iii) Sucrose concentration. The proliferation medium including 0.25 µM IBA was supplemented with 0.5, 1, 2, 3, 4, 6 and 8% (w/v) sucrose.

iv) Establishing optimum subculture duration: hairy root cultures were subcultured onto a medium including 0.25 µM IBA and 3% (w/v) sucrose every two, three, four or five weeks. For this experiment, the final evaluation was made after 10 weeks.

For all experiments, each hairy root clone was maintained for two subcultures in the tested culture conditions. The growth parameters acquired were root branching, defined as the number of lateral roots per cm of initial mother root (number of lateral roots/cm) and growth rate, defined as the average growth of lateral roots over 4 weeks of subculture (mm/day). The growth rate and branching were measured at the end of the second subculture. Ten root branches from five different Petri dishes (replicates) were evaluated for each culture condition. Vitrification was also

evaluated in two experiments using a scale of 0 to 5, where zero corresponded to non-vitrified roots and 5 to totally vitrified roots. Vitrification was characterized by translucent, thick roots with frequent callusing.

Analysis of hairy root morphological variability

Phenotype variability was analysed using 62 hairy root clones established for 6 months under the optimum culture conditions described in this work. Surprisingly, the optimized conditions enabled the proliferation of non-transformed roots for the first time. Three non-transformed root clones were therefore established from independent plants and used as non-transgenic controls.

For the assessment of root morphological characteristics, root images of initial 40 mm long branched root fragments from both hairy root lines and non-transformed roots (controls) were taken during two 3-week subcultures. The roots from three different Petri dishes, corresponding to three replicates were evaluated for each root line. The images were acquired by a scanner (HP ScanJet 6000) and were analysed using the software procedures of WhinRHIZO V5.0 (Instrument Regent, Quebec, Canada). For each root clone, the variables acquired were: number of lateral roots per cm of mother root to evaluate root branching, the total root length (cm) at the end of 3 weeks of subculture, and the percentage of fine roots (%) with a diameter less than 0.5 mm.

Statistical analysis

Data from the evaluation of culture condition assays were analysed by an ANOVA and mean values were compared using the Duncan test at $P = 0.001$ for light, auxin and sucrose concentration experiments, and at $P = 0.05$ for subculture duration experiments. Data from the analysis of phenotypic variability between axenic root clones showed a normal distribution, with the exception of growth rate, for which values were [log(x+1)] transformed to standardize variances prior to analysis. Data collected on the second subculture were analysed by an ANOVA with the clone factor considered as a classification criterion, followed by the Tukey multiple comparison at $P < 0.05$. All of the measurements taken after the second subculture were then compared to the corresponding measurements from the first subculture. The comparison was performed by a discriminant canonical analysis to determine if distribution and correlations between the variables observed would be affected by subcultures. The redundancy between the two sets of measurements enabled an evaluation of phenotype stability over time. All the statistical analysis were performed using Statistica software (2004, Statsoft, France).

Table 1. Effect of different exogenous auxins (IAA, IBA and NAA) on hairy roots of *Coffea arabica* cultured in the dark. Branching (number of lateral roots/cm); Growth rate of lateral roots (mm/day); Root vitrification index (determined by using the following scale 0 = none and 5 = totally vitrified). Values with different letters are significantly different at $P < 0.001$ according to the Scheffe test.

Auxin type	**Concentration** (μM)	**Branching** (number of lateral roots /cm)	**Growth rate** (mm / day)	**Vitrification** (0-5)
No auxin/control	0	2.3 ± 1.0^{b}	0.05 ± 0.06^{d}	0.0^{a}
IAA	0.125	8.8 ± 4.2^{ab}	0.48 ± 0.11^{abc}	3.0 ± 0.5^{c}
-	0.25	5.8 ± 2.0^{b}	0.50 ± 0.29^{abc}	3.2 ± 0.6^{c}
-	0.5	9.2 ± 3.8^{ab}	0.21 ± 0.11^{bcd}	3.6 ± 0.8^{bc}
-	5	14.8 ± 2.1^{ab}	0.14 ± 0.07^{cd}	$4.2 \pm .5^{c}$
IBA	0.125	5.0 ± 1.8^{b}	0.44 ± 0.15^{abcd}	0.2 ± 0.3^{a}
-	0.25	11.3 ± 3.1^{ab}	0.60 ± 0.1^{ab}	1.2 ± 0.6^{ab}
-	0.5	18.5 ± 5.0^{a}	0.68 ± 0.08^{a}	3.2 ± 0.6^{bc}
-	5	0.6 ± 0.1^{c}	0.0^{d}	5.0 ± 0.0^{c}
NAA	0.125	4.8 ± 1.2^{b}	0.28 ± 0.11^{abcd}	2.4 ± 0.9^{abc}
-	0.25	19.0 ± 6.4^{a}	0.55 ± 0.19^{abc}	4.0 ± 0.4^{c}
-	0.5	11.4 ± 5.8^{ab}	0.26 ± 0.15^{abcd}	4.6 ± 0.6^{c}
-	5	0.0^{c}	0.0^{d}	5.0 ± 0.0^{c}

Results

Effect of auxin type and concentration

The effects of adding different auxins (IBA, IAA and NAA) to the culture medium on the growth of *C. arabica* hairy roots were compared (Tab. 1). Little or no branching and growth were observed in the absence of exogenous auxin. Low concentrations of the three auxins enabled hairy roots to branch and grow. With IBA and NAA, the optimum concentration was 0.25 µM for both branching and growth. The 5 µM concentration inhibited those two parameters. However, with IAA, the best branching pattern was obtained for 5 µM, although the growth rate was very weak at that concentration. The incorporation of 0.5 µM IBA most efficiently stimulated the growth rate and branching of coffee hairy roots.

Root vitrification was enhanced by NAA and IAA, even at low concentrations (0.125 µM), while with IBA significant vitrification symptoms only occurred using higher concentrations (0.5 and 5.0 µM). Using 0.25 µM IBA was the best compromise between root growth and vitrification status, as it led to high growth intensity and branching rates along with the lowest vitrification rate. That auxin treatment was adopted in all the following experiments.

Effect of light intensity

Light conditions had significant and marked effects on the branching, growth rate and vitrification of *C. arabica* hairy roots (Table 2). The branching and growth rate of hairy roots in full light conditions were the weakest among the three conditions that were studied. Significant enhancement of root growth was observed with an intermediate light intensity (20 $\mu mol.m^{-2}.s^{-1}$). Darkness led to efficient branching but moderate light gave the highest root growth rate. None of the light conditions provoked marked vitrification symptoms in coffee hairy roots.

Effect of sucrose

Sucrose concentration moderately affected the growth of coffee hairy roots (Fig. 1). Optimum sucrose concentrations for branching were found in a wide range, between 1 and 4%. The highest growth rates were observed at low sucrose concentrations (1 and 2%) and higher concentrations caused progressive root growth depletion. It was decided to incorporate 2% sucrose in the proliferation medium for the following experiments.

Table 2. Effect of light intensity on root branching (number of lateral roots/cm), growth rate of lateral roots (mm / day) and root vitrification of *C. arabica* hairy roots in a MS medium containing 0.25 µM IBA. Vitrification was evaluated using a scale of 0 to 5, where zero corresponded to non-vitrified roots and 5 to totally vitrified roots. Means within a column followed by the same letter were not significantly different at $P < 0.001$ according to Duncan's multiple test.

Light intensity ($\mu mol.m^{-2}.s^{-1}$)	Branching (number of lateral roots/cm)	Growth rate (mm / day)	Vitrification (0-5)
0	11.3 ± 3.2^a	0.6 ± 0.09^b	1.2 ± 0.6^a
20	10.4 ± 2.7^a	0.76 ± 0.08^a	0.0 ± 0.0^b
50 *(full light)*	0.4 ± 0.4^b	0.01 ± 0.01^c	0.8 ± 0.3^a

Table 3. ANOVA analysis of morphological variability existing between 62 hairy root clones of *C. arabica* and 3 non-transformed root clones established *in vitro* for 6 months. All morphological parameters were analysed at the end of a 3-week subculture period. SE: mean standard error. (*) significant differences at $P < 0.001$ (Tukey test).

Variable	**Mean ± SE**	**F**	***P***
Branching (number of lateral roots cm^{-1})	2.1 ± 1.0	1.98	NS
Percentage of fine roots (diam. < 0.5 mm)	86.4 ± 9.5	9.52	*
Total root length (cm)	57.0 ± 1.2	5.42	*

Effect of subculture duration

Subculture duration also significantly affected the development of *C. arabica* hairy roots (Fig. 2). Although very frequent transfers (every 2 weeks) of hairy roots onto fresh culture media had positive effects on the growth rate, that timing was not an optimum condition for root branching. The best compromise between branching and growth rate was obtained by subculturing hairy roots every three weeks.

Phenotype variability among hairy root clones

Morphological differences could be visually observed between hairy roots (Fig. 3). The ANOVA of morphological variability among 62 hairy root clones and 3 non-transformed root clones of *C. arabica* showed significant differences between the clones for the total root length ($P < 0.001$) and for the percentage of fine roots ($P < 0.001$). The branching pattern expressed as the number of lateral roots per cm of mother root did not display significant differences between hairy root clones and was therefore not kept for the following analyses since it could not be used to characterize particular altered phenotypes.

An analysis of the percentage of fine roots data (Fig. 4A) showed that 89% of hairy root clones were significantly similar to the non-transformed controls for the proportion of fine roots. On the other hand, seven hairy root clones (amounting to 11% of the total observations) showed less than 70% of fine roots and were significantly different from the non-transformed control roots. For the total root length variable (Fig. 4B), it was found that only two hairy root clones (amounting to 3% of the population) were significantly different from the other clones. The transformed root clones tended to grow faster as the majority displayed a higher total root length (two to four times higher) than non-transgenic control roots.

A correlation analysis between principal components at the end of 3- and 6-week growth periods showed that canonical R was very high for the percentage of fine roots and total root length variables (r=0.93 and 0.80 respectively). The redundancy between the two sets, i.e. the share of variance explained by canonical analysis amounted to 89%. We then observed that for those two variables, the average behaviour of a clone after a 6-week growth period was similar to that of the same clone after 3 weeks. Consequently, we assumed that the phenotypes were stable over time.

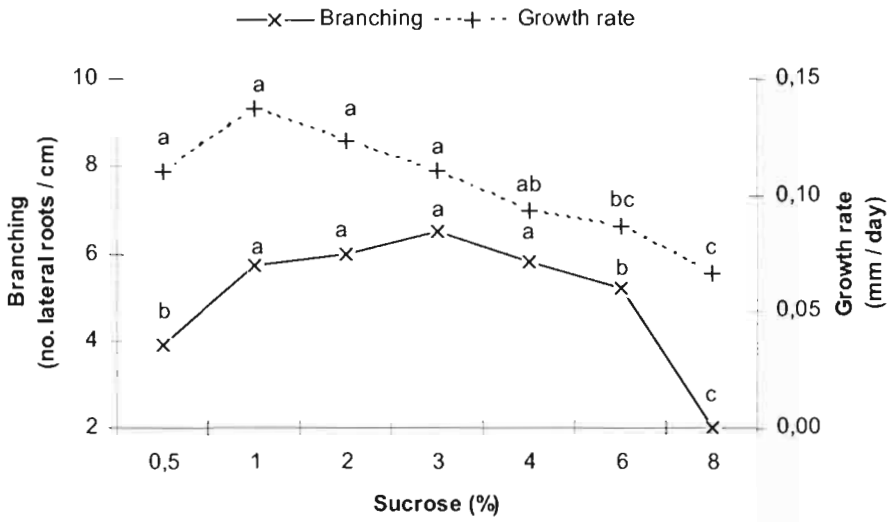


Figure 1. Effect of sucrose concentration on branching (number of lateral roots/cm) and growth rate (mm / day) of *C. arabica* hairy roots cultured on a MS medium containing 0.25 µM IBA in the dark. Values with different letters were significantly different at $P < 0.001$ according to Duncan's multiple test.

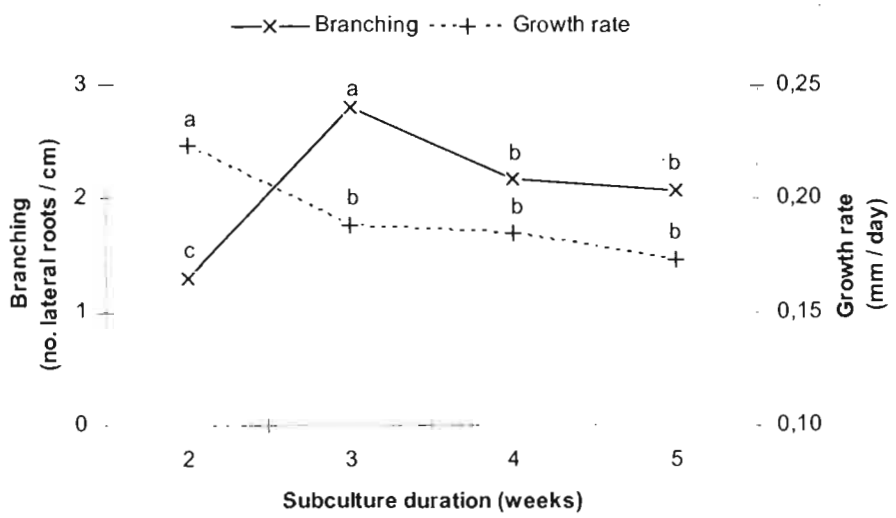


Figure 2. Effect of subculture duration on branching (number of lateral roots/cm) and growth rate (mm/day) of *C. arabica* hairy roots cultured on MS medium containing 0.25 µM IBA in the dark. Values with different letters were significantly different at $P < 0.05$ according to Duncan's multiple test.

The data array in Fig. 5 shows that the majority of root clones formed a cluster with an average total root length of between 20 and 90 cm and a percentage of fine roots between 75 and 95%. Clones characterized by a high percentage of fine roots (including those of the cluster) showed higher total root length than clones with thicker roots. The two root clones that were found to be significantly different from the non-transformed control roots for total root length (Fig. 4A), and seven others for the percentage of fine roots (Fig. 4B), were distantly positioned from the cluster of root clones showing a normal phenotype and could be considered as phenotypically altered.

Discussion

As soil nematodes are a major pest for coffee growing, the development of protocols for hairy root regeneration by *A. rhizogenes*-mediated transformation and for subsequent proliferation has become a priority in order rapidly to validate different nematode resistance genes such as *Mex-1* conferring resistance to *M. exigua*. Although coffee hairy roots are reported to have been obtained by different authors, the conditions for proliferation and maintenance were yet to be established.

Negative effects of light exposure for hairy root multiplication

We demonstrated that coffee hairy root cultures require an absence of light or low light intensity for successful proliferation. A review of the literature revealed contradictory information about the effect of the photoperiod and intensity of light on hairy root development. There have been few reports of such a negative impact of light exposure and for many species hairy roots have been grown under light conditions. Handa (1991) reported the successful maintenance of hairy roots from 30 different plant species under full light conditions. Vanhala et al. (1998) observed that darkness stimulated root biomass production in *yoscyamus* compared with a 12h photoperiod. Opposite results were found by Liu et al. (2002) on *Artemisia* sp. hairy roots who found higher growth rates under continuous light conditions and suggested that long periods of illumination accelerated the uptake of nutrients from the culture medium and accumulated carbohydrates in root axenic cultures. However, they noted that the hairy roots under continuous light became "green" after 4 weeks of culture. We also observed that coffee hairy roots under full light intensity became green and thicker, but exhibited few or no branching. Kollárová et al. (2004) demonstrated that exposure to light contributed to increase in *Karwinskia* sp. root biomass. They attributed that result to a higher water uptake that led to the appearance of callus-like cells. Coffee hairy root cultures required no light or low light intensity for normal proliferation.

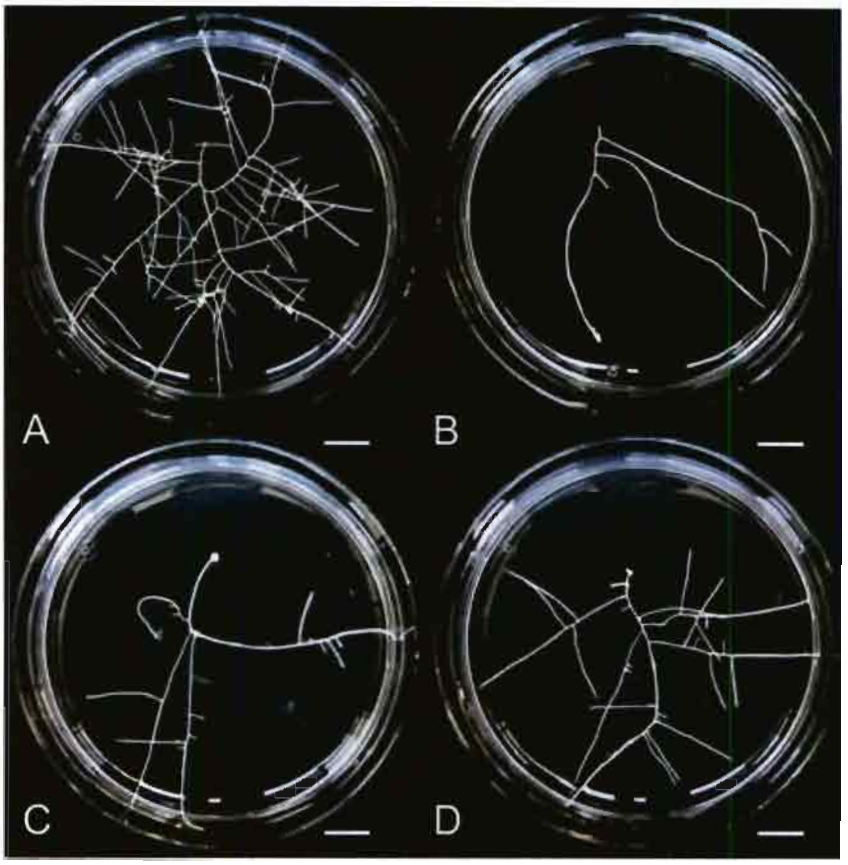
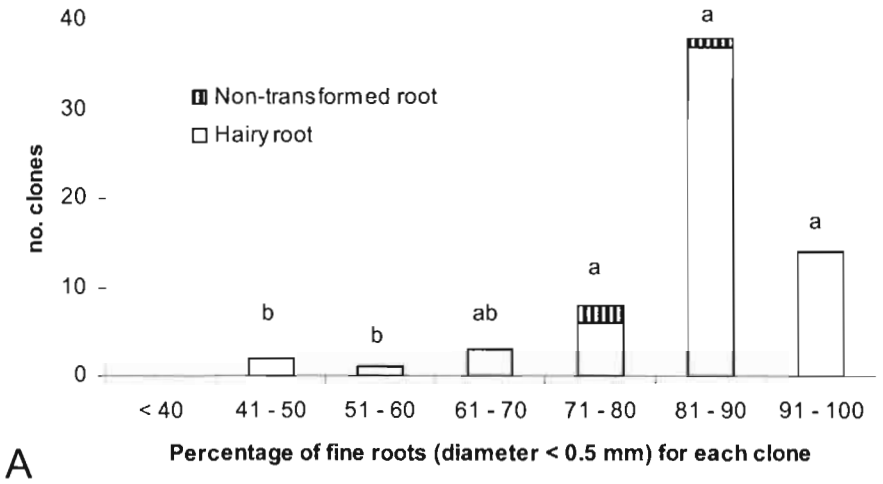


Figure 3. Different phenotypes of coffee axenic hairy root clones in comparison with non-transformed roots (control). **A:** X16 root clone showing a high growth rate and a large percentage of branched fine roots; **B:** non-transformed root clone (control) **C:** X43 root clone with low growth rate and small percentage of branched fine roots; **D:** X22 root clone exhibiting a normal phenotype with a large percentage of branched fine roots and an intermediate growth rate (scale bar: 10 mm).

