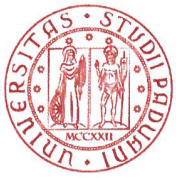

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SCUOLA DI DOTTORATO DI RICERCA IN: BIOSCIENZE E BIOTECNOLOGIE
INDIRIZZO: BIOTECNOLOGIE
CICLO XXV

Agrobacterium-mediated transformation of *Jatropha curcas* to overexpress a gene involved in the triglyceride biosynthetic pathway

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Index

Abstract

Negative environmental consequences of fossil fuels and concerns about petroleum supplies have spurred the search for renewable transportation biofuels. To be a valuable alternative, a biofuel should provide a net energy gain, have environmental benefits, be economically competitive, and be producible in large quantities without reducing food supplies.

Biodiesel is the most used alternative biofuel nowadays, along with bioethanol, in the world. Parent oil used in making biodiesel consists of triglycerides in which three fatty acid molecules are esterified with a molecule of glycerol. The trans-esterification produces methyl esters of fatty acids, which are biodiesel, and glycerol. The increasing demand of plant species producing oil rich seeds (i.e. *Brassica napus, Glycine max* and *Helianthus annuus*) is leading to the reduction of agricultural areas assigned to crops grown for human nutrition, and it is causing the increase in food prices.

The aim of the proposed project (in collaboration with Geneticlab S.r.l.) is to design reliable protocols to produce biomass and to induce lipid accumulation in cell cultures obtained from a plant species known as high oleic: *Jatropha curcas*. This species has attracted the interest of various developmental agencies in the tropics and subtropics due to its easy adaptability to semi-arid marginal sites, use of the oil as a diesel fuel substitute and its use in erosion control. *J. curcas* is monoecious, with male and female flowers on the same plant, and its center of origin are Mexico and Central America. In its natural area of distribution, the species is most abundant in tropical savanna and monsoon climates and in temperate climates without a dry season and with a hot summer.

The experimental approach followed two different directions: a) the establishment of protocols able to stimulate cells proliferation from leaf and seed explants; b) the search of easy and fast protocols useful to clonally propagate this species. Cell proliferation was obtained, on solid media and then in liquid media, in many different conditions of salt and phytohormone combinations. A suspension cell culture was characterized, and the biomass grew of seven fold in about three weeks, before entering

stationary/death phase.

On the other hand, *in vivo* propagation through cuttings was carried out from plants of different age and origin. The hardwood part of the plants is the most efficient in rooting and outliving. Organogenesis via a callus-mediated step and plant regeneration were achieved *in vitro* and acclimatization to natural environment was performed.

Agrobacterium-mediated transformation was planned in order to overexpress an enzyme involved in the plant triglycerides biosynthesis pathway, because it could be a potential application for the production of plant cell cultures accumulating triglycerides. The *Arabidopsis thaliana* gene coding for the diacylglycerol acyltransferase enzyme (DGAT) has been cloned into the delivery plasmid pGreen0029 (Kan^R), under the control of the constitutive CaMV 35S promoter and three plasmids with different constructs have been obtained: pG29Y, harbouring the gene coding for the YFP; pG29HD, harbouring the gene coding for the DGAT with the HA flag at the 5'-terminal; pG29HDY, harbouring the fusion product of the two coding sequences considered before.

Cell cultures transformed with pG29Y and pG29HDY allowed us to observe a different localized fluorescent signal, which could be explained with the expected insertion of DGAT in the ER membrane.

A quantification of storage lipid content was set up. This colorimetric assay was performed on seeds and the cultured cell lines in order to quantify the intracellular triglycerides content. The lipid test will be performed on transformed Jatropha calli and regenerated plants to evaluate the result on lipid accumulation in tissues other than seeds.

Some obtained results from this 3-years project have been applied with good results by Geneticlab company in the scale-up of cell cultures from laboratory to industrial level.

Abstract

Le conseguenze negative derivate dall'uso dei combustibili fossili e la preoccupazione sulla durata delle riserve petrolifere hanno stimolato la ricerca di fonti di energia rinnovabili. Un biocarburante, per essere considerato una valida alternativa, deve essere poco inquinante, economicamente competitivo e deve poter essere prodotto in grandi quantità senza intaccare le risorse alimentari.