Requirement of auxin supply for hairy root proliferation

In our study, we showed that auxins were indispensable for the proliferation of hairy root cultures in coffee and that the type and concentration of exogenous auxins strongly influenced root development. In several plant species, *A. rhizogenes*- transformed roots can proliferate after excision from the plant in media devoid of growth regulators. That autonomy is related to the integration and expression in transformed cells of *aux* and *rol* genes from the Ri plasmid that enable the production of endogenous auxin (Nilsson & Olsson, 1997). However, in some species including coffee, that situation does not occur and it becomes essential to add exogenous auxins to the culture media in order to obtain and/or stimulate hairy root development, although the presence of *aux* and *rol* genes has been established in transformed roots (Alpizar et al. 2006). Depending on the auxin concentration, positive or adverse effects on hairy roots growth have been described. Kim et al. (2003) and Kollárová et al. (2004) found that adding 0.25 µM IBA to culture media enhanced hairy root growth in *Panax* and *Karwinskia,* though higher concentrations induced callusing. Similarly, Park & Facchini (2000) in *Papaver* & Liu et al. (2002) in *Pueraria* found that IAA and IBA at 1.0 µM gave the strongest stimulation of branching but also caused higher inhibition of root growth, whereas both auxins at 0.1 µM, promoted better lateral root formation and growth. Conversely, even if high exogenous auxin concentration promoted hairy root branching by stimulating cell division in the pericycle, the process seemed to inhibit hairy root growth because an excessive accumulation of auxin in apex meristematic cells could inhibit meristematic tip activity (Finlayson et al. 1996) or lead to alterations in signalling response from other endogenous hormones involved in hairy root emergence and growth, such as polyamine (Ben-Hayyim et al. 1996), ethylene (Lorbiecke & Sauter, 1999) or ABA (De Smet et al. 2003). In our study, we found that the optimum concentration for root branching (0.25 µM IBA) also corresponded to an optimum for the growth rate. Moreover, that auxin treatment, when applied ton several subcultures, supported very satisfactory coffee hairy root proliferation over the long term.



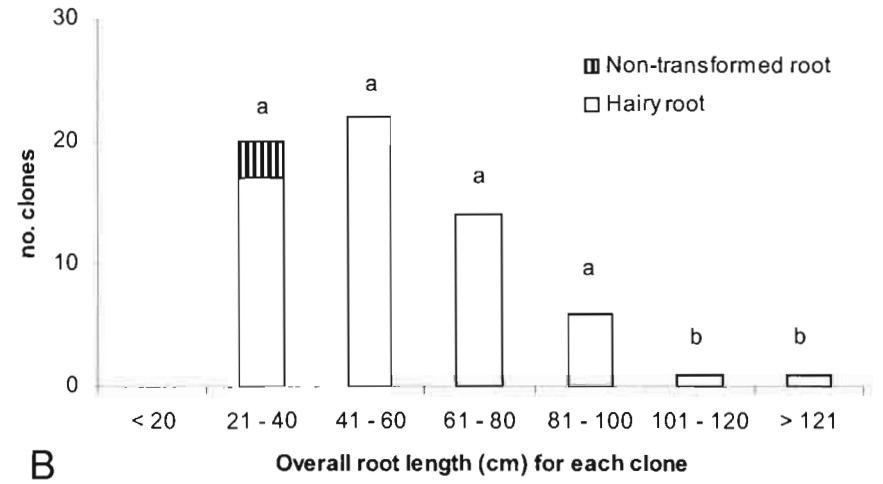


Figure 4. Morphological variability between hairy roots and non-transformed root clones of *Coffea arabica*. **A:** Classification of clones depending on the percentage of fine roots (diameter < 0.5 mm); **B:** Classification of clones according to total root length (cm) after three weeks' growth. Bars with different letters were significantly different at $P < 0.05$.

Sucrose supply and subculture duration

Optimization of the sucrose supply and subculture duration did not markedly affect the growth of coffee hairy root cultures when compared to light and auxin supply. Media with low sucrose concentrations were optimum for coffee hairy root proliferation. It had been previously shown that sucrose was necessary for adventitious root induction and needed to be continuously supplied for vigorous hairy root growth (Nilsson & Olsson, 1997). However, few data have been reported on the effects of sucrose concentration on hairy root growth. Romanov et al. (2000) observed that the effect of auxin on hairy root development in potato highly depended on the sucrose concentration in the culture media.

In our study, coffee hairy root growth was enhanced by frequent transfers every 3 weeks onto fresh culture media. Different authors have reported that the metabolic activity and rhizogenesis of hairy roots were stimulated shortly after sub-culturing. Nin et al. (1997) observed on *Artemisia* hairy roots that maximum root growth became exponential during the second week following subculturing, metabolic activity decreased after the third week and a stationary phase was observed in the fourth week of culturing. Liu et al. (2002) and Narayan et al. (2005), working on *Pueraria* and *Tylophora* hairy roots respectively, monitored the timing of lateral root appearance and revealed the presence of primordia only 2 days after subculturing. Both sets of authors described a maximum linear growth at 14-16 days after subculture. Arroo et al. (1995), measuring the uptake of labelled IAA by *Tagetes* hairy roots, found that all exogenous auxin was metabolized after 10 days of culturing. Those results confirm the importance of establishing the optimum subculture frequency in order to maintain optimum physiological activity.

Evidence of limited phenotypic variability among coffee hairy roots

The improvement of culture conditions for hairy root proliferation is commonly assessed by assessing weight increase, mainly because most hairy root research focuses on increased root biomass in order to stimulate the production of secondary metabolites. In the case of a functional analysis of genes involved in plant root/pathogen interaction, hairy roots can be of great interest but it is imperative to dispose of transformed roots that are morphologically similar to non-transgenic roots. An analysis of phenotypic variability thus becomes very important before using hairy roots in gene validation approaches.

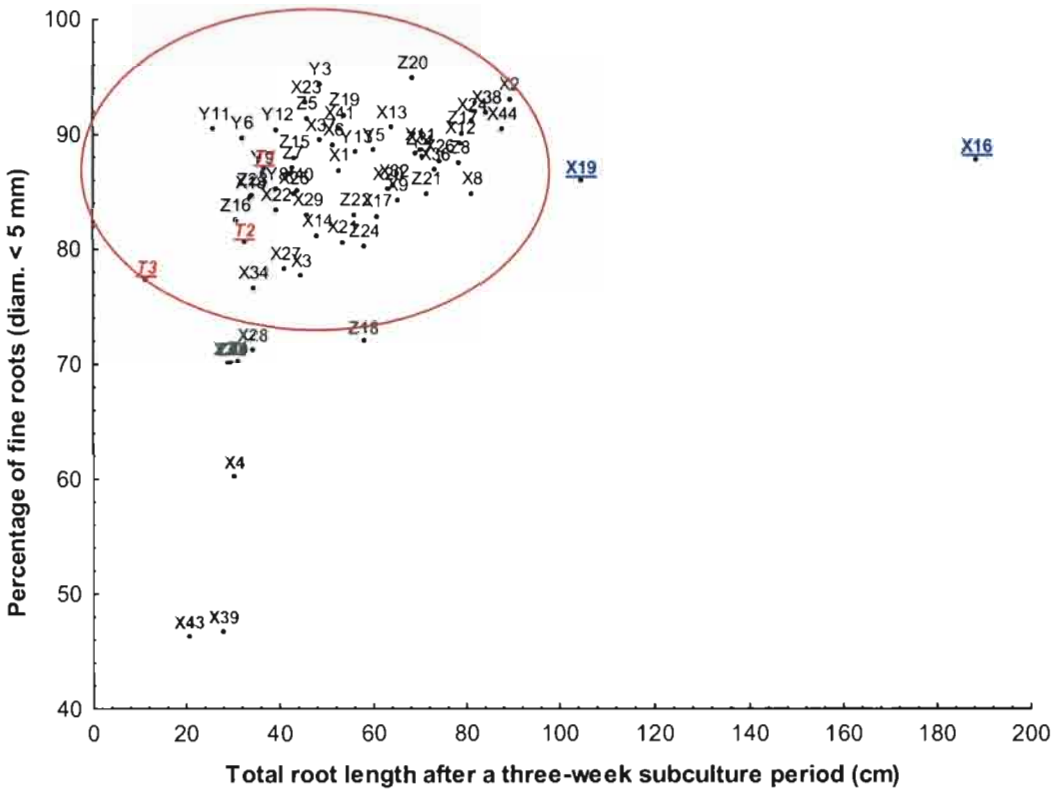


Figure 5. Relation between total root length (cm) after a 3-week subculture period and the percentage of fine roots for 62 hairy root clones and 3 non-transformed clones (controls) of *Coffea arabica*. Data from the control clones are shown in red and underlined (T1, T2 and T3). Hairy root clones significantly different from the controls for the percentage of fine roots (thick roots) are shown in green and those differing for the total root length in blue (fast growing). The hairy root clones within the ellipse were not significantly different from the controls for their morphology.

For the first time, our work describes culture conditions for coffee hairy root proliferation, which have led to the establishment of long-term axenic cultures of numerous transformed roots (62 lines) but also of some non-transformed roots. Macroscopic differences were observed between hairy roots. Since each hairy root is a cellular clone resulting from a single transformation event (Constantino et al. 1984), hairy root clone cultures and individuals regenerated from such roots retain the phenotype properties conferred by the specific T-DNA transferred to the plant cell (Meyer et al. 2000). The synergistic activity when all *rol*-gene products are simultaneously expressed is important in the induction of hairy roots and enhances development of the root *rol*-phenotype (Spena et al. 1987; Schmulling et al. 1988; Spanò et al. 1988). Both transformed and non-transformed root clones were used to characterize the morphological variability between clones derived from different transformation events, and to define the morphological variables that might enable an efficient distinction to be made between the different phenotypes. Such information would be useful in limiting the heterogeneity of hairy root material, with a view to using only those lines displaying less altered phenotypes in functional analysis studies of coffee root genes.

Phenotypic alterations such as short internodes, brittle and wrinkled leaves and stunted growth have been reported in *A. rhizogenes*-transformed coffee plantlets regenerated from somatic embryos of *C. canephora* (Spiral et al. 1993; Kumar et al. 2006) and from transformed roots of *C. arabica* (Sugiyama et al. 1995). However, no information has been provided about the phenotype of transformed roots. In other plant species, such morphological alteration has been reported for the root system and has been commonly described as root thickness, excessive branching and amount of biomass production (Handa, 1991; Nguyen et al. 1997; Nin et al. 1997; Narayan et al. 2005). This intra or inter-line variability among hairy root lines or the degree of phenotype alteration when compared to non-transformed roots could be major constraints for the utilization of hairy roots in functional analysis studies. Those constraints may lead to an increase in the number of individuals and replicates (Plovie et al. 2003) and, in the most extreme situation, to the *A. rhizogenes*-mediated method being replaced by transformation methods that do not induce phenotypic alterations (i.e. *A. tumefaciens*-mediated transformation).

Most of the literature on hairy roots highlights the degree of lateral branching, plagiotropism, presence of numerous hairs and the capacity of hairy roots to grow when isolated from the mother plant in hormone-free medium (Nilsson & Olsson, 1997). Our results revealed that coffee hairy

roots did not display most of those particular properties and that the majority of hairy root lines exhibited limited phenotype differences when compared to normal non-transformed root lines, and strong differences were restricted to some particular lines.

As shown in this work, both coffee hairy roots and non-transformed roots did not express growth autonomy in the absence of exogenous auxin, even though T-DNA from the root-inducing (Ri) plasmid from *A. rhizogenes* was previously confirmed to be integrated into the plant genome of transformed cells (Alpizar et al. 2006). Abundant lateral branching was often mentioned as one of the most typical traits within the altered phenotype of hairy roots (Tepfer, 1984; Spano et al. 1988; Guivarc'h et al. 1999). Coffee hairy roots showed a higher degree of lateral branching than non-transformed roots. However, surprisingly, a statistical analysis of root images of coffee root clones revealed high intra-clonal variability for the branching variable, which led to that variable being discarded for efficient differentiation between hairy root phenotypes. Probably, the average number of laterals per cm of mother root was not sufficient to characterize the root branching pattern. Previous work (Jourdan et al. 1995; Jourdan & Rey, 1997) showed that root branching could be modelled accurately by Markov chains, which consider the distance between laterals instead of the number of laterals itself. Future work should integrate that criterion for a more detailed root branching analysis.

Few authors have compared hairy root growth with that of non-transformed root cultures. In ours study, a majority of hairy roots displayed a root length between 2- and 6-fold higher than non-transformed roots. Fast growth of hairy roots has often been reported in the bibliography. Nevertheless, that difference remains slight when compared to other species (Bonhomme et al. 2000). Similar results, but expressed as fresh weight gain, were reported by Park & Parkcchini (2000) for poppy cultures on a medium enriched with 0.1 µM of IBA. However, Liu et al. (2002), on kudzu, observed that lateral root elongation was similar for both types of roots. Plagiotropic development was not characteristic of coffee hairy roots since non-transformed root cultures proliferated similarly when excised from the mother plant. Moreover, hairy roots belonging to a composite plant exhibited a normal positive geotropism when grown in soil (Alpizar et al. 2006).

The existence of morphological variability between transformed and non-transformed roots on the one hand, and between hairy root clones on the other, was revealed using two variables: the percentage of branched fine roots and the total root length. Both variables were confirmed to be

stable and to provide immediate application on the routine measurement through image acquisition and analysis of hairy roots to screen and discard aberrant phenotypes. Most of the coffee hairy root clones were morphologically similar. Using those two morphological variables, we found that the frequency of altered phenotypes was rather low and enabled the use of *A. rhizogenes*-mediated transformation systems for functional genomics of root genes. Hairy root clones underwent variable Ri T-DNA integration evens, reflected in the phenotype (Ambros et al. 1986; Jouanin et al. 1987; Mano et al. 1989). Further studies are under way to identify the genes from Ri T-DNA or molecular mechanisms responsible for the two altered phenotypes.

The existence of hairy root proliferation conditions for coffee offers new research perspectives. Hairy roots could be used for the production of plant secondary metabolites and for functional genomics studies of root genes. Hairy roots have been proposed as an easy system to screen for nematode resistance genes in crop plants and have been successfully used in tomato, sugar beet and potato (Kifle et al. 1999; Hwang et al. 2000; van der Vossen et al. 2000). In *Coffea arabica*, a localized genetic map of the chromosome carrying the major dominant *Mex-1* gene conferring resistance to *M. exigua* has been constructed (Noir et al. 2003) and physical mapping of the *Mex-1* region is under way. Hairy roots could be used to validate the *Mex-1* gene by functional complementation after elimination of aberrant phenotypes to increase the accuracy of the method.

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Chapter IV

Analysis of gene expression in transformed coffee roots

Expression des gènes rapporteurs GUS et GFP sous contrôle du promoteur CaMV 35S dans des racines de caféier transformées par *Agrobacterium rhizogenes* : mise en évidence d'une expression spatio-temporelle et utilisation de la GFP comme marqueur de sélection efficace

Introduction

Le caféier est l'arbre tropical le plus largement cultivé et représente le premier produit agricole d'exportation dans plusieurs pays en développement. La production commerciale repose sur deux espèces, *Coffea arabica* L. (75%) et *C. canephora* P. (25%). *Coffea arabica* qui est l'espèce la plus appréciée pour ses qualités sensorielles est très sensible à la plupart des parasites et en particulier, aux nématodes sédentaires endoparasites à galles (*Meloidogyne* spp.). Les *Meloidogyne* représentent l'une des contraintes agronomique majeures de la caféiculture et leur présence dans de nombreuses régions de production conduit à des baisses significatives de rendement et dans certains cas à l'abandon de la culture. Il est reconnu que la meilleure réponse au problème des nématodes chez le caféier est la lutte génétique. Jusqu'ici, une quinzaine d'espèces ont été caractérisées comme parasites du caféier (Carneiro et al. 2004). Des sources de résistance spécifiques ont été identifiées (Bertrand et al. 2001) telles que le gène *Mex-1*, introgressé de *C. canephora*, conférant la résistance à *M. exigua* qui est en cours d'isolement (Noir et al. 2003).

Le processus d'amélioration de la résistance aux nématodes passe par une meilleure connaissance des gènes de résistance (R) et des mécanismes associés. Une approche de dissection moléculaire de ces gènes de résistance et une meilleure compréhension des bases de la spécificité de leur résistance et de leur évolution devrait permettre d'en optimiser l'utilisation et d'augmenter la durabilité et l'utilité des gènes de résistance identifiés. Pour ces raisons, un procédé de transformation génétique par *Agrobacterium rhizogenes* a été récemment mis au point chez le caféier (Alpizar et al. 2006). Il permet la production rapide de racines transformées et de plantes composites ainsi que leur inoculation par *M. exigua* dans des conditions faciles et donc la validation de gènes R par complémentation fonctionnelle.

La compréhension de la régulation de l'expression des transgènes est primordiale pour le succès de la génomique fonctionnelle chez les ligneux. Bien que la transformation génétique soit rapportée chez un nombre croissant d'espèces ligneuses, la majorité des analyses détaillées sur l'expression de promoteurs se limite encore pour l'essentiel aux travaux sur *Populus* (Studart-Guimarães et al. 2005; Hawkins et al. 2006) mais également sur les Casuarinacées (Ahmadi et al. 2003; Obertello et al. 2005). Le promoteur CaMV 35S (Odell et al. 1985) a été le plus largement utilisé pour introduire des gènes étrangers dans des plantes, et s'est révélé très efficace chez les dicotylédones et chez quelques monocotylédones. Plusieurs études ont confirmé l'aptitude du promoteur 35S à contrôler l'expression de gènes chez des arbres tropicaux i.e. cacao (Maximova et al. 2006), *Hevea* (Blanc et al. 2006), *Citrus* (Cervera et al. 2000). Différent travaux ont montré que l'expression contrôlée par le promoteur 35S n'est pas toujours constitutive chez les ligneux, et des différences dans le profil, le niveau et l'inductibilité de l'expression du transgène ont été notées en conditions *in vitro* et *ex vitro* (Smouni et al. 2002 ; Hawkins et al. 2006). Chez le caféier, Van Boxtel et al. (1995) ont testé quatre promoteurs différents contrôlant l'expression du gène *uid*A et comparé son expression dans des embryons somatiques et des explants foliaires. De façon étonnante, aucune autre équipe n'a réalisé depuis ce type d'approche. Ainsi, bien que la construction chimérique 35S-GUS ait été le plus fréquemment utilisée et que son activité ait été confirmée, on ne dispose que de très peu d'information sur la spécificité de l'expression conférée par le promoteur viral 35S dans le caféier. En particulier, nous ne disposons d'aucune information sur son mode d'expression au niveau racinaire ni sur la stabilité de son expression.

La GFP (green fluorescent protein) trouvée chez l'algue *Aequorea victoria* présente une aptitude à émettre une fluorescence verte et peut également être exprimée dans un organisme étranger (Chalfie et al. 1994). Les différentes formes synthétiques de la GFP qui ont été développées depuis sa découverte ne sont pas toxiques pour les cellules et n'ont pas besoin de co-facteurs ou de substrats pour leur fluorescence. En conséquence, l'expression de la GFP peut être induite directement dans des cellules vivantes, ce qui permet l'identification visuelle des individus ou organes transformés et a été utilisée récemment comme système rapporteur pour optimiser les procédés de transformation (Belluci et al. 2003; Baranski et al. 2006). Chez *C. canephora*, Ogita et al. (2004) ont rapporté l'expression d'une forme synthétique de la GFP, en l'occurrence la sGFP, dans embryons somatiques transformés et les racines des semenceaux transformés par *A. tumefaciens*. Un premier objectif de ce travail consiste à vérifier que le gène rapporteur *gfp* s'exprime dans des racines de caféier transformées par *A. rhizogenes* et peut permettre une sélection visuelle par épifluorescence des racines transformées. Si c'était le cas, il serait alors

possible d'optimiser la méthodologie de production de plantes composites pour l'analyse fonctionnelle des gènes impliqués dans la physiologie des racines et en particulier dans la résistance aux nématodes.

Le deuxième objectif est de déterminer s'il est possible d'utiliser le promoteur CaMV 35S pour exprimer de manière constitutive des gènes de résistance aux nématodes et pouvoir sur- ou sous-exprimer leur expression dans le cadre d'études de génomique fonctionnelle. Nous avons choisi d'étudier en détail le mode d'expression conféré par la séquence pCaMV 35S après transformation génétique par *A. rhizogenes* de racines de *C. arabica*. Pour cela, nous avons étudié l'activité GUS et GFP sur une cinquantaine de clones de racines transformées cultivées en condition *in vitro* ainsi que sur des galles de *M. exigua* issues de racines transformées provenant chez des plantes composites cultivées en serre.