Il biodiesel è uno dei biocarburanti più utilizzati al mondo e l'olio da cui deriva è costituito di trigliceridi (TAG), ovvero molecole di glicerolo esterificate con tre acidi grassi. La trans- esterificazione con metanolo di questi trigliceridi produce metil esteri di acidi grassi, che costituiscono il biodiesel vero e proprio, e glicerolo. La crescente domanda di specie in grado di produrre semi oleaginosi (come *Brassica napus*, G*lycine max* e *Helianthus annuus*) sta causando la riduzione delle aree agricole destinate alla coltivazione di colture edibili, causando l'aumento dei prezzi degli alimenti.

L'obiettivo del progetto, in collaborazione con Geneticlab S.r.l., è l'ottenimento di protocolli in grado di indurre la produzione di biomassa e l'accumulo di lipidi in colture cellulari derivate da espianti di *Jatropha curcas*. Questa specie si adatta facilmente a climi semi aridi, è in grado di crescere su suoli poveri e marginali, è molto utile per combattere l'erosione e accumula molti trigliceridi nei semi. *J. curcas* è monoica, con fiori maschili e femminili sulla stessa infiorescenza; è una pianta originaria del Mexico o comunque del Centro America. Questa specie viene coltivata nelle zone tropicali e sub-tropicali e nei climi temperati caratterizzati da un'estate calda e l'assenza di una stagione secca.

L'approccio sperimentale ha seguito due diverse direzioni: a) la messa a punto di protocolli in grado di stimolare la proliferazione cellulare a partire da diversi tipi di espianto; b) la ricerca di protocolli veloci e ripetibili utili alla propagazione della specie. La proliferazione cellulare è stata ottenuta, sia su mezzo solido che liquido con diverse concentrazioni di sali e di fitoregolatori. Le colture cellulari in sospensione sono state caratterizzate

dimostrando che la biomassa ottenuta può aumentare fino a più di 7 volte, rispetto all'inoculo iniziale, in 3 settimane .

La propagazione per talea è stata eseguita a partire da esemplari di diversa età e origine e la parte legnosa della pianta si è dimostrata essere la più efficiente nella radicazione. L'organogenesi da callo e la rigenerazione dell'intero individuo sono state ottenute *in vitro*; le piante così rigenerate riescono ad acclimatarsi all'ambiente naturale.

La trasformazione mediata da *Agrobacterium tumefaciens* è stata programmata per sovraesprimere un enzima coinvolto nella via biosintetica dei trigliceridi. Questa strategia potrebbe permettere l'ottenimento di colture cellulari in grado di accumulare trigliceridi in grandi quantità. Il gene codificante la diacilglicerolo aciltransferasi (DGAT) di *A. thaliana* è stato clonato nel vettore pG0029 sotto il controllo del promotore costitutivo 35S e, in particolare, sono stati preparati tre diversi vettori plasmidici: nel plasmide pG29Y è inserita la sequenza codificante per la proteina fluorescente YFP; nel plasmide pG29HD quella codificante per l'enzima DGAT e nel plasmide pG29HDY quella codificante per il prodotto di fusione DGAT::YFP.

Colture cellulari trasformate con i plasmidi pG29Y e pG29HDY hanno permesso di osservare un segnale di fluorescenza con una diversa localizzazione sub-cellulare per i due costrutti; è infatti noto da dati presenti in letteratura che l'enzima DGAT svolge la sua funzione nella membrana del reticolo endoplasmatico.

È stato inoltre ottimizzato un saggio utile alla quantificazione dei trigliceridi intracellulari che ha permesso di valutare l'accumulo di TAG nei semi e nelle colture cellulari di *J. curcas*. Questo saggio verrà eseguito sulle colture cellulari e sui diversi tessuti delle piante trasformati.

Alcuni dei risultati ottenuti durante i 3 anni di questo progetto sono stati applicati con buoni risultati dalla ditta Geneticlab S.r.l., permettendo il trasferimento di tecnologia dalla scala di laboratorio a quella industriale.

1 Introduction

1.1 Biofuels

High energy prices, concerns about petroleum supplies, and a greater recognition of the environmental consequences of fossil fuels increased the interest in biofuels, especially for transportations. To understand if alternative fuels provide benefits over the fossil fuels requires thorough accounting of the direct and indirect inputs and outputs for their full production and use life cycles. To be a valuable substitute, an alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, but should be economically competitive with it and producible in sufficient quantities to have an important impact on energy demands; it should also provide a net energy gain over the energy sources used to produce it. Moreover, it should be obtained in large quantities without reducing food supplies.

Among current food-based biofuels, soybean biodiesel has major advantages over corn grain ethanol. Biodiesel provides 93% more usable energy than the fossil energy needed for its production, reduces greenhouse gases (GHG) by 41% compared with diesel, reduces several major air pollutants, and has minimal impact on human and environmental health through N, P, and pesticide release. Corn grain ethanol provides smaller benefits through a 25% net energy gain and a 12% reduction in GHGs, and it has greater environmental and human health impacts because of increased release of air pollutants and nitrate, nitrite, and pesticides (Hill *et al*., 2006). Biodiesel can be mixed with petroleum diesel and directly used in engines with no need of modifications. Compare with petroleum diesel, biodiesel has less carbon monoxide emission (about 50%), reduces hydrocarbon, aldehydes, fume, and suspension particle by about 95%, 30%, 80%, and 30%, respectively. Biodiesel also gives no sulfide emission (Kasim *et al*., 2009).

Parent oil used in making biodiesel consists of triglycerides in which three fatty acid molecules are esterified with a molecule of glycerol. In making biodiesel, triglycerides react with methanol in a reaction known as trans-

esterification or alcoholysis (Figure 1-1). Trans-esterification produces methyl esters of fatty acids, that are biodiesel, and glycerol. The reaction occurs stepwise: triglycerides are first converted to diglycerides, then to monoglycerides and finally to glycerol. Trans-esterification requires 3 moles of alcohol for each mole of triglyceride to produce 1 mole of glycerol and 3 moles of methyl esters. Industrial processes use 6 moles of methanol to be sure that the reaction is driven in the direction of methyl esters. Trans-esterification is catalyzed by alkalis, acids and lipase enzymes. Alkali-catalyzed trans-esterification is about 4000 times faster than the acid catalyzed reaction. Consequently, alkalis such as sodium and potassium hydroxide are commonly used as commercial catalysts at a concentration of about 1% by weight of oil. Use of lipases offers important advantages, but is not currently applied because of the relatively high cost of the catalyst. Alkali-catalyzed trans-esterification is carried out at approximately 60 °C under atmospheric pressure, as methanol boils off at 65 °C at atmospheric pressure. Under these conditions, reaction takes about 90 minutes to complete. A higher temperature can be used in combination with higher pressure, but this is quite expensive. Methanol and oil do not mix, hence the reaction mixture contains two liquid phases. Other alcohols can be used, but methanol is the least expensive. Biodiesel is recovered by repeated washing with water to remove glycerol and methanol (Chisti, 2007).

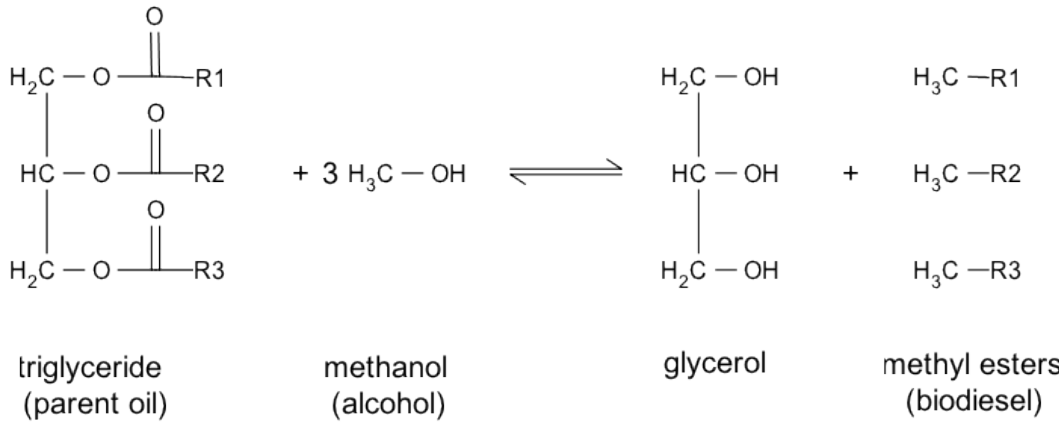

Figure 1-1. Trans-esterification of oil to biodiesel. $R_{1\text{-}3}$ are hydrocarbon groups.

Seeds, rich in oil, of several plant species are the main source of plant triglycerides used for the biodiesel production.