Matériel et Méthodes

Matériel biologique

Des graines provenant de deux variétés de *C. arabica*, Caturra and IAPAR-59, ont subi une stérilisation superficielle et une élimination superficielle de la parche. Après une imbibition de 48-72h, les embryons ont été extraits en conditions stériles. La germination a été obtenue en cultivant les embryons zygotiques sur milieu semi-solide GER (Etienne, 2005) dans des boîtes de Pétri de 5.5 cm de diamètre pendant 8 semaines à 27°C et à l'obscurité.

Souche bactérienne et vecteurs binaires

La souche A4RS d'*Agrobacterium rhizogenes*, de type agropine mannopine (Jouanin et al. 1986), a été utilisée dans toutes les expérimentations. Cette souche est dérivée de la souche sauvage A4 modifiée par l'introduction d'une résistance aux antibiotiques rifampycine et spectinomycine. Les trois vecteurs pBIN19 35S-*uidA*, pBIN19 35S-*gfp4* et pCAMBIA2300 35S-*gfp4* ont été utilisés dans les différentes expériences après avoir été introduits dans la souche A4RS par la méthode d'électroporation (Sambrook, 1989). Le plasmide binaire pBIN19 35S-*uidA* porte sur son ADN-T le gène bactérien *uidA* (GUS) codant pour la β-glucuronidase sous contrôle du promoteur CaMV 35S du virus de la mosaïque du choux fleur (CaMV) avec un intron additionnel permettant une expression spécifique dans les cellules eucaryotes (Vancanneyt et al. 1990). Le plasmide binaire pBIN19 35S-*gfp4* (Haseloff et al. 1997) porte le gène de la *gfp* (green fluorescent protein) sous contrôle du CaMV p35S. Enfin, le plasmide pCAMBIA2300 35S-*gfp4* porte également le gène

gfp sous contrôle du promoteur CaMV 35S. Par ailleurs, les deux plasmides pBIN portent le gène nptII (neomycin phosphotransferase) sous contrôle d'un promoteur *nos*, alors que le vecteur pC2300 porte le gène nptII sous contrôle du promoteur CaMV 35S. Les souches armées de *A. rhizogenes* ont été cultivées sur milieu LB semi-solide avec les antibiotiques appropriés: 50 $mg.l^{-1}$ rifampycine, 500 $mg.l^{-1}$ spectinomycine, 50 $mg.l^{-1}$ kanamycine at 28°C pendant 48h avant leur utilisation en transformation génétique.

Transformation génétique

Les embryons germés ont été infectés avec *A. rhizogenes* en blessant les hypocotyles avec un scalpel préalablement plongé dans une culture bactérienne de 48h. La co-culture a été conduite pendant 14 jours à 20°C en plaçant les embryons infectés dans des boîtes de Pétri de 50mm de diamètre à l'obscurité sur milieu MS (Murashige & Skoog, 1962) additionné de 30 $g.l^{-1}$ saccharose et solidifié en ajoutant 2.5 $g.l^{-1}$ phytagel (Sigma). Les embryons ayant subi la co-culture ont été décontaminés par immersion dans un milieu MS avec cefotaxime (500 $\mu g.ml^{-1}$) puis ont été repiqués toutes les 3 semaines sur des concentrations décroissantes de cefotaxime (500, 200, 100 $\mu g.ml^{-1}$). Les racines transformées sont apparues au site de blessure après 4-8 semaines.

L'efficacité de transformation a été calculée comme le pourcentage d'embryons inoculés qui ont régénéré au moins une racine transformée au site de blessure. Le pourcentage de racines co-transformées, GUS- ou GFP-positives, a été évalué 12 semaines après transformation. Pour les plantes transformées par la souche A4-pBIN19-35S-*gus* cette évaluation se fait par trempage d'une pointe racinaire appartenant à un système bien ramifié dans une solution de coloration (X-Glu). Pour les plantes transformées par les souches A4-pBIN19-35S-*gfp4* et A4-pCAMBIA2300-35S-*gfp4*, l'identification des racines co-transformées et l'évaluation du pourcentage de co-transformation se fait au stéréomicroscope à fluorescence sur matériel vivant. Les racines GUS- ou GFP négatives sont éliminées et une seule racine bien ramifiée est conservée sur la tige non-transformée pour produire des plantes composites co-transformées. Les embryons transformées et les plantes composites sont repiquées toutes les 3 semaines sur milieu MS et maintenues sous une photopériode de 14h (20 $\mu mol\ m^{-2}s^{-1}$) à 26°C jusqu'à l'acclimatation. Des fragments racinaires de 3 cm peuvent être prélevés sur les systèmes racinaires co-transformés bien ramifiés, pour initier des cultures axéniques entretenues. Ces lignées 'hairy roots' sont conservées *in vitro* à long-terme à l'obscurité et à 26°C par des sub-cultures de 3 semaines sur un milieu MS contenant 0.3% saccharose et 0,25 µM AIB (Acide indole 3-butyrique) comme défini dans le chapitre précédent.

Dosages de l'activité GUS

Test histochimique GUS: Pour vérifier et localiser l'activité ß-glucuronidase, des racines transformées sectionnées ont été immergées dans une solution colorante contenant 1 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (Xgluc) dans des microplaques multi-puits et incubées une nuit à 37◦C selon les recommandations de Jefferson (1987). Afin de confiner la localisation de la coloration bleue, 0,5 mM K3Fe(CN)6 et 0,5 mM K4Fe(CN) ont été ajoutés à la solution Xgluc.

Dosage fluorimétrique de l'activité ß-glucurinidase: Des racines en culture ont été broyées avec du tampon d'extraction (50 mM KPO_4 buffer, pH 7.0, 1 mM EDTA et 10 mM ß-mercaptoethanol) dans des tubes épendorf. De façon à obtenir une concentration finale de 0,44 mg/ml, du 4-Methyl-umbelliferyl-ß-D-glucoronide (MUG) a été ajouté au tampon de dosage fluorimétrique ß-glucuronidase (GUS) (50 mM tampon $NaPO_4$, pH 7.0, 10 mM ß-mercaptoethanol, 10 mM EDTA, 0.1% [w/v] sodium lauryl sarcosine et 0.1% [w/v] Triton X-100. Les dosages ont été réalisés sur des extraits de tissus végétaux de 50 µl pendant 3 h à 37°C et arrêtés avec 10× le volume de 0,2 M Na_2CO_3. Un spectrophotomètre à fluorescence a été utilisé pour quantifier la montant de 4-methylumbelliferone (MU) produit à partir du MUG. La concentration de protéine a été déterminée en accord avec le protocole décrit par Bradford (1976) avec le BSA comme standard.

Fluorescence de la GFP

L'épifluorescence de la GFP dans des racines de caféier vivantes a été détectée en utilisant un stéréomicroscope à fluorescence, à travers un jeu de filtres GFP2 [λ excitation: 480±40 nm et λ excitation stop: 510 nm]. Pour l'analyse au CLSM (confocal laser scanning microscopy), les racines ont été immergées dans l'iodure de propidium (5 μg/ml) pendant 15 min. Ce produit permet de marquer les parois cellulaires et de les révéler par fluorescence. Un microscope de type Zeiss LSM 510 META équipé d'un module de déconvolution spectrale a été utilisé pour effectuer les observations à des longueurs d'onde d'excitation de 488 nm pour la GFP et 543 nm pour l'iodure de propidium. Les émissions de la GFP et de l'iodure de propidium ont été collectées dans des canaux séparés (505–530 nm et 560–615 nm respectivement) et ensuite recouverte afin de créer des images composites.

Préparations histologiques

Des coupes histologiques des racines transformées et non transformées ont été observées pour une comparaison anatomique mais également pour comparer les réactions des tissus aux nématodes. Des fragments racinaires d'1 cm ont été prélevés à 2 cm de l'apex pour l'étude anatomique de racines différenciées. Pour l'étude de l'infection par des nématodes, 3 ou 4 galles ont été sélectionnées pour chaque motif transgénique ou non transgénique. Les échantillons racinaires ont été fixés dans du FAA (formaldéhyde, alcool, acide acétique et eau distillée) pendant 48 h. Ils ont été graduellement déshydratés dans une série de bains d'éthanol (70–100%), 1 h dans chaque bain, inclus dans l'historésine 7100 (LKB), à 4°C une nuit et ensuite inclus dans la résine. Des sections transversales ou longitudinales de 3 µm d'épaisseur ont été coupées et colorées avec du PAS (periodic acid Schiff), qui colore les polysaccarides en rouge (parois et amidon), et NBB (naphthol blue black) qui met en évidence par une coloration bleue les protéines solubles et insolubles (Fisher, 1968).

Dispositif expérimental et analyse des données

1) *Efficacité des vecteurs*. Les efficacités de transformation et de co-transformation des 3 vecteurs pBIN19-35S-*uidA*, pBIN19-35S-*gfp4* et pCAMBIA2300-35S-*gfp4* seront comparées chez la variété Caturra en inoculant 36 embryons zygotiques (3 répétitions de 12 embryons) pour chaque vecteur.

2) *Anatomie des racines transformées vs non transformées*. Dans une étude précédente (Chap. 3), nous avons montré l'existence de 3 phénotypes parmi les racines transformées par *A. rhizogenes*. Ces phénotypes sont : 89% de clones au phénotype similaire à celui des racines non transgéniques caractérisé par une croissance intermédiaire et une proportion élevée de racines fines (diam.< 0,5mm) ; 3% de clones au phénotype altéré à forte proportion de racines fines mais à croissance rapide ; 11% de clones au phénotype altéré à faible proportion de racines fines et à croissance lente. Des échantillons ont été prélevés sur 4 clones représentatifs de chacun des ces phénotypes ainsi que sur des clones de racines non transgéniques. De plus, pour un même clone, des échantillons ont été prélevés *in vitro* (culture axénique) et *ex vitro* (sur plante composite correspondante cultivée sur substrat horticole) pour pouvoir distinguer l'influence de l'état transgénique et de l'environnement de culture. Dix coupes histologiques transversales ont été réalisées pour chaque échantillon pour comparer l'anatomie racinaire. Les mesures de rayons du cylindre central et du parenchyme cortical sont des moyennes portant sur une vintaigne de

Table 1. Mise en évidence du phénomène d'extinction des transgènes chez des clones de racines de *C. arabica* var. Caturra transformés par *A. rhizogenes* à travers le suivi au cours du temps de l'expression des gènes rapporteur *gus* et *gfp*.

	4 months after transformation (July 2005)	19 months after transformation (October 2006)	
	No. clones with (+) reporter gene expression	No. clones with (+) reporter gene expression	Gene silencing frequency (%)
pBIN19-p35S-*gfp4*	12	5	58
pBIN19-p35S-*uidA*	22	18	18

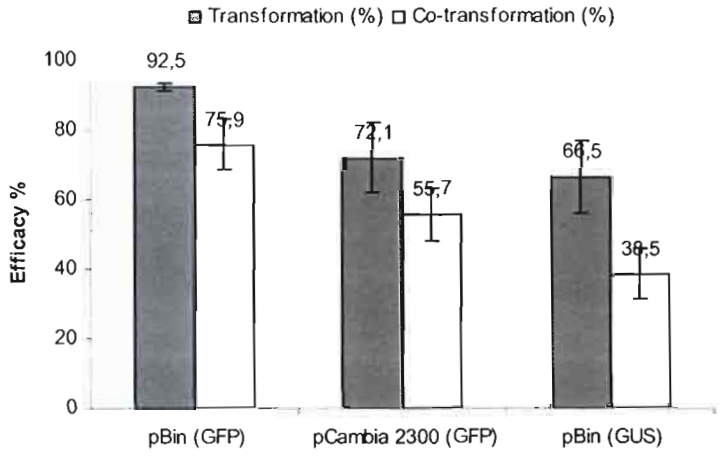

Figure 1. Efficacité des vecteurs binaires pBIN19-35S-*uidA*, pBIN19-35S-m*gfp4* et pCAMBIA2300-35S-*gfp4* pour la production de racines de caféier transformées par *A. rhizogenes*. Mesure des efficacités de transformation et de « co-transformation » 12 semaines après transformation par l'expression des gènes rapporteurs *uidA* et *gfp*.

mesures. De même, des galles dues à l'infection par *Meloidogyne exigua* ont été prélevées sur des plantes composites correspondant à ces mêmes phénotypes (3 galles par phénotype) pour comparer les réactions des tissus lors de l'infection par le nématode.

3) *Expression du gène rapporteur* uidA

L'activité GUS a été vérifiée sur 2 pointes racinaires pour les 19 clones transgéniques. Le dosage de l'activité ß-glucuronidase pour un même clone a été réalisé sur les racines provenant de 3 boîtes de Pétri correspondant à différentes répétitions. Chaque valeur est donc une moyenne assortie d'un écart-type.

4) *Expression du gène rapporteur* gfp

Six clones racinaires transformés par le vecteur A4-pBIN19-35S-*gfp4* présentant des niveaux d'intensité plus ou moins forts d'épifluoresence ont été étudiés en microscopie confocale pour affiner la localisation de l'expression GFP aux niveaux des tissus de la racine ainsi qu'au niveau des compartiments cellulaires (cytosol, vacuole, noyau, paroi cellulaire..). Trois galles de *Meloidogyne exigua* provenant des racines transformées par le même vecteur appartenant à des plantes composites de *C. arabica* var. Caturra ont également été observées.

Résultats

1) *Efficacité des vecteurs binaires.*

Des fréquences élevées de transformation – supérieures à 65% - ont été obtenues pour les 3 vecteurs binaires pBIN19-35S-*uidA*, pBIN19-35S-*gfp4* et pCAMBIA2300-35S-*gfp4* (Fig. 1). Les deux gènes rapporteurs *gus* et *gfp* s'expriment bien dans les racines transformées de caféier. Les fréquences de « co-transformation » évaluées grâce à l'expression des gènes rapporteurs *gus* et *gfp* sont inférieures d'environ 15-25% mais restent relativement élevées (39-75%) et donc compatibles avec une utilisation en analyse fonctionnelle. Les deux vecteurs binaires pBIN19 et pCambia2300 insérés dans la souche A4 d'*A. rhizogenes* représentent donc des vecteurs efficaces de transformation pour le caféier. Le vecteur PBIN19 armé du gène rapporteur *gfp* permet d'obtenir les meilleures fréquences de transformation et de co-transformation. Cependant, les niveaux élevés de co-transformation obtenus pour la première fois avec un vecteur pCambia (pCAMBIA2300-35S-gfp4) permettent d'envisager l'utilisation en routine de ces vecteurs qui sont largement plus utilisés et plus stables que les vecteurs pBIN.

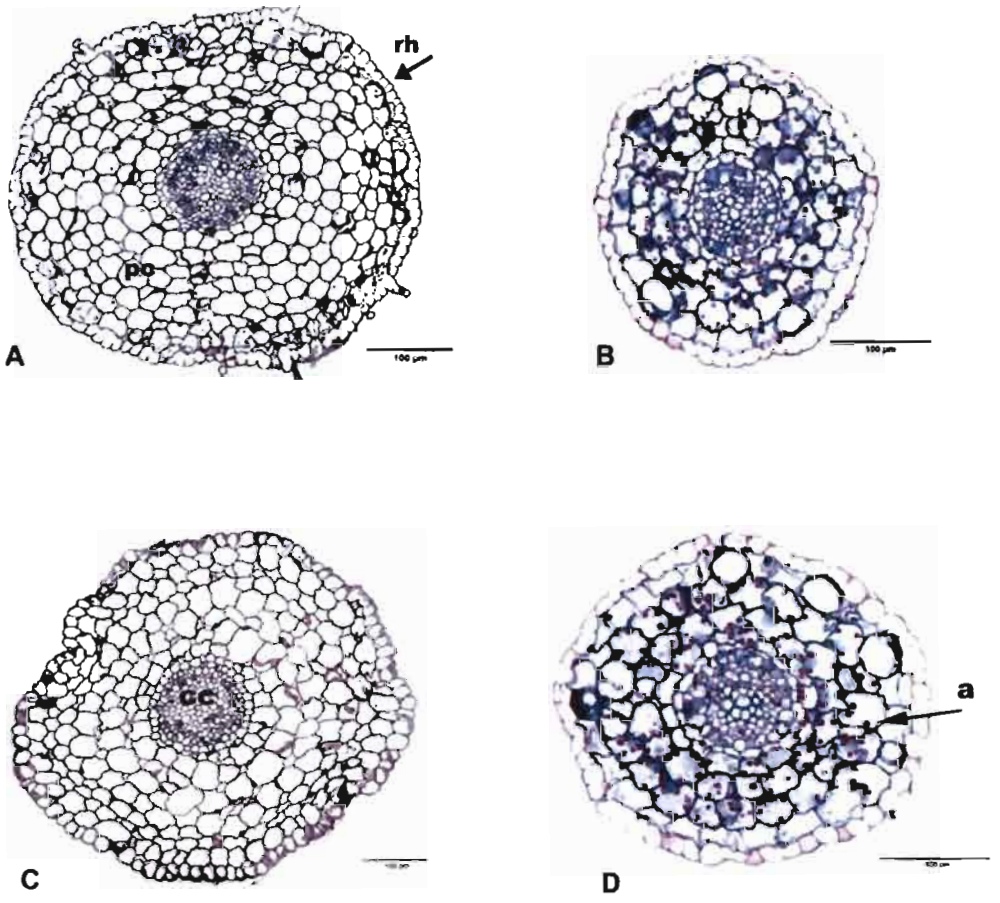

Figure 2. Influence des conditions de culture *in vitro* et *ex vitro* sur l'anatomie des racines transformées par *A. rhizogenes*. (**A** et **B**) Coupes transversales d'un clone au phénotype non altéré (CR-38) en conditions *ex vitro* (**A**) et *in vitro* (**B**). (**C** et **D**) coupes transversales d'un clone au phénotype altéré à croissance rapide (CR-13) en conditions *ex vitro* (**C**) et *in vitro* (**D**). cc= cylindre central, a= amidon, pc= parenchyme cortical, rh= rhizoderme. La barre représente 100 µm.

2) *Anatomie des racines transformées par* A. rhizogenes

La possibilité de prélever un fragment racinaire sur une plante composite avant de l'acclimater et de le cloner *in vitro* (cultures 'hairy roots') a rendu possible la comparaison de la morphologie d'un même clone racinaire *in vitro* et *ex vitro*. Cette étude a montré que l'environnement de culture a un effet important sur l'anatomie des racines de caféier, qu'elles soient transgéniques ou non. Les racines cultivées en terre (Fig. 2A,C) sont environ deux fois plus grosses que celles maintenues *in vitro* (diam. 150 µm vs 290 µm) (Fig. 2B,D). Cette modification du diamètre est pour l'essentiel liée à une augmentation de l'épaisseur du parenchyme cortical (Fig. 3A,B) associée à la présence d'un plus grand nombre d'assises cellulaires chez les racines cultivées en terre (7-11 vs 4-7) (Fig. 2). Elle est également liée, dans une moindre mesure, à l'augmentation du diamètre du cylindre central. Par contre le ratio des diamètres du cylindre central / diamètre racinaire n'est pas affecté et reste similaire *in vitro* et *ex vitro* autour d'une valeur de 26-30% (donnée non présentée). On trouve également d'importantes réserves amylacées dans des tissues différenciés *in vitro* que l'on ne retrouve pas chez les racines cultivées *ex vitro* (Fig. 2). De plus, les racines des clones cultivées *in vitro* semblent moins différenciées que leurs homologues en terre : il semble en effet qu'il y ait au niveau du cylindre central moins de pôles de xylème dans les racines *in vitro* / *ex vitro*.

L'analyse histologique n'a pas permis de mettre en évidence de différence anatomique entre racines transformées et non transformées cultivées dans les mêmes conditions (Fig. 4). Même les clones racinaires pour lesquels des phénotypes altérés ont été précédemment mis en évidence, sur des critères de développement tels que la vitesse de croissance ou la proportion de racines fines ne présentent aucune particularité anatomique. L'organisation des tissus est la même ainsi que les proportions des différents tissus. Ainsi le rayon du cylindre central et du parenchyme cortical ne varie pas significativement entre racines transformées et non transformées (Fig. 3A,B).