1.2 Oil plants

In many plants, TAGs are the major energy storage compartment, that constitute a highly reduced form of carbon and serve as an important energy reserve in seeds for subsequent germination and seedling development. These storage lipids are of great nutritional and nutraceutical value, and thus a common source of edible oils for human consumption. They are also becoming increasingly important raw materials in the petrochemical industry for the production of paints, detergents, lubricants, biofuels, and nylon precursors; moreover, it is an undisputable fact that non-renewable crude oil reserves are rapidly being exhausted. Because of our heavy reliance on plant oils in the food and petrochemical industries, increasing the seed oil content and generating desirable fatty acids (FAs) in oilseed crops are considered aims of tremendous socioeconomic value (Lung and Weselake, 2006).

TAGs are present in small subcellular spherical oil bodies (OBs) of approximately 1 μm in diameter (Figure 1-2). Each OB has a matrix of TAGs wrapped by a layer of phospholipids (PLs) and structural proteins called oleosins. The small size of OBs provides a large surface area per unit of TAG, which would facilitate lipase binding and lipolysis during germination. OBs in mature seeds or in isolated preparations are considerably stable and do not aggregate or fuse. This stability is in contrast to the instability of artificial liposomes made from amphipathic and neutral lipids; the liposomes gradually coalesce after formation. The stability of seed OBs is caused by the fact that surface is shielded by a layer of oleosins. In maturing seeds, TAGs, phospholipids (PLs), and oleosins are synthesized in the endoplasmic reticulum (ER), from which budding OBs are released. Oleosins in seeds are small proteins of about 15 to 26 kD. An oleosin molecule can be divided into three portions according to its amino acid sequence. The N-terminal portion can be short or long and is hydrophilic. The central portion is a long hydrophobic stretch of 72 residues. The C-terminal portion can be short or very long and its

approximately 30 residues adjacent to the central hydrophobic stretch; can form an amphipathic α-helical structure that interacts horizontally with the charged phosphate and choline groups of the PL layer on the OB surface. They completely cover the surface of the subcellular OB. They can be abundant in seeds with a high proportion of oils and small OBs (therefore more OB surface area). The ratio of oils to oleosins determines the size and even the shape of the OBs (Hsieh and Huang, 2004).

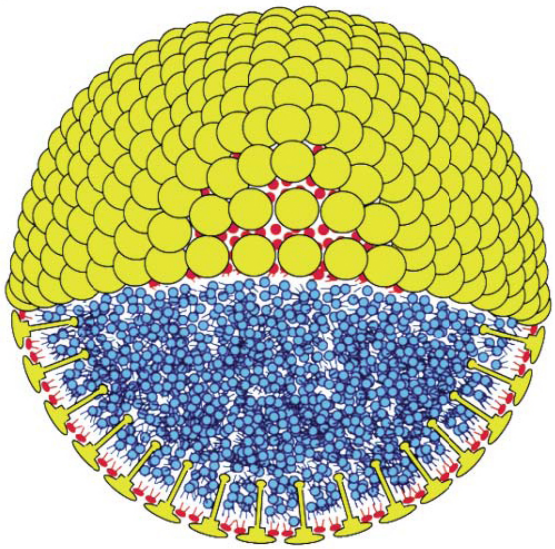

Figure 1-2. Representation of an oil body. Oleosin are indicated in yellow, phospholipid layer in red and triacylglycerol in blue (Hsieh and Huang, 2004).

TAGs accumulate during seed maturation and are then stored in the seed until germination, after which it is used to support seedling growth. During seed maturation TAGs are synthesized within the endoplasmic reticulum (ER) and are packaged into immature oil bodies, which bud off from the ER to form the mature organelle. Depending on the plant species, storage protein and carbohydrate reserves (most frequently in the form of starch) also accumulate. These storage reserves are typically insoluble compounds that can remain intact in dry seeds for extended periods. Upon seed germination these reserves need to be quickly converted to soluble metabolites that can be transported throughout the seedling and used to support growth, which enables photoautotrophism to be achieved before reserves are exhausted. The mobilization of storage oil involves the coordinated induction of a number of biochemical pathways in different

subcellular locations. The first step in oil breakdown is catalyzed by lipases, which hydrolyze TAGs to free fatty acids (FAs) and glycerol. The FAs then enter single membrane–bound organelles termed glyoxysomes where β-oxidation and part of the glyoxylate cycle occur. Glyoxysomes are structurally similar but metabolically distinct from the more ubiquitous peroxisomes; glyoxysomes contain two enzymes that are unique to the glyoxylate cycle, malate synthase (MLS) and isocitrate lyase (ICL). β-oxidation converts FAs to acetyl-CoA, which is subsequently condensed into 4-carbon compounds via the glyoxylate cycle. These 4-carbon compounds are then transported to the mitochondria, where they can be converted to malate and transported to the cytosol for gluconeogenesis, or used as substrates for respiration (Graham, 2008).

Plant oils are becoming one of the promising renewable feedstocks for biodiesel production and have become more attractive recently because of their environmental benefits. Due to being renewable, with energy content similar to diesel fuel, usable after undergoing some chemical modifications, vegetable oils are becoming a promising alternative as a substitute for diesel fuel. The advantages of plant oils are renewability, liquid nature-portability, higher heat content (about 88% of diesel fuel), biodegradability, and lower sulphur and aromatic content. Edible oils from canola, soybean, rapeseed, sunflower and corn have been used for biodiesel production and have proved to be good as a diesel substitute. The non-edible oils from *Madhuca indica*, *Jatropha curcas* and *Pongamia pinnata* have also found to be suitable. More than 95% of biodiesel production feedstock comes from edible oils in developed countries because the properties of biodiesel produced from these oils are very similar to petroleum-based diesel. In view of their several advantages, plant oils have a great potential to replace petroleum-based fuels in the long run (Koh and Ghazi, 2011).

Oils and fats are the most important renewable raw materials of the chemical industry. They make available fatty acids in such purity that they may be used for chemical conversions and for the synthesis of chemically pure compounds. Their interesting industrial applications are the usage as

environmentally friendly fluids and lubricants, insulating fluid for electric utilities such as transformers and additive to asphalt. Modern methods of synthetic organic chemistry including enzymatic and microbial transformations were applied extensively to fatty compounds for the selective functionalization of the alkyl chain (Metzger and Bornscheuer, 2006). The possibility to use plant oils in many different fields has given a relevant importance to this sector; for this reason both production and price of oil plants have dramatically increased in the last 10 years (see Table 1-1).

	2000-2001			2010-2011		
	Seeds	Oil	Meal	Seeds	Oil	Meal
Soya	175.7	26.7	116.1	264.2	41.2	174.4
Cotton	33.5	3.5	11.3	43.5	5.0	14.9
Peanut	31.1	4.3	5.5	35.4	5.1	6.2
Sunflower	23.1	8.2	9.3	32.7	12.2	13.0
Rape (Canola)	37.3	13.4	21.2	60.4	23.3	34.8
Palmkernel	7.0	3.0	3.6	12.6	5.7	6.7
Copra/coconut	5.8	3.6	1.9	5.9	3.7	1.9
Olive		2.5			3.0	
Palm		24.3			47.9	
Fish			5.7			4.7
Total production	313.9	89.8	174.7	454.7	147.2	256.7
Total exports	66.8	30.8	48.5	107.9	60.3	77.1

Table 1-1. Production of oilseeds, oils and fats, and oil meals in 2000/2001 and 2010/2011. Data from USDA expressed in million tonnes (mt).

Seed and meal markets are dominated by soybeans. Soya beans, grown mainly in North and South America, are the most important source of protein meal used mainly for animal feed and the second largest source of oil after palm oil. In 2010/11, these two oils represented 61% of the nine major vegetable oils detailed by USDA (United States Department of Agriculture) compared with 57% ten years ago.

In 2010/2011 four out of nine oils covered the 84.7% of the total market (palm, soy, rape and sunflower) with 124.6 million tonnes produced. Three minor oils (cotton, peanut and olive) covered the 8.9% of the market with a

production of 13.1 million tonnes while the two lauric oils (palm kernel and coconut), distinguished from other oils by their characteristic fatty acid composition, covered the 6.4% of the market with a production of 9.4 million tonnes. The production is represented by about 24% of seeds, 41% of oils, and 30% of oil meals. Over the ten years between 2000/10 and 2010/11, it is apparent that total production of plant oils increased of about the 64% (Gunstone, 2012).

The high prices for vegetable oils recently attracted a good deal of media attention. From the 1950s until the 2007/08 surge in prices, the real cost of most oils and oilseeds declined by about 3% per year, corresponding to a halving of price each 20-25 years.