3) *Expression du gène rapporteur* uidA

Le dosage fluorimétrique de l'activité glucuronidase montre qu'il n'y a aucune activité enzymatique détectable chez les clones non transgéniques. Les niveaux d'activité GUS mesurés chez les clones repérés positifs par le test histochimique (3 boîtes par clone) révèlent des variations assez importantes (Fig. 5), avec des différences autour d'un facteur 10 entre le clone GUS positif présentant la plus forte activité (cr-13) et celui montrant la plus faible activité (Z22).

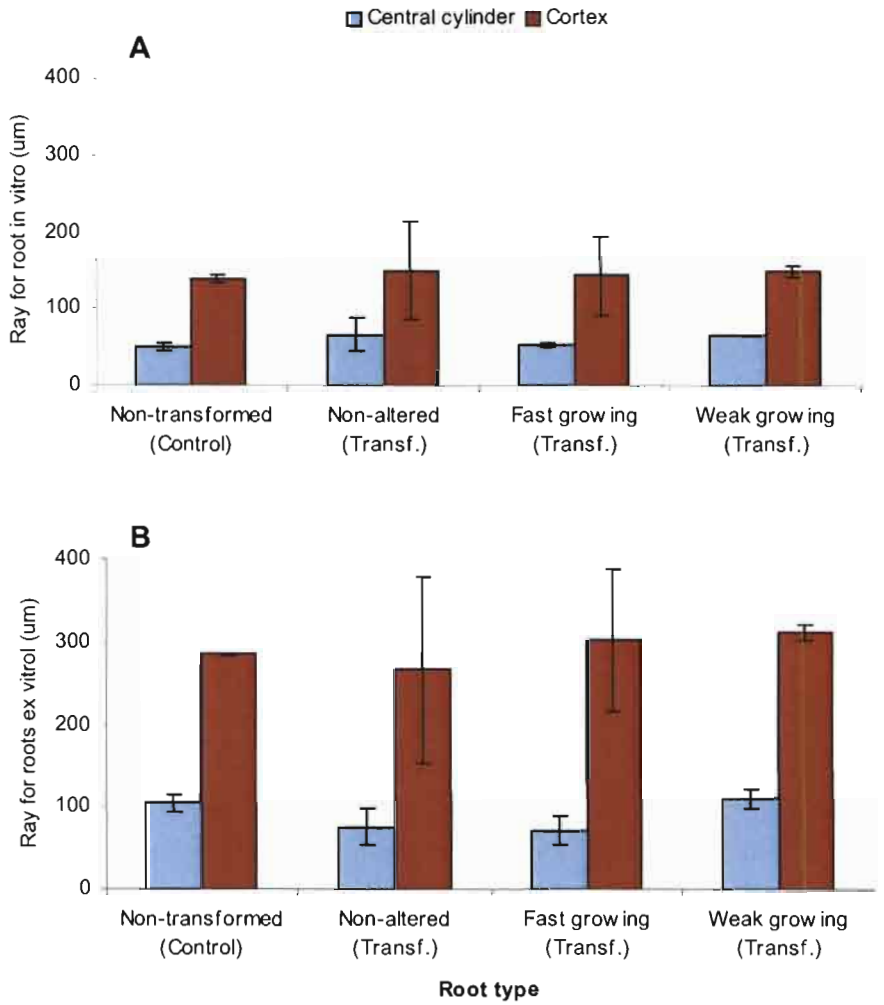

Figure 3. Comparaison des rayons du cylindre central et du parenchyme cortical pour des racines transformées ou non par *A. rhizogenes* en conditions *in vitro* (**A**) ou *ex vitro* (**B**). Les conditions *ex vitro* correspondent à des racines connectées à des plantes 'composites' cultivées en sol horticole. Les clones de racines ont été établis à partir de fragments de racines prélevés sur ces mêmes plantes composites et conservées à long-terme *in vitro* sous forme de cultures 'hairy roots' en boîtes de Pétri sur milieu semi-solide. Chaque valeur correspond à 20 mesures sur des coupes histologiques réalisées sur 5 clones par type de racine étudié. Trois phénotypes de racines génétiquement transformées ont été comparés. Deux phénotypes sont morphologiquement altérés : 'croissance rapide' et 'croissance lente/faible proportion de racines fines'. (Chap. 3).

Le profil d'expression GUS est très variable d'un clone racinaire à l'autre (Fig. 6). La localisation de l'activité GUS peut être limitée aux seules pointes racinaires ou être observée également dans le cylindre central. Dans quelques cas, l'expression GUS peut affecter l'ensemble des tissus de la racine (Fig. 6E,F). Dans ce cas, la coloration dans le parenchyme cortical semble assez hétérogène et se présente sous forme d'une pigmentation. Toutefois, bien que l'expression GUS présente des profils variables d'un clone à l'autre, elle est homogène au sein d'un même clone racinaire où ces profils sont conservés dans l'ensemble des ramifications racinaires et dans les racines primaires comme secondaires (Fig. 6A,C,E). L'analyse qualitative (détection histochimique de GUS) de 22 clones racinaires '35S-*uidA*' révèle (Table 1) que l'expression du transgène *uidA* est stable pendant la période comprise entre juillet 2005 et octobre 2006. Seulement 18% des clones n'expriment plus le transgène après 15 mois.

4) *Expression du gène rapporteur* gfp

Les observations en épifluorescence de la GFP permettent de mettre en évidence très tôt et très efficacement la néoformation des racines transformées au site d'inoculation. Dès la troisième semaine après transformation, une activité GFP est facilement détectable au site de blessure (Fig. 7A). Dès leur apparition, les racines transformées sont identifiables par leur fluorescence par rapport aux autres tissus de l'embryon (Fig. 7B,C,D). L'activité GFP permet aisément de distinguer et d'identifier sous stéréomicroscope à fluorescence les racines transformées de celles qui ne le sont pas (Fig. 7E,F). Dans les racines transformées, les observations en épifluorescence montrent une expression GFP uniforme et forte dans l'ensemble de la racine. Toutefois, des variations dans l'intensité de la coloration verte permettent de distinguer les clones pour lesquels la GFP s'exprime plus fortement (Fig. 7G,H).

Les analyses en microscopie confocale ont permis de préciser le profil d'expression dans des racines de caféier du gène rapporteur *gfp* sous contrôle du promoteur CaMV 35S. L'utilisation de 2 canaux, spécifiques de l'iodure de propidium (iP) et de la GFP, permet d'attribuer la fluorescence verte à la seule activité GFP, la fluorescence rouge étant spécifique de l'iP. Aucune auto-fluorescence correspondant au spectre d'émission de la GFP n'est observable dans les racines non transformées génétiquement (Fig. 8A). Il semble que la localisation de l'expression de la GFP sous contrôle du promoteur CaMV 35S soit liée à l'état de différentiation des tissus. Dans des tissus jeunes indifférenciés tels que le méristème ou les tissus proches du méristème, le mode d'expression est uniforme et l'activité GFP forte (Fig. 9A,B).

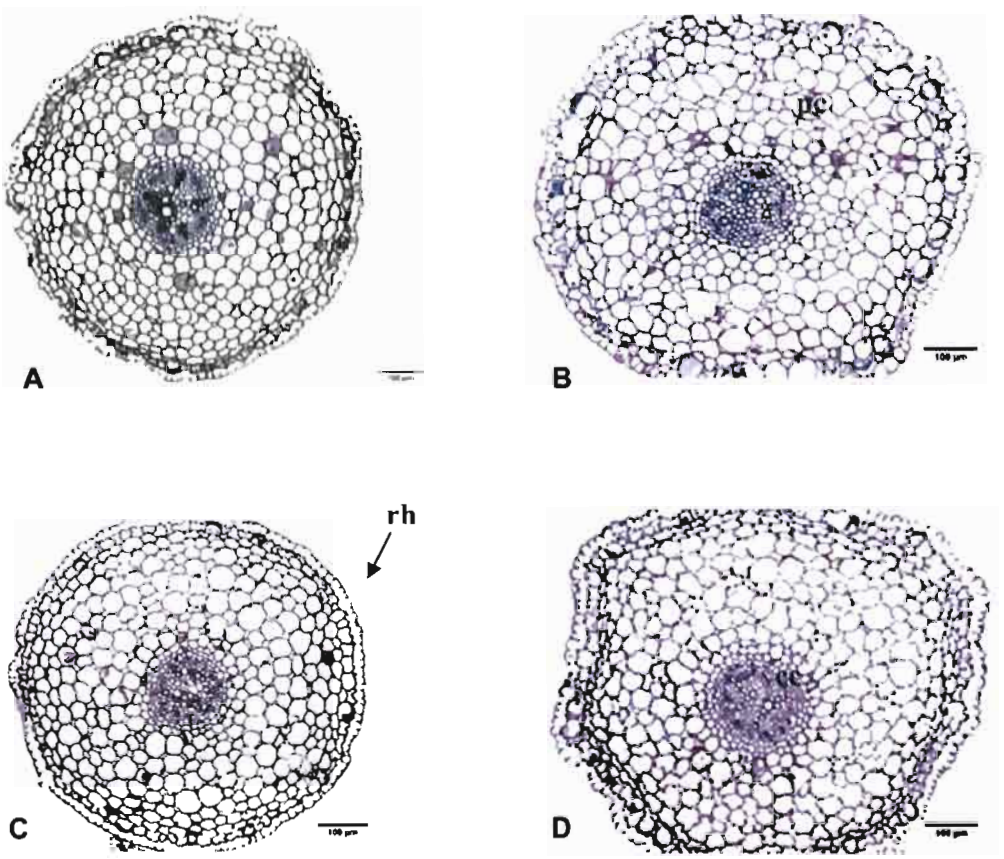

Figure 4. Comparaison de l'anatomie des racines transformées et non transformées génétiquement par *A. rhizogenes*. Coupes transversales sur différents clones cultivés en terre sur plantes composites et présentant des phénotypes altérés ou non. **A**) Racine non transformée. **B**) racine transformée au phénotype non altéré (CR-3). **C**) racine transformée au phénotype altéré à croissance rapide (CR-32). **D**) racine transformée au phénotype altéré à croissance lente / faible proportion de racines fines (diam. < 0,5mm) (CR-6). cc= cylindre central, rh= rhizoderme, pc= parenchyme cortical, x= xylème. La barre représente 100 µm.

Dans les tissus différenciés, plus éloignés du méristème, cette activité est confinée dans certaines zones encore indifférenciées tels que le future xylème et apparaît ailleurs de manière sporadique. Contrairement à l'impression donnée par les observations en épifluorescence, en microscopie confocale l'expression GFP apparaît en fait hétérogène. La fluorescence n'est pas observée de manière constitutive dans toutes les cellules (Fig. 8). Par contre, l'activité GFP est observable en fonction des racines transformées dans tous les tissus, que ce soit dans le parenchyme cortical (Fig. 8B), le rhyzoderme (Fig. 8C), le péricycle (Fig. 8E), le cylindre central (Fig. 8E). L'appréciation de l'intensité de l'expression GFP établie lors de l'analyse en épifluorescence est trompeuse parce que ce type de lumière arrive au-dessus du plan focal et en conséquence, toute l'épaisseur de la racine apparaît fluorescente. Avec le microscope confocal, uniquement la lumière qui vient du plan observé est analysée. Les clones racinaires transgéniques repérés pour leur forte expression ont systématiquement une expression GFP assez forte en périphérie au niveau de l'épiderme (Fig. 8C) ou de l'assise sous épidermique alors que l'expression dans les autres tissus peut être très limitée (cas de Fig. 8D).

Au niveau intracellulaire, cette expression de la GFP est également hétérogène. La fluorescence verte peut être observée dans différents compartiments cellulaires, mais la plupart du temps, elle est visible au niveau du cytosol plaqué contre la paroi cellulaire (Figs. 8B,C,E). Dans les tissus encore indifférenciés, l'activité GFP est systématiquement observée dans le cytosol (Fig. 9A,B). Des analyses en trois dimensions (xyz) ont permis de confirmer que la GFP pouvait parfois s'exprimer dans l'ensemble de la cellule, y compris dans la vacuole centrale (Fig. 10A). Très rarement, l'expression a pu être mise en évidence au niveau du noyau par analyse de la co-localisation des activités iP et GFP caractérisée par une fluorescence jaune, grâce à des analyses bi- (Fig. 10B) et tri-dimensionnelles (Fig. 10C,D). Par contre, aucune fluorescence de la GFP n'a pu être caractérisée au niveau de la paroi cellulaire (Fig. 10E).

De façon surprenante, l'détection de l'épifluorescence de la GFP chez 12 'clones 35S-*gfp*4' transformés âges de 15 moins (Table 1) indique que le pourcentage de clones ayant perdu l'expression du transgène *gfp4* est de 58%. Ce pourcentage calculé après la même période d'évaluation (15 mois) apparaît plus élevé que celui obtenu lors de l'évaluation de l'expression du transgène *uidA* (18%). Cependant, ce résultat reste très préliminaire et limité à un faible nombre d'évènements de transformation. Une expérimentation à plus grande échelle va être initiée pour mieux apprécier la stabilité de l'expression de ce transgène.

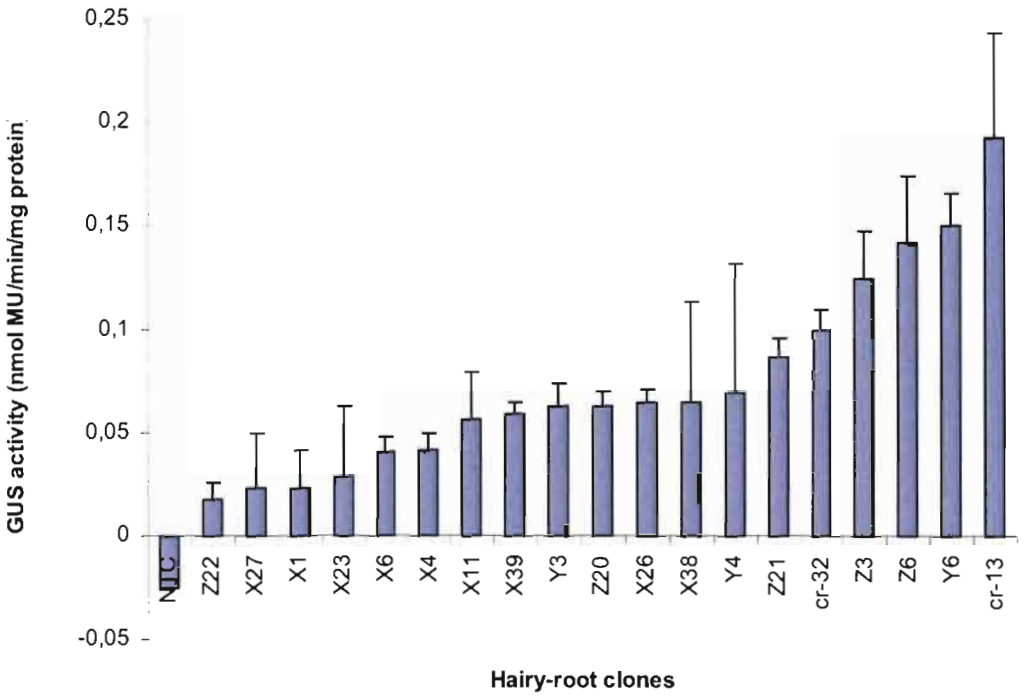

Figure 5. Activité GUS chez des clones de racines de *C. arabica* non transformées (NTC) et transformées par *A. rhizogenes*, souche A4-pBIN19-pCAMV 35S-*uid*A, en utilisant le 4-methylumbelliferyl-ß-D-glucuronide (MUG) comme substrat. Les barres représentent la moyenne ± ES de 3 mesures indépendantes dans différentes boîtes de Pétri.

5) *Expression des gènes rapporteurs dans les galles de* M. exigua

L'examen histologique des galles présentes sur des racines transformées par le gène p35S-GFP4 ne révèle aucune anomalie au niveau du site nourricier où des femelles et des cellules nourricières peuvent être observées dans une configuration normale (Fig. 11A,B). L'expression GUS est très forte spécifiquement au niveau des sites nourriciers (Fig. 11C,D). L'observation par microscopie confocale de l'activité GFP dans les galles de *M. exigua* confirme l'expression forte du gène rapporteur au niveau des sites nourriciers ainsi qu'une forte concentration dans les parois des œufs.

Discussion

La GFP est un marqueur de sélection efficace

Une des contraintes majeures des procédés de transformation génétique est liée à l'utilisation presque incontournable de gènes marqueurs qui sont le plus souvent des antibiotiques ou des herbicides. Outre le problème bien connu associé à la création d'organismes résistants à ces produits et à leur possible impact sur l'environnement, leur utilisation présente également des contraintes importantes pour les procédures de transformation. En effet, le processus de sélection basé sur la résistance aux antibiotiques ou aux herbicides est lourd, gêne et ralentit le développement des tissus transformés et allonge donc les temps de régénération. De plus, il n'est pas totalement efficace puisqu'un certain pourcentage d'individus non transformés parvient à se développer (notion d'échappements). Un des objectifs majeurs en transformation génétique est de se passer de ces marqueurs de sélection conventionnels en développant différentes alternatives telles que la sélection positive basée sur l'utilisation de sucres normalement non assimilables (xylose, mannose...) (Haldrup et al. 1998; Joersbo, 2001) ou la sélection sans gènes marqueurs (DeVetten et al. 2003). Cette dernière approche est la plus attrayante mais requiert une efficacité de transformation importante, une très bonne maîtrise des processus de régénération et une expression performante des gènes rapporteurs dans les tissus transformés. Nous avons montré dans le présent travail que cette option est viable pour la production de racines transformées par *A. rhizogenes* et de plantes composites. Les vecteurs pBin19 et pCambia2300 testés ont permis la régénération d'un grand nombre de racines adventices montrant une intense fluorescence verte qui permet une identification aisée, peu coûteuse et non destructrice pour les tissus analysés. Des résultats comparables ont été récemment publiés par Baranski et al. (2005) chez la carotte pour la sélection de racines transformées par *A. rhizogenes*. La proportion de racines de caféier exprimant la GFP (co-transformées) est très élevée – entre 55 et 76% - et supérieure à celle rapportée par ces

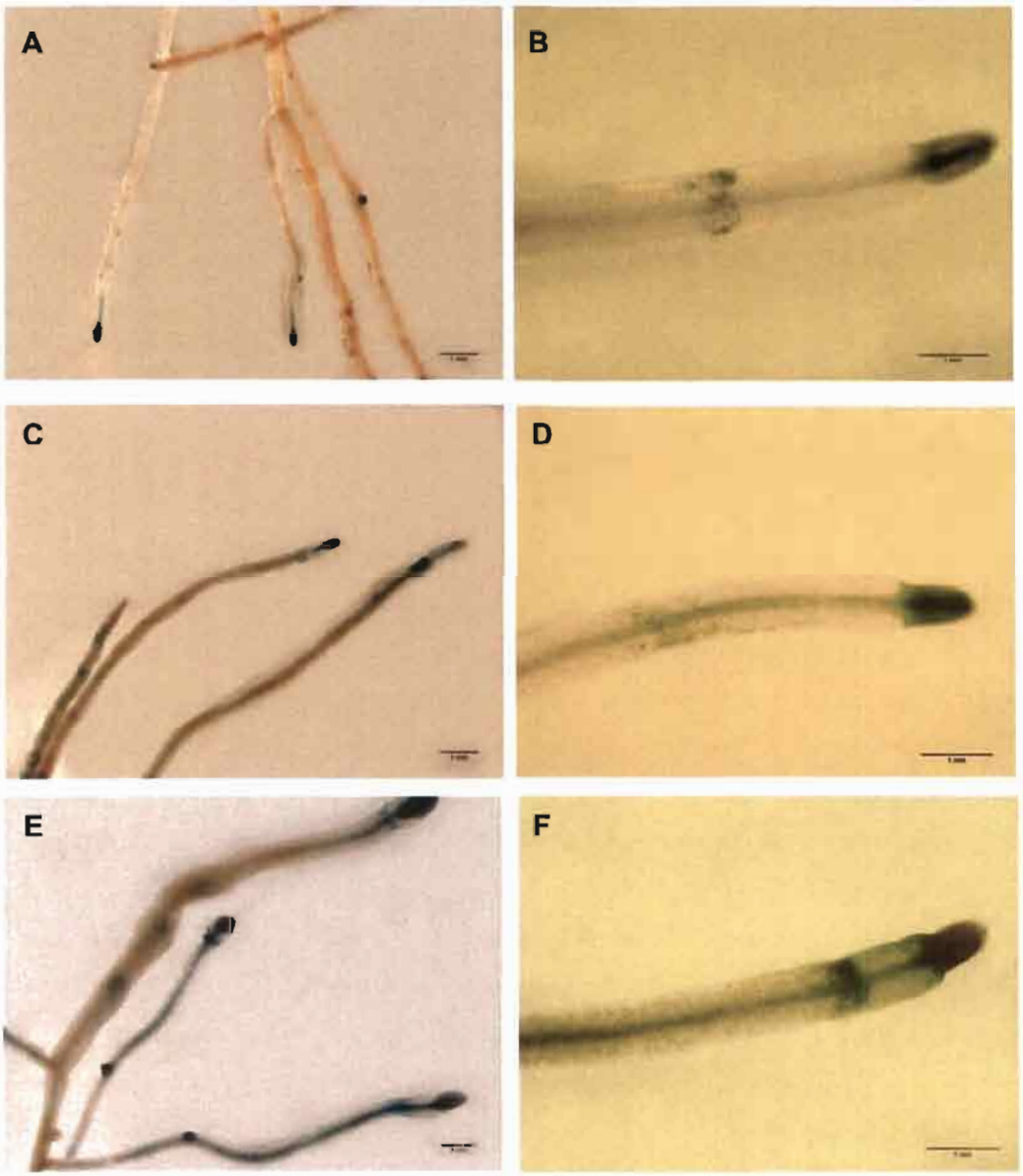

Figure 6. Localisation histochimique de l'expression du gène de la ß–glucuronidase (GUS) dans des racines de *C. arabica* transformées par l'agrobactérie A4-pBIN19-35S-*uidA*. Les tissus exprimant activement le gène GUS sont colorés en bleu. Des niveaux d'expression variables sont obtenus en fonction des clones racinaires. Racines transgéniques avec une forte coloration limitée aux méristèmes (**A**, **B**), dans les méristèmes et le cylindre central (**C**, **D**), ou étendue également au parenchyme cortical (**E**, **F**). La barre représente 1 mm.

auteurs (20-25%) ainsi que la proportion de racines exprimant l'enzyme GUS (35-45%). De plus, Baransky et al. (2005) ont doublé la sélection visuelle d'une sélection à la kanamycine (gène *nptII*), ce qui a pour effet d'augmenter la proportion de racines exprimant la GFP. A l'inverse, notre procédé n'est basé que sur la sélection visuelle.