	Rapeseed	palm	sunflower	soyabean
2000/01	372	235	428	336
2001/02	451	329	587	412
2002/03	588	421	592	534
2003/04	670	481	663	633
2004/05	660	392	703	545
2005/06	770	416	635	573
2006/07	852	655	846	771
2007/08	1410	1058	1639	1327
2008/09	868	633	837	826
2009/10	927	793	956	924
2010/11	1367	1154	1404	1306

Table 1-2. Annual average vegetable oil prices (US$/tonne) between 2000/01 and 2010/11 (USDA February 2012).

It is apparent from Table 1-2 that, in the recent past, prices have fluctuated markedly, sometimes falling and sometimes rising quite sharply. Price changes result from a mismatch of supply and demand influenced by many factors as: growing demand from a rising population with increasing wealth, which is increasingly urbanized; there are also other sources that are outside the usual listings of commodity oils such as used frying oils, oils from new vegetable sources such as jatropha and algal oil; there is an increase in the cost of agricultural production and in storage and transport costs resulting from the severe rise in the price of oil; oilseed yields have

fallen through poor climatic conditions in many parts of the world; whenever prices are changing rapidly speculators interfere in the market and make profits for themselves (Gunstone, 2012).

It is almost clear that plant oil market has rapidly increased in the last years, and is expected to grow more. For this reason there is the need of new alternatives, economically more advantageous. In parallel to oils derived from soy, rape, sunflower and palm it is emerging the production of oils from non-edible species as *Madhuca indica*, *Pongamia pinnata* and *Jatropha curcas*; thanks to their high content in unsaturated fats, more than 70% (see Table 1-3), and to the lack of competition with the market and the land use of edible species they are very interesting species (Koh and Ghazi, 2011).

Fatty acid	jatropha	pongamia	sunflower	soybean	palm
Lauric (C_{12}/0)	-	-	0.5	-	-
Myristic (C_{14}/0)	-	-	0.2	0.1	-
Palmitic (C_{16}/0)	14.2	9.8	4.8	11.0	40.3
Palmitoleic (C_{16}/1)	1.4	-	0.8	0.1	-
Stearic (C_{18}/0)	6.9	6.2	5.7	4.0	3.1
Oleic (C_{18}/1)	43.1	72.2	20.6	23.4	43.4
Linoleic (C_{18}/2)	34.4	11.8	66.2	53.2	13.2
Linolenic (C_{18}/3)	-	-	0.8	7.8	-
Arachidic (C_{20}/0)	-	-	0.4	0.3	-
Behenic (C_{22}/0)	-	-	-	0.1	-
Saturates (%)	21.1	16.0	11.6	15.5	43.4
Unsaturates (%)	78.9	84.0	88.4	84.5	56.6

Table 1-3. Fatty acid composition for different vegetable oils (Koh and Ghazi, 2011).

The possibility of studying and take advantages from these plants, applying molecular and genetic engineering technologies, will be fundamental to define their productivity and economic success. The main objectives are to power the seed oil content and to improve the quality of the fatty acid content, especially in poorly known species as *J. curcas*. Moreover, it is very important to avoid, as much as possible, the competition for agricultural areas between edible and non-edible species.

1.3 *Jatropha curcas* L.

Jatropha curcas (Linnaeus), also known as physic nut, belongs to the E*uphorbiaceae* family and it is a species belonging the genus *Jatropha*, that contains about 175 known species. The botanist Carl Von Linnaeus was the first to name the physic nut *J. curcas*. The genus name Jatropha derives from the greek words *jat´ros* (doctor) and *troph´e* (food), which reveals its medicinal uses (Divakara *et al.*, 2010).

Many scientists have attempted to define the origin of physic nut, but the source remains controversial. Martin and Mayeux (1984) identified the Ceara state in Brazil as a centre of origin but without giving any arguments. According to other sources, jatropha seems to be native to Central America as well as to Mexico where it occurs naturally in the forests of coastal regions. From the Caribbean, this species was probably distributed by Portuguese seafarers via the Cape Verde Islands and former Portuguese Guinea (now Guinea Bissau) to other countries in Africa and Asia (Heller, 1996). Today it is cultivated in many countries as shown in Figure 1-3.

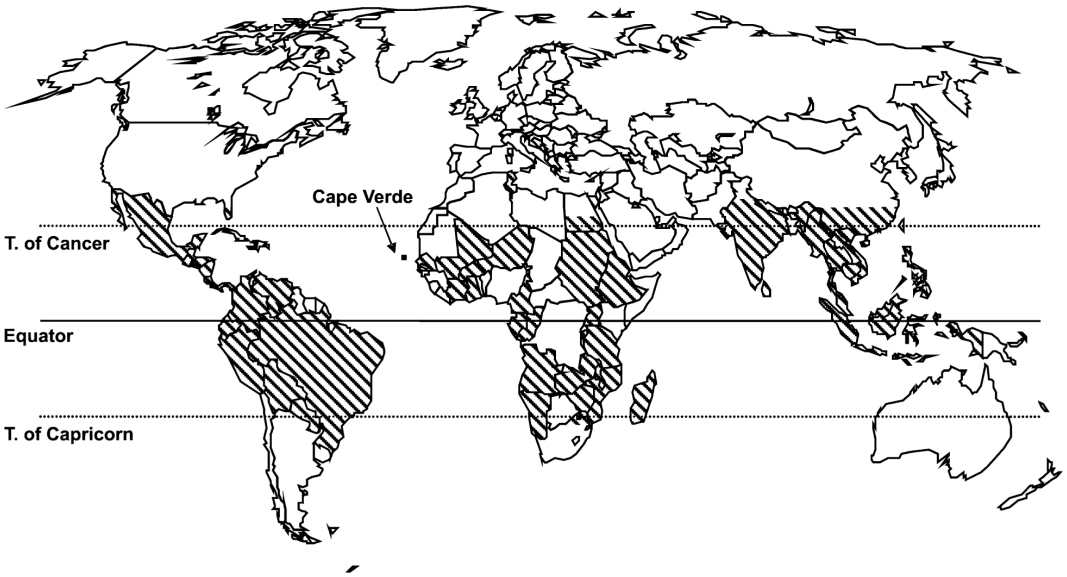

Figure 1-3. Approximate global distribution of *J. curcas*. Shaded regions indicate areas in which *J. curcas* is found (King et al., 2009).

Jatropha grows in tropical and sub tropical regions, with cultivation limits at 30°N and 35°S. It also grows in lower altitudes of 0-500 metres above sea

level. Jatropha is not sensitive to day length (flowering is independent of latitude) and may flower at any time of the year. It is a succulent shrub that loses its leaves during the dry season, with deep roots that make it well suited to semi-arid soils. While jatropha can survive with as little as 250 to 300 mm of annual rainfall, at least 600 mm are needed to flower and set fruit. The optimum rainfall for seed production is considered between 1000 and 1500 mm, which corresponds to subhumid ecologies. While jatropha is able to grow with 3000 mm of rainfall, higher precipitation is likely to cause fungal attack and restrict root growth in all but the most free-draining soils. *Jatropha curcas* is not found in the more humid parts of its area of origin, Central America and Mexico. Rainfall induces flowering and, in areas of unimodal rainfall, flowering is continuous throughout most of the year. Optimum temperatures are between 20°C and 28°C, average minimum temperatures above 8–9°C, indicating a lack of tolerance to frost, and average maximum temperatures between 35°C and 45°C. Very high temperatures can depress yields. The plant is well adapted to conditions of high light intensity and is unsuited to growing in shade (Brittaine and Lutaladio, 2010; Contran *et al.*, 2013).

Its high ecological adaptability allows *J. curcas* to grow in a wide range of climatic conditions from semiarid to humid. Anyway, it does not naturally occur in regions with annual rainfall less than 950 mm and more humid environmental conditions result in a higher productivity.

J. curcas is able to grow in a wide range of soil types, ranging from alluvial soil to red lateritic soil, even on gravelly, sandy, and saline soils. Neutral (pH 6.0–8.0), well-drained, and aerated soils are preferred, whereas soils with risk of ephemeral water logging, such as Vertisols or other heavy clay soils, are not suitable.

J. curcas strength as a crop results from its ability to be well adapted to poor quality soils. However, soil properties have an important effect on *J. curcas* productivity, and a poor nutrient level leads to an increase of seed development failure (Contran *et al.*, 2013).