Le processus de sélection utilisant la GFP consiste à identifier et conserver une seule racine transformée pour générer rapidement une plante composite destinée aux bio-essais pour évaluer la résistance aux nématodes. Plusieurs racines transformées étant généralement présentes au site d'inoculation de l'agrobactérie (3 en moyenne), il est possible d'opérer une seconde sélection sur les critères morphologiques d'intensité de croissance et de bonne ramification. En effet, il est généralement admis qu'un certain pourcentage des racines transformées par *A. rhizogenes* présente une inaptitude à la croissance. Par exemple, chez la carotte Guivarc'h et al. (1999) n'ont établi seulement que 30 clones racinaires à partir de 160 pointes racinaires excisées. Nous avions déjà montré précédemment que l'expression de l'enzyme GUS permettait aussi une sélection visuelle sur ces mêmes critères (Alpizar et al. 2006). Cependant, ce processus de sélection était beaucoup plus lourd car l'identification par le test histochimique GUS étant destructif, il s'agissait de prélever et de repérer autant de pointes racinaires pour évaluation qu'il y avait de racines présentes. Il fallait également attendre que ces racines soient très bien développées pour que l'élimination d'une pointe racinaire n'hypothèque pas leur développement. La sélection basée sur l'expression de la GFP est non destructive et se fait rapidement en une seule étape sous le stéréo-microscope à épifluorescence, sans même devoir ouvrir les récipients de culture. L'impact sur la quantité de travail et sur sa qualité est important.

Par ailleurs, il ne semble pas y avoir de problème de toxicité lié à la production de la GFP. Les couples histologiques mettent en évidence une organisation normale au niveau du site nourricier (femelles et cellules géantes) et les images de microscopie confocale montrent des masses d'œufs se développant normalement dans des sites nourriciers exprimant la GFP. Par ailleurs, les bio-essais ont montré des taux de multiplication de *M. exigua* similaires sur racines transformées exprimant ce gène rapporteur et sur plantes normales non transformées (résultats non présentés). L'utilisation du gène rapporteur gfp4 peut donc être envisagée en génomique fonctionnelle pour étudier l'interaction caféier/nématodes à galles.

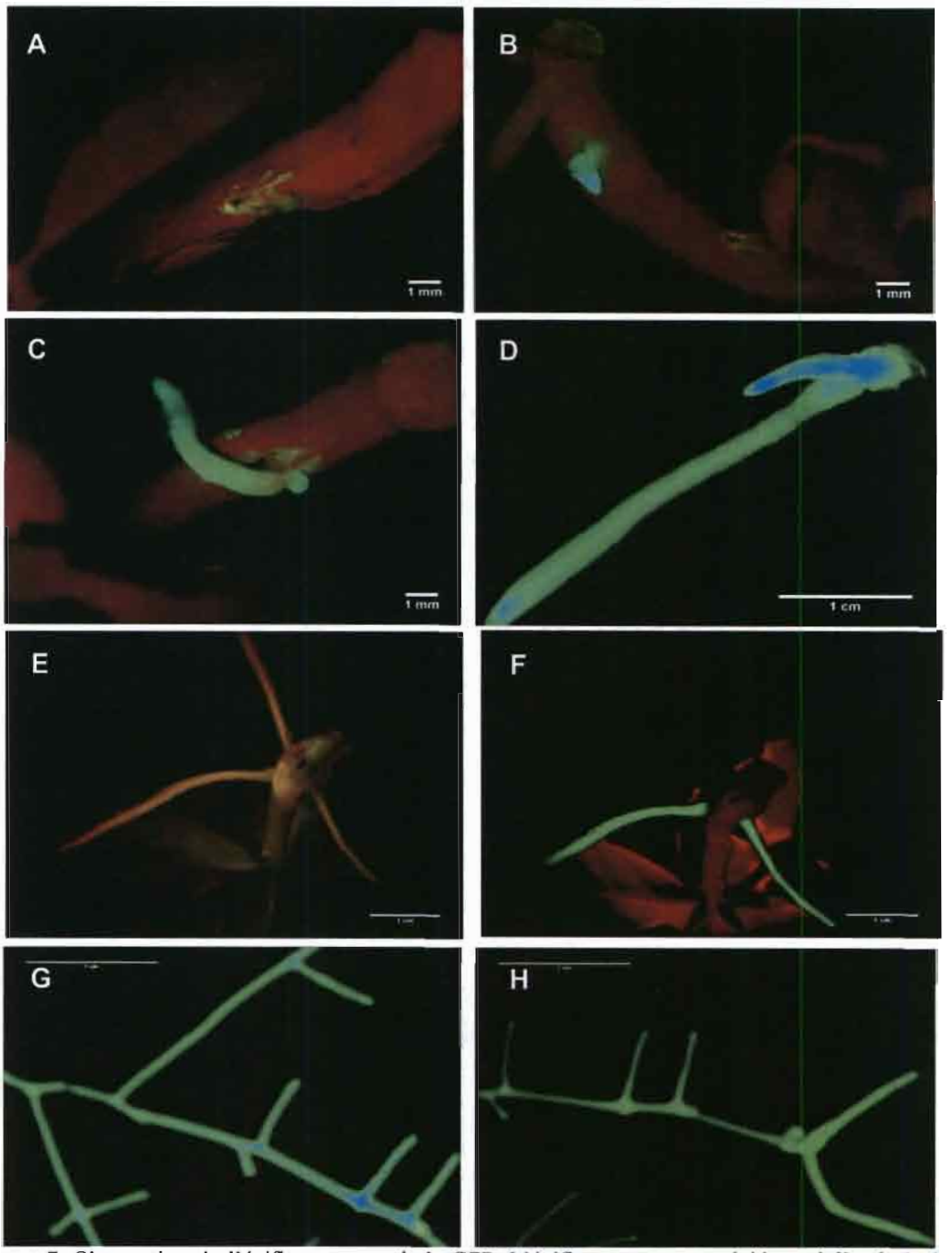

Figure 7. Observation de l'épifluorescence de la GFP. L'épifluorescence est évidente à l'endroit de la blessure 3 semaines après transformation (**A**), et continue d'être identifiée pendant tout le processus de formation de la racine transformée (**B,C,D**). L'activité de fluorescence permet d'identifier et de sélectionner efficacement au stéréoscope les racines co-transformées avec le gène *gfp4*, (**E,F**). Dans les racines ramifiées, la fluorescence est uniforme mais permet cependant d'identifier des différences d'intensité dans la coloration verte entre différents clones (**G,H**).

Le mode d'expression du promoteur 35S n'est pas constitutif

Les trois vecteurs utilisés ont comme caractéristique commune de posséder une construction chimérique avec la séquence codante d'un gène rapporteur, *gfp* ou *uidA*, sous contrôle du promoteur 35S du virus de la mosaïque du choux fleur. Ce promoteur a été le plus utilisé jusqu'ici car son expression est forte et réputée constitutive. Chez le caféier, à l'exception des travaux de Leroy et al. (2000) et Perthuis et al. (2005) qui ont utilisé le promoteur EF1α d'*Arabidopsis* pour contrôler l'expression d'un gène codant pour une protéine insecticide de *Bacillus thuringiensis* pour la résistance à la mineuse des feuilles, le p35S a été presque systématiquement utilisé sans que son mode d'expression soit précisé. Travaillant sur l'interaction caféier/nématodes à galles et préparant la validation fonctionnelle du gène de résistance *Mex-1* (Noir et al. 2003), il nous est apparu indispensable de caractériser cette expression dans les racines de caféier. En nous appuyant sur l'activité de deux gènes rapporteurs, nous montrons dans le présent travail que l'expression du p35S n'est pas constitutive dans les racines de caféier. Les résultats obtenus avec les 2 gènes rapporteurs convergent pour montrer que cette expression est très variable d'un événement de transformation à l'autre. Cette variabilité affecte à la fois l'intensité de l'expression et sa localisation. Cette dernière est hétérogène dans les tissus différenciés, de type mosaïque et l'activité du gène rapporteur est limitée à certaines cellules, et même, à certains compartiments cellulaires. La microscopie confocale à permis d'observer que l'activité GFP s'exprimait préférentiellement dans les tissus les plus indifférenciés (méristème, cylindre central, galles) et au niveau intracellulaire préférentiellement dans le cytosol. La localisation histochimique de l'activité GUS était également située dans les pointes racinaires et le cylindre central.

La possibilité pour le promoteur 35S de contrôler l'expression des transgenes a été démontrée chez beaucoup d'espèces pérennes. La plupart des travaux ont rapporté l'expression des constructions 35S-GUS et 35S-GFP, et finalement, très peu de détails sont disponibles sur la spécificité de l'expression conférée par le promoteur viral 35S. De façon similaire à nos résultas, Smouni et al. (2002) and Obertello et al. (2005) ont montré chez *Casuarina glauca* que dans les racines, le promoteur CaMV 35S entraînait une forte expression GUS dans les apex et les tissus vasculaires. Hawkins et al. (2006), sur le peuplier ont démontré que l'expression de 35S-GUS était constitutive et stable dans tous les organes de la plante, après 7 ans de vie en plein champ. L'analyse détaillée de coupes histologiques a permis de localiser l'expression GUS dans le xylème et le cambium vascularisé (tissus vivants) alors que les cellules mortes (vacuoles et fibres) n'ont montré aucune coloration. Chez *Hevea brasiliensis*, l'expression de 35S-GUS est forte

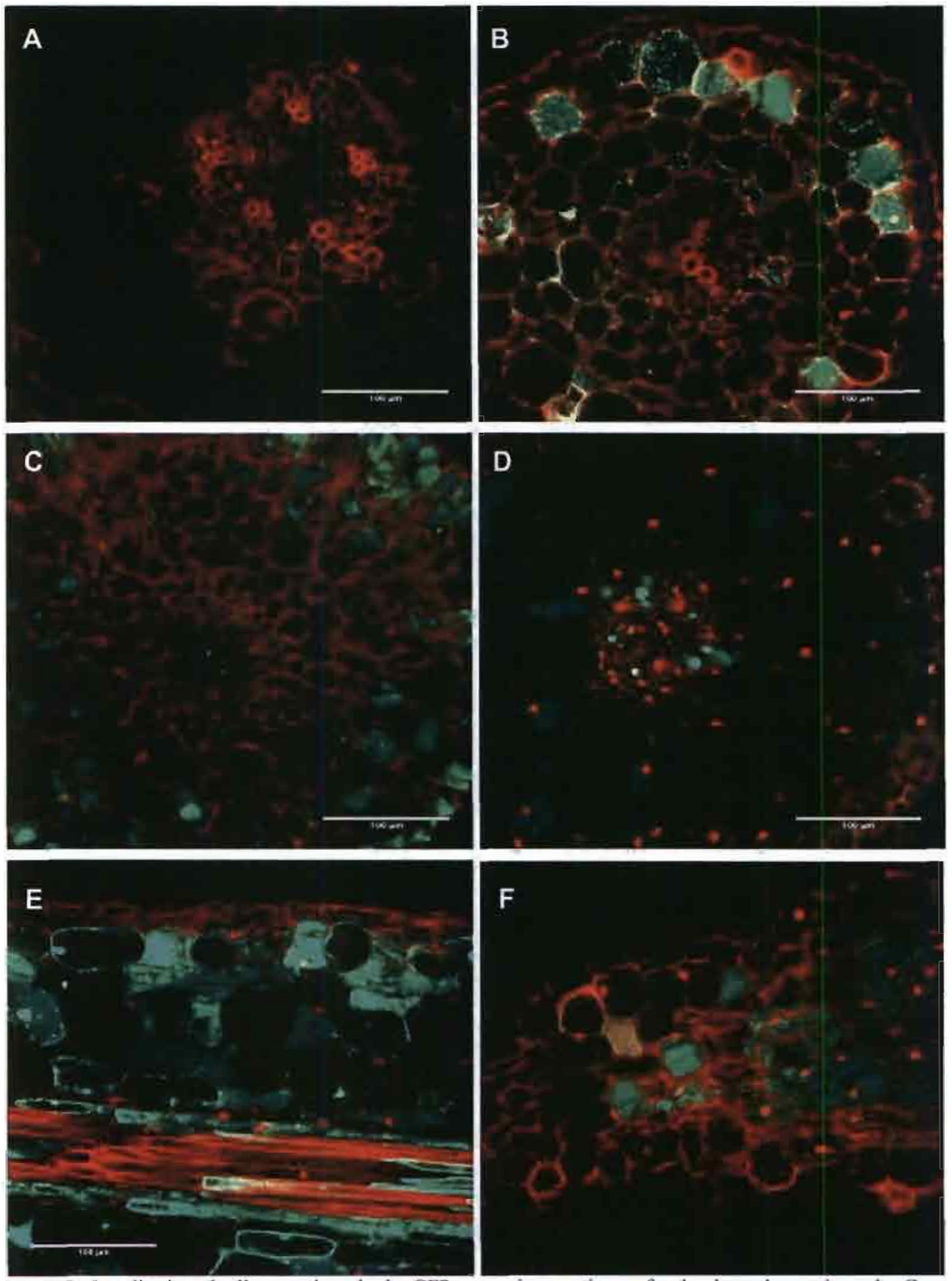

Figure 8. Localisation de l'expression de la GFP par microscopie confocale dans des racines de *C. arabica* génétiquement transformées par *A. rhizogenes*. La souche A4-pBin19-p35S-GFP4 a été utilisée. (**A**) Coupe transversale dans une racine non transformée confirmant l'absence d'activité GFP. (**B**, **C** et **D**) coupes transversales dans des racines transformées montrant un mode d'expression de type mosaïque et hétérogène entre les évènements de transformation. (**E**) coupe longitudinale montrant une forte expression dans le cortex et le cylindre central. (**F**) coupe longitudinale proche de l'apex révélant une forte activité GFP au niveau du méristème racinaire.

essentiellement dans les tissus actifs des différents organes de la plante (feuille, tige, racine) et plus particulièrement dans les faisceaux vasculaires présents (Blanc et al. 2004; Marteaux, 2004).

Quel promoteur pour l'étude des interactions caféier/nématodes ?

La plupart des publications sur la transformation génétique du caféier ont décrit des mises au point technologiques et aucun gène de caféier n'a encore fait l'objet d'une étude en analyse fonctionnelle. Cependant, depuis quelques années, la génomique du caféier connaît un essor spectaculaire dans le but d'identifier des gènes d'intérêt agronomique. Le gouvernement brésilien a financé un 'Programme de Génome du Caféier' très ambitieux avec comme objectif d'établir une banque de données de plus de 200 000 EST (expressed sequence tags) qui pourrait conduire à l'identification de plus de 30 000 gènes. Récemment, une équipe mixte Nestlé-Cornell University a publié une banque de données d'EST d'environ 47 000 clones cDNA, correspondant à 13.175 unigenes (Lin et al. 2005). Parallèlement, des banques BAC chez les deux espèces de caféier, *C. arabica* et *C. canephora*, ont été établies (Noir et al. 2004, Leroy et al. 2005). De telles cartes sont d'une importance stratégique pour la sélection assistée par marqueurs, pour avancer sur le clonage positionnel de gènes d'importance agronomique, et pour l'analyse de la structure et de la fonction des gènes. Enfin, un réseau de génomique international sur le caféier (ICGN) a été crée en 2005 pour accélérer les recherches dans ce domaine. Quelques gènes d'intérêt agronomique pour la résistance à des stress abiotiques (sécheresse, froid) et biotiques (nématodes, rouille orangée, maladie des baies du caféier.) ont été identifiés et sont en cours de clonage (Lashermes et al. 2005; Andrade et al. 2006).

Des projets de génomique similaires existent chez d'autres arbres et ont conduit à l'identification de nombreux gènes. Jusqu'ici, à part le promoteur 35S et son dérivé, le 70S ou e35S, peu de promoteurs ont été testés chez des espèces pérennes transformées de façon stable en comparaison avec des plantes annuelles et ceux qui l'ont été proviennent généralement d'espèces herbacées et de virus (Jouanin & Pilate 1997; Ahuja, 2000). Très peu de promoteurs ont été isolés et caractérisés chez les plantes pérennes.

Nos résultats révèlent des contraintes pour l'utilisation du promoteur 35S liées à son mode d'expression particulier chez le caféier. Ce mode d'expression est très variable d'un clone racinaire à l'autre et dans nombre de cas, cette expression concerne essentiellement les tissus les plus indifférenciés et une certaine proportion de cellules dans les zones différenciées. Malgré cela, pour trois raisons le promoteur CaMV 35S semble adapté pour la validation fonctionnelle

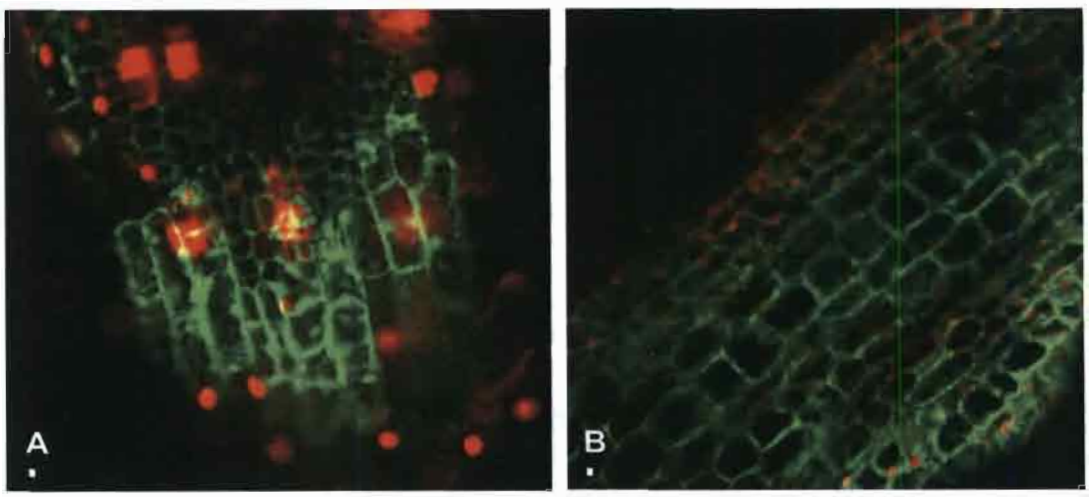

Figure 9. Localisation de l'expression de la GFP par microscopie confocale dans des tissus indifférenciés proches des méristèmes chez des racines de *C. arabica* transformées par *A. rhizogenes*. (**A**) localisation de l'activité de la GFP dans le cytosol. (**B**) l'expression est forte et uniforme dans l'ensemble des tissus. Localisation essentiellement dans le cytosol.

des gènes de résistance aux *Meloidogyne*: i) les jeunes racines et plus particulièrement les premiers centimètres des racines où l'activité du promoteur 35S est forte correspondent aux zones de pénétration et de reproduction des nématodes à galles; ii) l'expression du promoteur 35S est stable dans le temps puisque des lignée de près de deux ans continuent à présenter une forte activité GUS ou GFP; iii) enfin, au niveau du site nourricier, l'expression du 35S est fortement stimulée. Goverse et al. (2000) ont confirmé cette observation et ont associé cette sur-expression locale à une augmentation temporaire de la teneur en auxine et à la sensibilité des cellules à cette hormone. Ces observations indiquent que l'utilisation du promoteur CaMV 35S peut donc être envisagée pour la validation du gène *Mex-1* par complémentation fonctionnelle.

Plus généralement, la caractérisation de gènes d'intérêt chez le caféier va s'accélérer et il devient souhaitable d'identifier des promoteurs plus spécifiques et plus adaptés aux plantes pérennes. Récemment, le promoteur du gène de la métallothionéine a été isolé chez *C. glauca* et introduit chez des espèces annuelles mono- et dicotylédones, *Nicotiana tabacum* et *Oriza sativa* (Ahmadi et al. 2003). Les auteurs ont montré que P*cgMT1* peut être une séquence adéquate pour contrôler l'expression de transgènes dont les produits des gènes sont préférentiellement nécessaires dans les racines. Ce promoteur pourrait être également utilisé chez le caféier. Des essais préliminaires ont montré qu'il s'exprimait fortement dans les racines de caféier. Cependant, l'idéal serait d'identifier un promoteur racine-spécifique et assez fort parmi les promoteurs natifs des gènes qui vont être validés chez le caféier.

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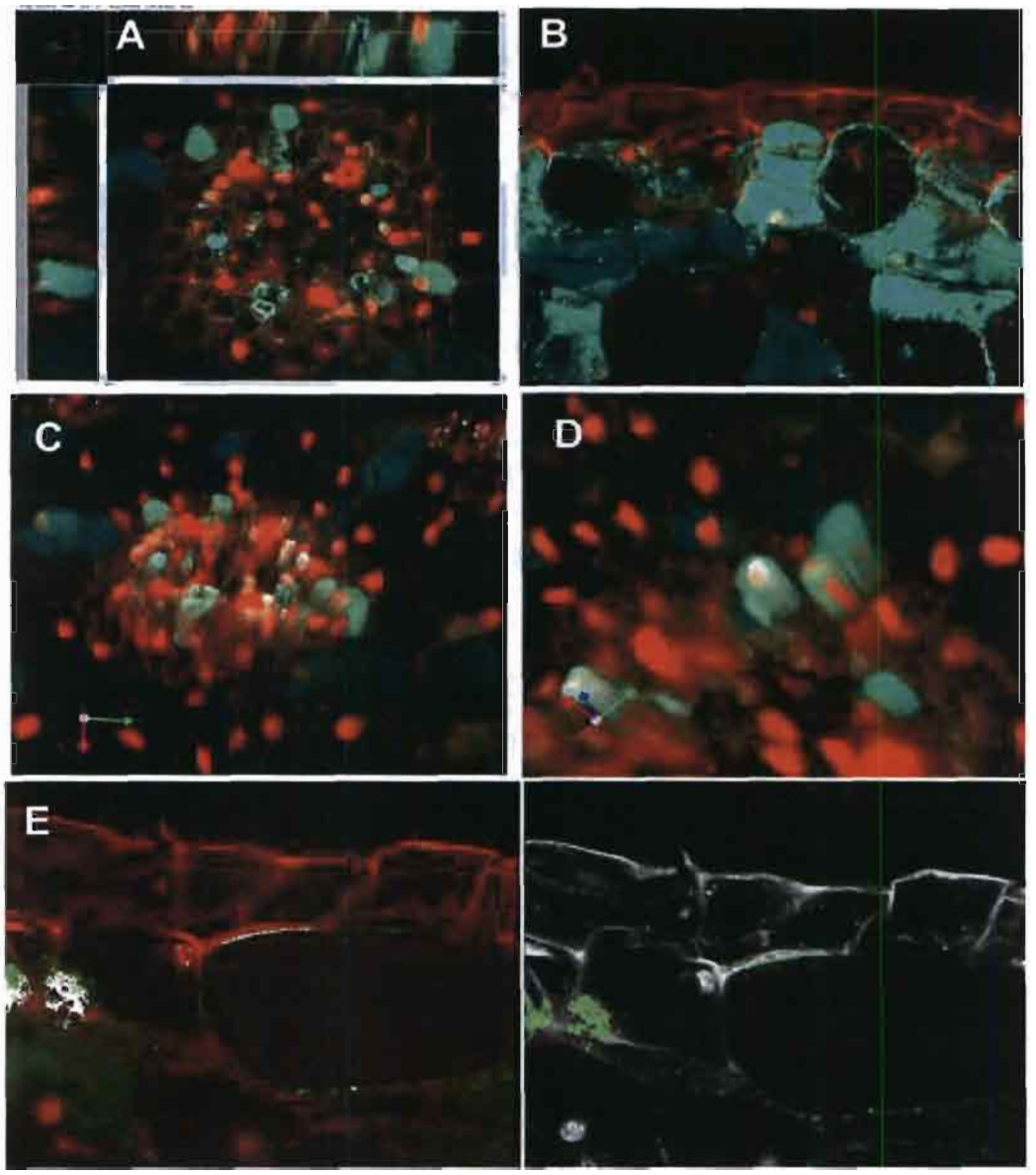

Figure 10. Localisation de l'expression de la GFP au niveau cellulaire par microscopie confocale chez des racines de *C. arabica* transformées par *A. rhizogenes* souche A4-pBin19-p35S-GFP4. (**A**, **C**, **D**) Confirmation par analyse tri-dimensionnelle de la localisation de la GFP dans l'ensemble de la cellule, y compris le noyau (en jaune) et la vacuole (fluorescence verte). (**B**) Co-localisation des activités iP et GFP dans le noyau (en jaune) [étude réalisée à l'aide du logiciel de traitement d'image Volocity®]. (**E**) Mise en évidence de l'absence d'activité GFP au niveau pariétal par la non co-localisation des activités iP et GFP (sélection des pixels allumés en analyse d'image).

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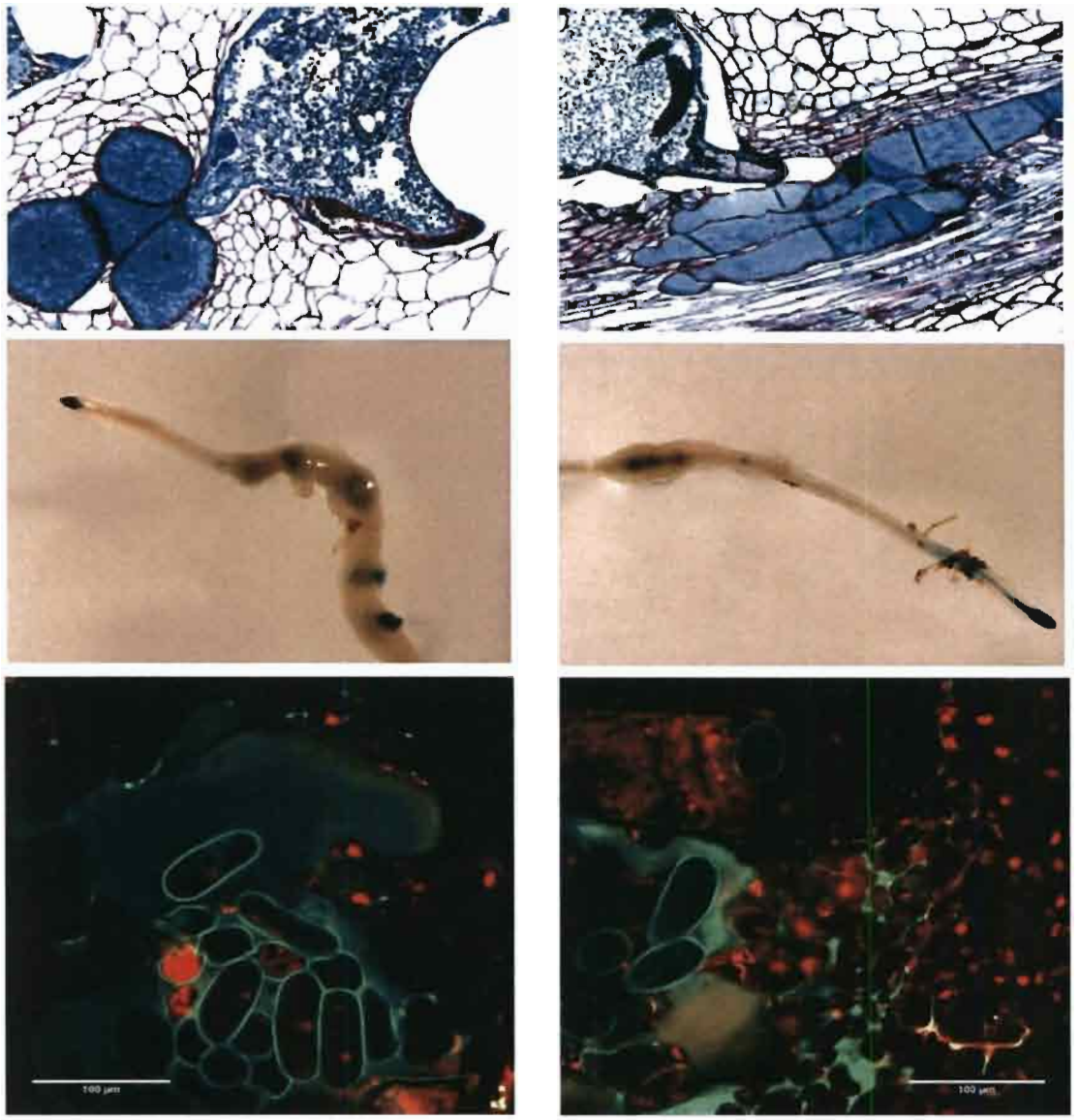

Figure 11. Mise en évidence de l'expression des gènes rapporteurs *uidA* et *gfp* au niveau des sites nourriciers du nématode à galles *M. exigua* dans des racines de caféier transformées par *A. rhizogenes*. (**A** et **B**). Coupes histologiques montrant la femelle et les cellules nourricières dans des racines transformées par la souche A4-pBin19-p35S-GFP. (**B** et **C**) Expression de l'activité GUS dans des galles (sites nourriciers) sur racines transformées par la souche A4-pCambia2300-p35S-GUS. (**D** et **E**) Activité GFP dans des galles de racines transformées par la souche A4-pBin19-p35S-GFP. Forte concentration de la GFP dans les parois des œufs. cn= cellules nourricières, f= femelle, o= œufs.

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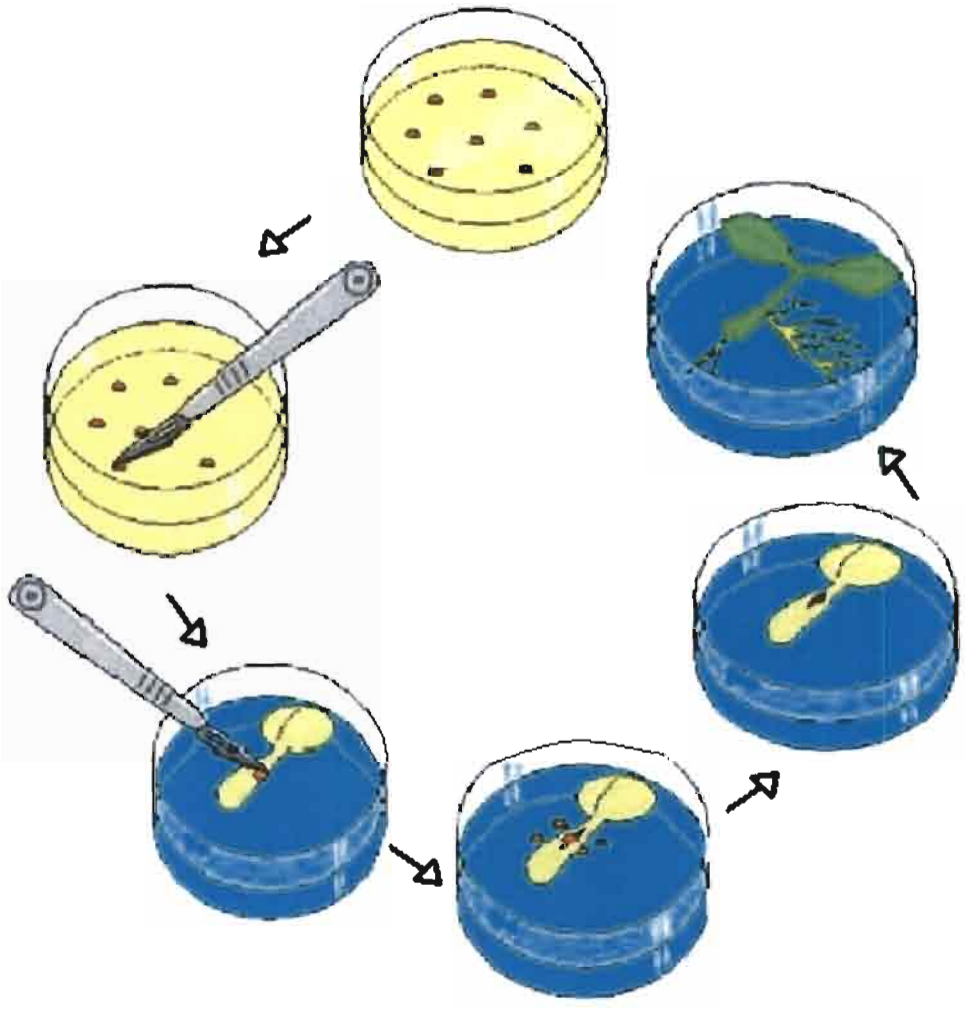
Chapter V

General discussion and perspectives



Scheme 1. Relative incidence of different parameters on the efficiency of *Agrobacterium rhizogenes*-mediated transformation protocol of *Coffea arabica*, developed for the functional analysis of *Mex-1* gene. The factors possessing strong influence on transformation efficacy are described in green and those that not exhibited significant effect, in red color.

Improvement of coffee transformation protocol

The procedures of coffee transformation reported in the literature before this study were complex, tedious and inefficient in order to be used for the functional analysis of putative genes (e.g. 0.3% transformation efficiency reported by Leroy et al. 1997, 3% by Kumar et al. 2006). In that context, we developed an efficient *Agrobacterium rhizogenes*-mediated protocol that enables efficient and rapid regeneration of transformed roots and composite plants exhibiting a suitable phenotype to be acclimatized within 4 months following the transformation (Scheme 1).

Although the developed protocol (described in Chapter 3) give results that are adequate for the functional validation of *Mex-1* gene, in this section we aim to give some insight and perspective that could help to improve its efficacy in the future. (a) The development of larger binary vectors (e.g. BIBAC Hamilton et al. 1996, TAC Liu et al. 1999) that allow the cloning and transfer of large genomic-DNA fragments (< 150 kb) allows the possibility to test them in coffee in terms of compatibility with the A4RS *Agrobacterium*. They could be useful to accelerate the cloning and validation of agronomically important genes. (b) The addition of acetosyringone, a plant phenolic compound that is reported in other plant species to affect the level of transcription of the *vir* genes of *Agrobacterium*, was tested at different concentrations both during agrobacteria culturing and co-cultivation with the plant, however no increase of transformation efficiency was found. (c) Neither the elimination of antibiotic selection marker in the last culture of the agrobacteria during its multiplication nor, (d) the inoculation of the agrobacteria at diluted concentration or, (e) the simultaneous co-inoculation of *A. rhizogenes* + *A. tumefaciens*, increased the transformation efficiency (data not shown). (f) The response of different organs from zygotic embryo to *A. rhizogenes* infection significantly influenced the transformation efficiency (hypocotyls produced more transformed roots than leaves or roots). In the future the physiological status of the embryo, conditioned in particular by the time of seed storage and the duration of the germination period previous to agroinfection should be studied carefully in order to maintain or increase the transformation efficiency achieved to date. (g) The results obtained from different co-cultivation periods and (h) co-cultivation temperature experiments showed a strong influence of these parameters on transformation efficiency. Both parameters should be adjusted again if different *Coffea* sp. must to be transformed.

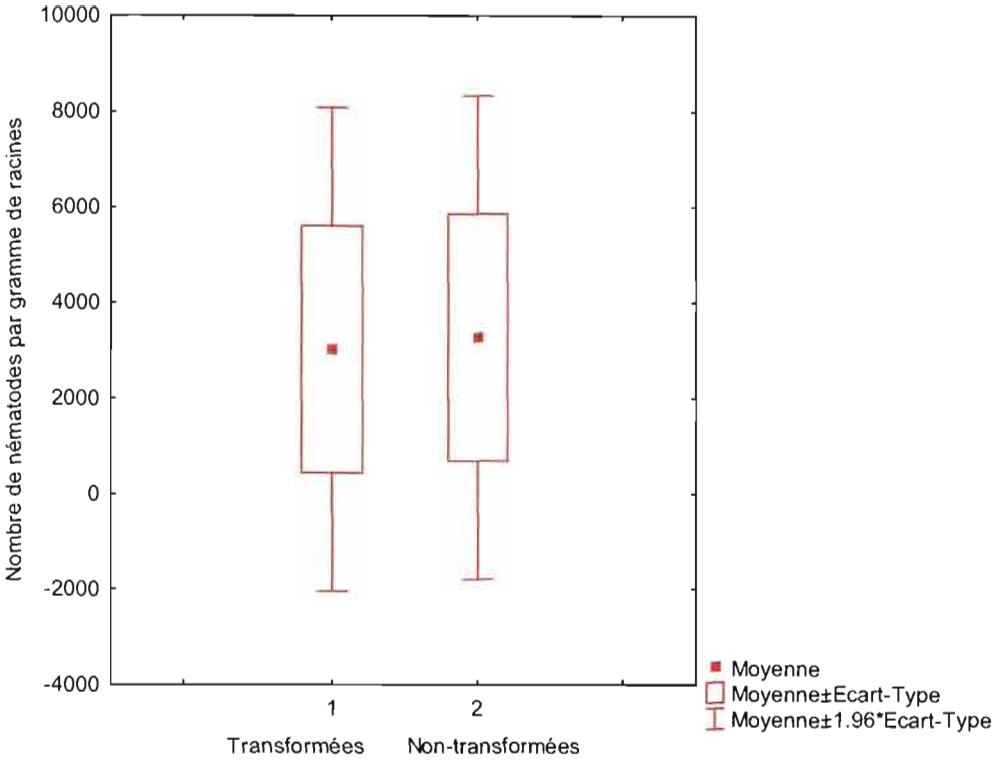


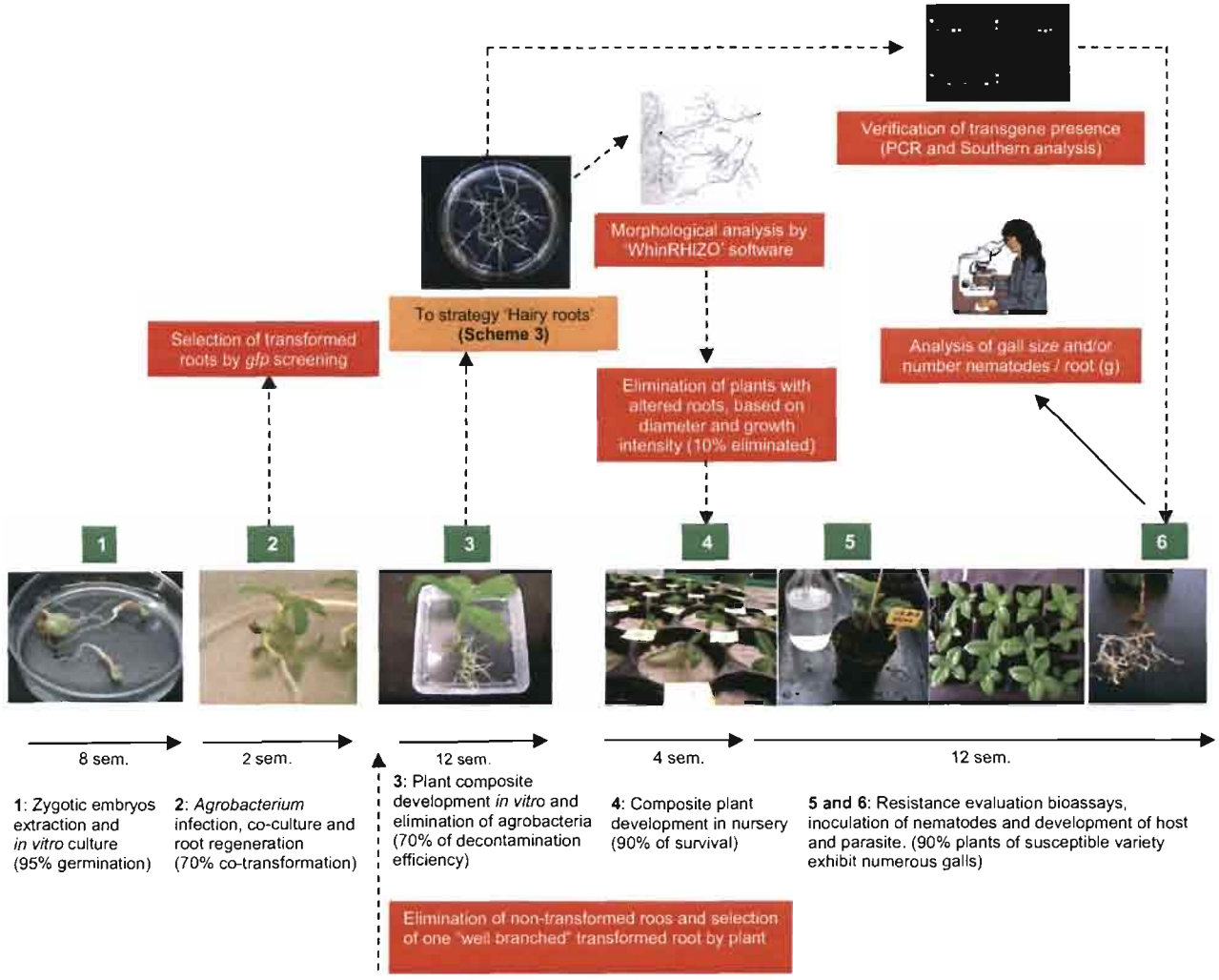
Figure 1. Comparison between the numbers of *M. exigua* individuals per gram of fresh root extracted from *A. rhizogenes*-transformed vs. normal roots in susceptible Caturra variety. Extraction was done four months after *M. exigua* inoculation. Each value represents the mean of three replicates from 34 transformed roots and 11 normal roots. Values were not significantly different at $P \leq 0.001$ (Neuman–Keuls test).

Figure 2. Illustration of *C. arabica* var. Caturra normal roots (control) and transformed root with *A. rhizogenes* pBIN19.35S-*uidA* four months after inoculation of *M. exigua* nematodes.

Use of composite plants for nematode bioassays

Different important conditions had to be respected before using the *Agrobacterium*-mediated transformation procedure for the functional validation of R genes to nematodes: i) the transformed roots should be morphologically and anatomically similar to non-transformed roots to ensure that the effect of oncogenes present on the Ri-T DNA on root development is limited. This verification is important to ensure that the influence of other factors than the expression of the resistance from the candidate gene is limited and does not disturb the nematode penetration and multiplication within transformed roots, ii) the composite plants should constitute a reliable system for the bioassays done with the aim of testing the resistance to the root-knot nematodes conferred by the candidate R gene (this work constitutes the first time composite plants have been used to study plant/nematode interactions); iii) the substrate where root and nematode interact, should allow the normal development of both roots and pathogens (multiplication rates similar to those obtained in natural conditions).

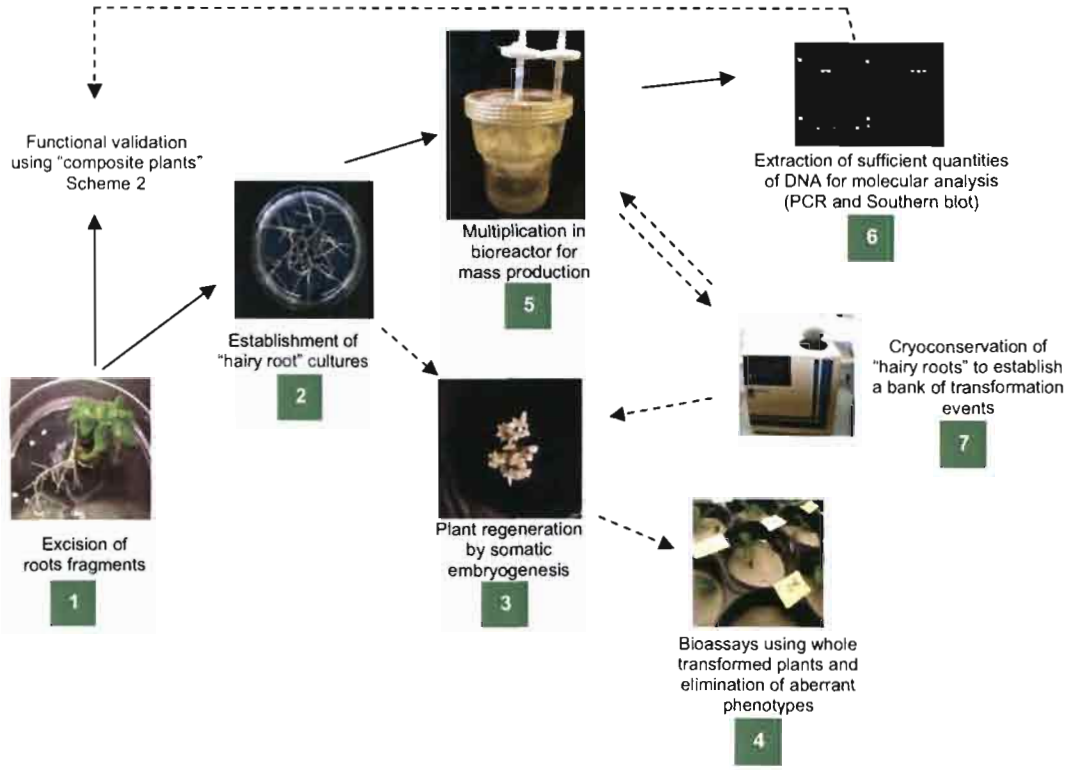
The average number of nematodes extracted from transformed roots of the susceptible "Caturra" variety (3001±2687 nem. g. root^{-1}, F=1.91, *P*=0.61) compared with non-transformed roots (3258±2200 nem. g. root^{-1}, F=1.91, *P*=0.61), was not significantly different according to ANOVA test (Fig. 1). These results prove that *M. exigua* nematodes multiply normally on transformed roots and that their transgenic status does not represent a problem to develop studies on the interaction between coffee/*M. exigua*. A similar trial is under work with the resistant Iapar-59 variety in order to verify on a larger scale that the resistant character is not perturbed. The results above also showed that reproduction levels varied largely between different transformed roots as well as for control plants. Nematode multiplication variability has been reported in *A. rhizogenes* mediated-transformed roots of sugar beet (Cai et al. 2003) and tomato (Plovie et al. 2003) and similarly, in coffee resistance tests to *M. exigua* carried in nursery conditions on normal non-transgenic seedlings under natural tropical conditions of substrate, temperature and humidity (F. Anthony, pers. comm.). We hypothesized three causes that may induce this variability, the first two ones are specific to the bioassay on composite plants: i) the fragility and small size of the plant material induce development delay and losses during the acclimatization to *ex vitro* conditions, ii) problems related to the horticultural management: inconsistencies in substrate composition, limited root biomass, timing and localization of nematode inoculum, iii) a variability related specifically to the living material (i.e. maintenance of nematodes virulence).



Scheme 2. Production and utilization of composite plants for functional validation of resistance genes to root-knot nematode

Based on the results from the experiments carried out during this thesis work, an efficient protocol for nematode bioassay was proposed in the Scheme 2. This protocol allows the evaluation of the resistance conferred by candidate sequences of the *Mex-1* gene seven months after transformation of the embryos. Different soil substrates were evaluated with the aim to find the most suitable to replace the native tropical soil conditions. The previous represented one of the most serious tasks for developing a reliable nematode bioassay in controlled conditions. All the commercially available substrates were too rich in organic matter and presented enormous different physical proprieties compared with the tropical counterpart. Moreover, when the commercial substrates were mixed with regular sand, a progressive compactness was observed limiting the water drainage, enhancing algae proliferation and inhibiting the normal growth of roots. In another bioassay, where the substrate was mixed either with vermiculite or mashed cork, an enormous variability in the nematode multiplication was recorded (in both transgenic and non transgenic control plants), probably due to fact that porosity spaces were too wide in order for nematodes to achieve normal penetration of the root. Recently, a culturing substrate composed of a mixture of pure silica sand and water-absorbent synthetic polymer proposed by Reversat et al. (1999) allowed us to resolve considerably the drawbacks mentioned above. These authors demonstrated that sand particles represent adequate supports that allow nematode penetration.

The use of this alternative substrate led to a considerable reduction of the mortality rate of composite plants (from 50 to less than 20%) as the time of acclimatization (from 12 to 7 months). More than one hundred composite plants of the Caturra variety have been inoculated at the six leaf pair stage in these improved conditions; at this stage the root system from these composite plants exhibits an exponential growing phase and the formation of nematode galls is observed three weeks after the inoculation of 500 nematode individuals per plant. We propose the gall size analysis as a complementary method for screening the transformed plants. This visual evaluation can be done rapidly during nematode extraction and counting analysis and would provide complementary qualitative data to increase the accuracy of the determination of the resistant/susceptible character. According to the experience accumulated from several inoculations in controlled conditions, *M. exigua* maintained in tomato or pepper often exhibited lost of virulence to multiply in either transgenic or non-transgenic coffee roots. The nematode multiplication on those species is often used since is quicker than coffee. Therefore, an important improvement for future bioassays is to maintain the nematode inoculum in susceptible coffee plants at least 4 months before carrying out the experiment.

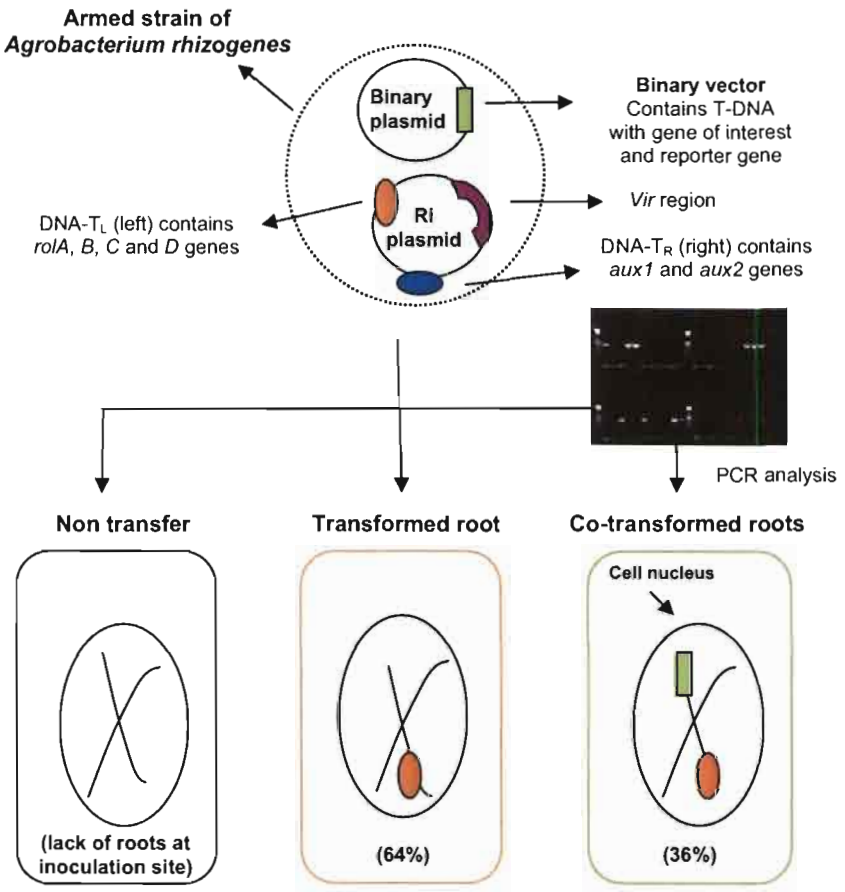


Scheme 3. Strategy of utilization of 'hairy roots' to help the functional validation of resistance genes to nematodes (discussed in Scheme 2). Full arrow: already developed techniques, dotted arrow: still to develop. A excised fragment from each transformed root (1), is transferred and maintained in axenic culture conditions (2), from which can either (3) be regenerated whole transformed plants through somatic embryogenesis procedures, (4) enabling then the detection of any aberrant phenotype at the foliar level which later can be discarded to functional analysis of *Mex-1* gene. From the axenic cultures of transformed root clones it is also possible (5) to induce an intense proliferation of root biomass in bioreactor in order to (6) verify by PCR or Southern analysis the integration in plant cells of the candidate *Mex-1* gene and assess the number of gene copies. This activity allows the molecular study of transformed roots from composite plants thanks to the important quantities of roots that can be obtained with hairy roots. Samples of all transformed clones could also be maintained in cryo-conservation (7) in order to keep a bank of different transformation events of *Mex-1* gene for ulterior complementary studies.

Use of hairy roots morphological analysis to screen for aberrant root phenotypes

As mentioned above, another major technical challenge facing the functional validation of *Mex-1* by *A. rhizogenes*-mediated transformation is the production of transgenic roots exhibiting desirable and stable phenotypes (the most similar as possible to non-transformed roots). The results of the image analysis of morphological parameters confirmed that approximately 90% of coffee roots transformed by *A. rhizogenes* are morphologically similar to non-transformed roots (Chapter 3). For the other 10% showing aberrant phenotype (fast growing or thick diameter) no correlation was found between the phenotypic alterations and the absence/presence of some oncogenes (*rol* and *aux* genes) from the Ri plasmid assessed through PCR analysis (Table 2). All the clones show a similar pattern for the oncogenes and the few differences remarked among clones seem independent of the morphological variability observed. Interestingly, we noted that the T_R-DNA from the Ri plasmid bearing the *aux1* and *aux2* genes was never integrated in coffee cells although the T_L-DNA bearing the *rol* genes are always transferred. The absence of *aux* genes probably explains why the hairy root clones are unable to grow without an exogenous auxin addition in the culture medium (see chapter 3). These genes are involved in the autonomous growth of roots transformed by *A. rhizogenes*. In this perspective, it is planned to quantify the auxin concentration for each root clone. The morphological variability could also be explained by other genes present on the T_L-DNA of the Ri-plasmid. Other *non-rol* genes of the T_L-DNA (i.e. *ORF3n*, *ORF8* and *ORF13*) have been characterized for their capacity to synthesize auxin or to modify the sensitivity to these hormones in different organs of the plant or altering the plant morphogenesis (Lemcke and Schmulling, 1998). This analysis could permit to understand if the morphological variability of transgenic roots is linked to variable hormone levels (auxin, cytokinin, abcsisic acid).

On the other hand, our results demonstrated that the absence of *rolA* gene of T_L-DNA fragment in a few independent hairy root clones does not affect its developmental fate, suggesting that only *rolB* and *rolC* gene integration is indispensable for the regeneration of the coffee transformed root. Similar results were published for other plant species i.e. tobacco (Chriqui et al. 1996) and *Populus* (Nilsson and Olsson, 1997) who found that *rolB* presence was determinant for hairy root induction whereas *rolC* might be involved in HR elongation. Nevertheless, since 10% of the clones showing altered phenotypes could not be explained by presence/absence of particular oncogenes, it may be explained by different expression levels of one or various oncogenes from the T_L region (as observed for the *uidA* gene in Chapter 4) or by an epigenetic control.

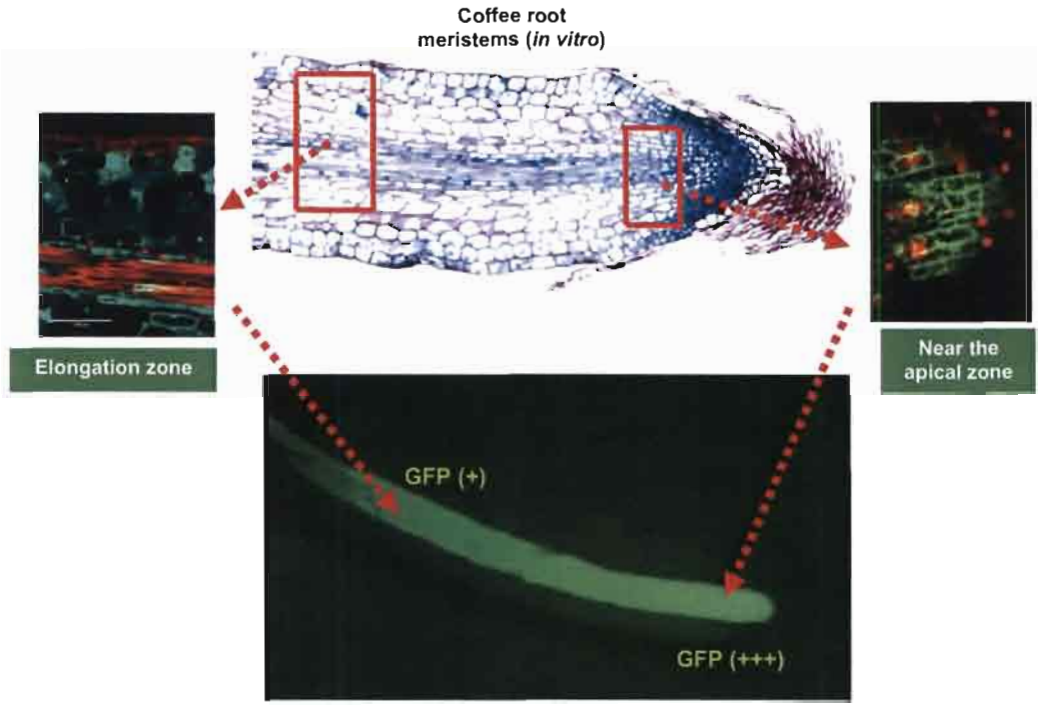


Scheme 4. Possible events of transfer of **Ri** and **binary plasmids T-DNA** from armed *A. rhizogenes* A4RS strain into coffee plant cells. The results from 55 different transformation events, where the *virD* gene from the Ri plasmid was absent, demonstrated that the system allowed a high production of transformed roots [up to 64% of total transformation events] with a satisfactory proportion of co-transformed roots [up to 36% of roots exhibiting positive histochemical expression of GUS gene (in green)]. The PCR analysis revealed that *aux1* and *aux2* genes from the T_R-DNA sub-fragment of the Ri plasmid (in blue) were never inserted in the cell, and that *rolB* and *rolC* genes from the T_L-DNA sub-fragment (in orange) were systematically present in all transformed roots. The *rolA* gene was absent in 15% of the root clones. The frequencies of the different events in the experiment are indicated below the scheme.

Interestingly, no anatomical differences were found through histological annalyses neither between transformed roots exhibiting different morphological phenotypes in *in vitro* and *ex vitro* culture conditions, nor between transformed roots and non-transformed roots. Different research teams working with hairy roots in other plant species like carrot or tomato have reported anatomical differences (Guivarch's et al. 1999; Cho et al. 2000). To our knowledge, the detailed analysis of the integration of Ri T-DNA transferred by *A. rhizogenes* is the first reported for coffee plant. In addition, the strategy developed in Scheme 3 to screen composite plants for aberrant root phenotypes with the aim to discard them before functional analysis of a candidate gene is the first reported for any plant species.

Decontamination of the agrobacteria

One of the main problems using *A. rhizogenes*-mediated transformation is the difficulty to eliminate the agrobacteria after co-cultivation period. It is well known that this difficulty is particularly strong with the A4 strain (Kumar et al. 2006). Conventionally, decreasing concentrations of the bacteriostatic cefotaxime in culture medium is used. However, with this protocol, a significant percentage of transgenic roots still contain the bacteria. The previous poses a significantly problem since infected root clones cannot be used in functional analysis studies because of the impossibility to screen through large scale and reliable molecular analysis (i.e. PCR test) whether the presence of the reporter gene or transgene belongs to the transformed plant or to bacteria. The assessment of the efficiency of the decontamination is conventionally realized through PCR analysis checking for the presence of the *virD* gene from the Ri plasmid of the bacteria in a region outside the T-DNA. By consequence, the hairy roots bearing this gene are supposed to be contaminated by the agrobacteria. At the beginning of our experiments, using cefotaxime treatment, the efficiency of decontamination achieved was 60% (evaluation assessed on 62 root clones). We developed a new decontamination treatment (Scheme 2) using a mix of cefotaxime and PPM® (Plant Cell Technology, WA, USA) which is known to have an affect on wide spectrum of pathogens (bacteria and fungi). The application of this treatment resulted in a significantly increase of the decontamination efficiency; only 11.3% of clones exhibited presence of *virD* gene (Table 2). Recently, Kumar et al. (2006) described a mix of potassium clavulanate, a β-lactamase inhibitor at 100 mg l^{-1} along with cefotaxime in the culture medium. We have planned to test and compare the efficiency of this other "mix" with our protocol in further experiments.



Scheme 5A. Spatial control of GFP expression driven by the 35S promoter in transformed roots of *C. arabica*. Confocal laser scanning microscopy enabled detailed examination of *gfp* expression patterns in developing roots. In general, GFP activity was stronger and homogeneous near the root apical zone, whereas cell type-specific or "mosaic" patterns were systematically observed in the epidermis, cortical parenchyma and central cylinder of the elongation zone.

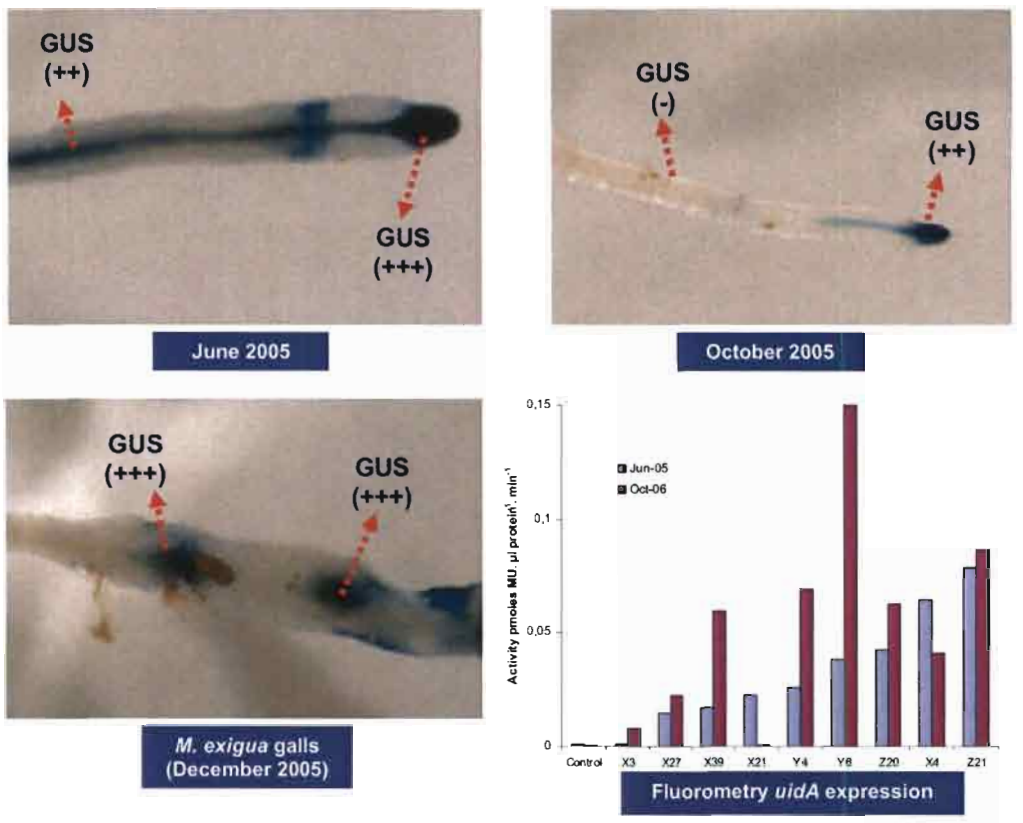
Expression of 35S promoter in coffee roots

For *Mex-1* complementation analysis, the choice of the promoter is highly dependent upon its capacity to drive effective expression of the transgene (putative R gene to *M. exigua*) in roots and particularly in nematode feeding sites. In all the experiments, we used as promoter the CaMV35S since it was previously reported in coffee that it drove efficient expression of transgenes in different plant organs (Spiral et al. 1993; Kumar et al. 2006; Ribas et al. 2006a), although a precise characterization of its expression pattern in roots has never been done in this plant.

In transformed roots of *C. arabica* the analysis of the expression pattern of *uidA* gene over three years and *gfp* gene over 1 year showed that the promoter CaMV35S provides a spatio-temporal regulation of expression for both reporter genes:

- Spatial, because both *uidA* and *gfp* expression was induced preferably and strongly in root meristems and giant cells of the nematode feeding sites compared with differentiated regions of the root system (Scheme 5A, B). Also at the cell level, the expression driven by the 35S promoter is observed essentially in the cytosol.
- Temporal, because in half of the roots harboring 35S-*gfp* construct, after an initial strong fluorescence in the whole root, a substantial decrease was noticed in the following months. However, this reduction is weak in the roots harboring the 35S-*uidA* construct (Table 1, Chapter 4). The re-activation of the 35S-*uidA* construct in feeding sites is another demonstration of this temporal control within clones with low expression levels (see after).

We illustrated in the scheme 5B that in transformed coffee roots for a same root clone (X4) of the susceptible Caturra variety, *uidA* expression driven by 35S promoter varied throughout the time. It evolved from a strong staining in both central cylinder and meristematic region in June 2005 to a staining restricted to the meristematic regions in October 2005. This root clone was inoculated with *M. exigua* in October, and in December of the same year the *uidA* expression has increased again in the nematode feeding sites. Based on the previous results, we hypothesized that although part of the visual expression of the reporter gene is lost within the time; 35S promoter is latent and continuously inducing *de novo* up regulation of the transgene in undifferentiated root sites where a rise of auxin occurs.



Scheme 5B. Spatial and temporal regulation of 35S-*uidA* gene in transformed roots (clone X4) of *C. arabica* var. Caturra. The reporter gene exhibited a decrease of the general expression pattern in October compared with June 2005; however GUS staining was recovered again in December 2005 in the nematode feeding sites in roots that have been inoculated with *M. exigua* three weeks before. Numerous similar observations indicate that a coffee transformed root clone that showed a low frequency of GUS activity (restricted only to root tips) after several months of culture, will be able to exhibit a marked increase of GUS staining in the feeding site after nematode infection.

A literature review indicates that one research team has discarded the use of the 35S promoter for the validation of resistance genes to cyst and root-knot nematodes in potato, because of the down-regulation of 35S-GUS expression observed during the time of exposure with the pathogen (Urwin et al. 2001; Lilley et al. 2004). On the other hand, the GUS activity driven by 35S promoter has been found to be variable in different feeding sites of *Meloidogyne incognita* within a same root system of transformed tobacco (Bertioli et al. 1999). Our results in coffee roots disagree with those of the previous authors since we observed that a strong GUS staining is revealed to be stable since early events of feeding site development and until well after the final moult of female in all galls of different inoculated roots analyzed. We hypothesized that the same will occur with the level of expression of *Mex-1* gene in transformed root of the susceptible variety; however we can not explain the appearance of transformation events where resistance is not expressed although the presence of the resistance gene is confirmed by molecular tools. Such events could be induced by silencing process. Therefore it is important to dispose of a protocol to preserve in axenic conditions all the transformation events in order to study the causes of unexpected low levels of resistance (i.e. insertion sites and elevated copy number of inserted genes, presence of extra-border sequences, morphological alterations and high auxin level). To our knowledge, our results are the first to show the clear activity of the 35S promoter inside the giant cells within the feeding sites initiated by *Meloidogyne* spp. in a tree species and would contribute in the near future to an increase in our knowledge about the pathway and mechanism of resistance of coffee to root-knot nematodes.

Finally, we propose for the near validation of *Mex-1* gene the use of a construct without selection marker gene (antibiotic or herbicide) and with the candidate sequence of *Mex-1* placed near to the right border of the T-DNA driven by the CaMV35S promoter. In addition, we suggest the use of the *gfp* gene as visual selection marker fused to the left of the *R*-gene candidate sequence, so that *gfp* expression guaranties the integration in the plant cell of the *Mex-1* sequence. It should be recalled that as with *A. tumefaciens*, with *A. rhizogenes* the T-DNA transfer is carried according to a polarized process from the right edge towards the left edge. In addition, we recommend the use of the pCambia2300 binary vector instead of pBin19 that was commonly used until now in coffee genetic transformation for two reasons: i) the pBin vectors are reputed to be unstable (frequent recombinations within the T-DNA have been observed; M. Royer, pers. comm.) compared to the pCambia family, and ii) the actual progress and wide availability in the pCambia vectors.

Table 2 Molecular characterization of 35S-*uidA* in transformed coffee hairy roots. Analysis of insertion (presence/absence) of *uidA* reporter gene and T_R (*aux1* and *aux2*) and T_L (*rolA*, *B*, *C*, *D*) sub-fragments of the Ri plasmid determinate by PCR (each clone was analyzed three times)

	PCR test							
Clone	*uidA*	*rolA*	*rolB*	*rolC*	*rolD*	*aux1*	*aux2*	*virD*
Control								
A4C1	+	+	+	+	+	+	+	+
x1	+	+	+	+	+			
x2	+	+	+	+	+			
x3		+	+	+	+			
x4	+	+	+	+	+			
x6	+	+	+	+	+			
x8		+	+	+	+			
x9	+	+	+	+	+			
x11	+	+	+	+	+			
x12		+	+	+	+			
x13		+	+	+	+			
x14		+	+	+	+			
x16		+	+	+				
x17	+	+	+	+	+			
x18		+	+	+	+			
x19		+	+	+	+			
x20	+	+	+	+	+			
x21	+	+	+	+	+	+	+	+
x22		+	+	+				
x23	+	+	+	+	+			
x24		+	+	+	+			
x25	+	+	+	+	+			
x26	+	+	+	+	+			
x27	+	+	+	+	+	+	+	+
x28		+	+	+	+			
x29		+	+	+				
x30		+	+	+	+			
x32	+	+	+	+	+			
x34		+	+	+	+			
x36		+	+	+	+			

Table 2 Molecular characterization of 35S-*uidA* in transformed coffee hairy roots. Analysis of insertion (presence/absence) of *uidA* reporter gene and T_R (*aux1* and *aux2*) and T_L (*rolA*, *B*, *C*, *D*) sub-fragments of the Ri plasmid determinate by PCR (each clone was analyzed three times)

	PCR test							
Clone	*uidA*	*rolA*	*rolB*	*rolC*	*rolD*	*aux1*	*aux2*	*virD*
x37	+	+	+	+				
x38	+	+	+	+				
x39		+		+				
x40		+	+	+				
X41	+	+	+	+	+			
x43	+	+	+	+	+			
x44	+	+	+	+	+			
y3	+		+	+	+			
y4	+		+	+	+	+	+	+
y5	+	+	+	+	+			
y6	+		+	+		+	+	+
y8		+	+	+	+			
y9	+	+	+	+	+			
y11	+	+	+	+	+	+	+	+
y12		+	+	+	+			
z1		+	+	+	+			
z3	+	+	+	+	+			
z5			+	+				
z7	+	+	+	+	+			
z8			+	+				
z14	+	+	+	+	+			
z15		+	+	+	+			
z16		+	+	+	+			
z17		+	+	+	+	+		+
z18	+		+	+	+			
z19			+	+	+			
z20	+	+	+	+		+	+	+
z21	+	+	+	+	+			
z22	+		+	+				

General conclusion

The purpose of this thesis was to develop efficient tools (transgenesis and bio-assays) for the functional analysis of coffee tree resistance genes (R genes), using a transformation procedure based on *Agrobacterium rhizogenes*. This work was part of a project being implemented by a research team seeking to assess biodiversity with a view to optimized exploitation of genetic resources. A clearer understanding of genetic diversity and of the way in which R genes evolve is essential for better conservation and exploitation of genetic resources. The transfer of R genes to cultivars needs to be rational, not only in terms of sustainability, but also controlled, in order to limit undesirable effects – particularly by uncontrolled introgression of *C. canephora* genes, which have a negative impact on beverage quality. By mastering transgenesis – which was the main aim of this thesis – it should be possible to validate the functionality of the *Mex-1* gene identified by our team. That gene of resistance to *Meloidogyne exigua*, a root-knot nematode that is widespread in Latin America, is currently undergoing cloning.

During this thesis work, an attempt was made to fulfil the main objective and the specific objectives that were fixed. In this final section, the main results are recalled and discussed in relation to the issues raised. Prospects are indicated for using the technologies developed, whilst also trying to enhance knowledge of transformation mechanisms.

Attempts have been made to back up the discussion with diagrams and, sometimes, results not published in the thesis are mentioned to support the statements made.

1/ Reminder of the main results and their implications

In the first section, a comprehensive bibliographical review is developed on the cultivation and breeding of coffee and more specifically on genetic transformation. A trait-wise deliberation concerning the principal achievements of donor gene sources, methods employed, selection of transformed tissue, regeneration of whole plants, and the future prospects of transgenesis for functional validation of *Mex-1* are described.

In the second section, it is confirmed that incomplete dominant expression of the *Mex-1* gene exists in heterozygous genotypes and entails an intermediate resistance that reduces the development of nematode juveniles into females, and not necessarily penetration. We showed that such intermediate resistance was stable under high pest pressure under both controlled and field conditions. It was also demonstrated that gall size was significantly smaller on heterozygous *C. arabica* genotypes than on susceptible homozygous genotypes. Those results suggest a possible dosage effect of the *Mex-1* gene.

In the third section, the setting-up of an *Agrobacterium rhizogenes*-mediated protocol is described for transformation of *Coffea arabica* that enables efficient and rapid production of composite plants (transformed roots induced on non-transformed shoots). It was shown that transformed roots under nursery conditions retained the resistance/susceptibility phenotype to *M. exigua* of the varieties from which they were derived.

In the fourth section, a description is given of the culture conditions for efficient proliferation of hairy roots. The similarity of phenotypes between hairy root clones and non-transformed roots was revealed. Using a reliable routine image measurement protocol, altered hairy root phenotypes were efficiently discarded before a functional analysis of root genes.

In the fifth section, it is demonstrated that the *A. rhizogenes*-mediated transformation protocol developed in the third section was reliable with three different binary vectors (pBin19-p35S-*uidA*, pBin-p35s-*gfp*, pCambia2300-p35s-*gfp*) showing high levels of transformation efficacy. By histological analysis it was demonstrated that the anatomy of *A. rhizogenes* transformed roots was similar to non-transformed roots under both *in vitro* and *ex vitro* culture conditions. We showed that glucuronidase activity was heterogeneous and showed variable patterns of expression between different transformed roots with the *uidA* gene. Similarly the epifluorescence expression of *gfp* gene was heterogeneous between different transformed roots. The analysis performed byconfocal microscopy led to the definite conclusion that the pattern of expression of the CaMV35 promoter was mosaic and heterogeneous between the transformed root clones. It was determined that expression was mosaic (expressed only in a few root tissues and cells) and localized almost exclusively in the cytosol of the cell.

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Etude de la résistance de *Coffea arabica* au nématode *Meloidogyne exigua* conférée par le gène *Mex-1* et mise au point des outils pour son analyse fonctionnelle

Résumé En Amérique Latine, les nématodes à galles (*Meloidogyne* sp.) sont des parasites fréquents sur les racines des caféiers (*Coffea arabica*) et provoquent des baisses de rendement importantes. Concernant *Meloidogyne exigua*, la lutte génétique consiste à introgresser dans les variétés cultivées des gènes de résistance (gènes R) provenant de l'espèce *C. canephora*. Le gène nommé *Mex-1*, identifié récemment est en cours de clonage. Cette thèse avait pour objectif de développer des outils (transgénèse et « bio-essais ») pour réaliser la validation fonctionnelle des gènes candidats de résistance à *M. exigua*. Différentes expérimentations en pépinière et en champs ont permis de prouver que l'expression du gène *Mex-1* pouvait être dominant incomplète. Une méthode de transformation basée sur l'utilisation d'*Agrobacterium rhizogenes*, a été mise au point permettant la régénération rapide et efficace de racines transformées sur des hypocotyles d'embryons zygotiques et la production consécutive de plantes composites co-transformées (50-60%) sur lesquelles peuvent être appliqués les tests de résistance au nématode en conditions contrôlées. Les conditions d'entretien à long terme des racines transformées 'Hairy roots' ont été définies. La caractérisation morphologique de 62 clones transformés a permis l'identification de deux phénotypes altérés. L'utilisation des gènes rapporteurs *gus* et *gfp* a permis une sélection visuelle des racines transformées et a également permis de préciser le mode et la stabilité de l'expression du promoteur CaMV35S au niveau racinaire. Les résultats sont discutés pour apprécier l'efficacité des outils développés et la stratégie pour la validation fonctionnelle du gène *Mex-1*. L'exploitation de cet outil est également envisagée pour l'analyse fonctionnelle d'autres gènes de résistance aux nématodes à galles, ou bien de gènes de résistance aux stress abiotiques (sécheresse) ou impliqués dans le développement de la racine.

Mots clés: *Mex-1* gène, analyse fonctionnelle, *Agrobacterium rhizogenes*, hairy roots, *Meloidogyne exigua*, *Coffea arabica*

Study of the resistance of *Coffea arabica* to *Meloidogyne exigua* root-knot nematode conferred by the *Mex-1* gene and development of tools for its functional analysis

Abstract In Latin America, root-knot nematodes (*Meloidogyne* sp.) are frequent in the roots of the coffee-trees (*Coffea arabica*) and cause important falls of outputs. Concerning *M. exigua*, the genetic fight consists in the introgression of resistance genes (*R*-genes) introgressed from *C. canephora* species in the cultivated varieties. The *Mex-1 R*-gene was recently identified and cloning is under work. The objective of this thesis was to develop tools (transgenesis and bioassays) to carry out the functional analysis of the candidate genes of resistance to *Meloidogyne exigua*. We demonstrated through experiments in nursery and field conditions that *Mex-1* could induce incomplete dominant expression. A transformation process based on the use of *Agrobacterium rhizogenes* was developed. This process allows the fast and effective production of transformed roots from the hypocotyls of germinated zygotic embryos and the subsequent production of co-transformed composite plants (50-60%) on which the tests of resistance to the nematode can be carry in controlled conditions. The conditions of long-term maintenance of the transformed roots 'hairy root' were defined. The morphological characterization of 62 transformed clones allowed the identification of two altered phenotypes. The use of the reporter genes *gus* and *gfp* enabled a visual selection of transformed roots and to specify the pattern and the stability of the expression of the CaMV 35S promoter at the root level. The results are discussed to appreciate the effectiveness of the developed tools. The strategy for the functional validation of the *Mex-1* gene is discussed. The exploitation of this genetic transformation process is also considered for the functional validation of other resistance genes to nematodes, or resistance genes to abiotic stresses (drought) or involved in root development.

Key words: *Mex-1* gene, functional analysis, *Agrobacterium rhizogenes*, hairy roots, *Meloidogyne exigua*, *Coffea arabica*