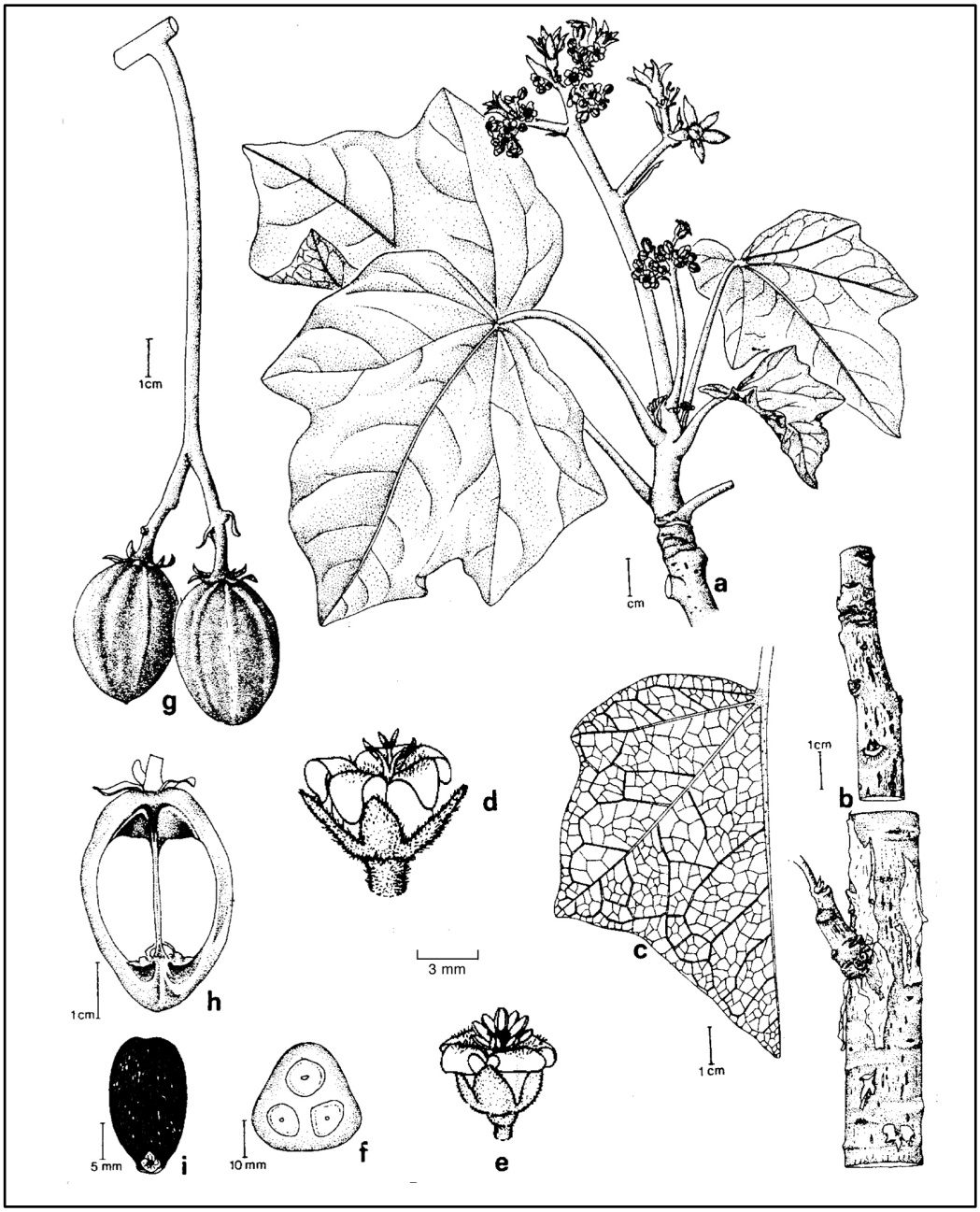

Figure 1-4. Important parts of the plant *J. curcas*. a: flowering branch; b: bark; c: leaf veinature; d: pistillate flower; e: staminate flower; f: cross-cut of immature fruit; g: fruits; h: longitudinal cut of fruits; i: mature seed (Heller, 1996).

J. curcas is a perennial large shrub or small tree, with a life expectancy of up to 50 years (Heller, 1996). The plant can reach a height of 3 m, but under favorable conditions it can grow even up to 5–6 m. *J. curcas* shows articulated growth, with a morphological discontinuity at each internode, and its dormancy is caused by rainfall and temperature fluctuations (Heller, 1996). The plant presents terminal and axillary small buds and

branches from the ground. Stem is straight, with thin pale-brown bark and numerous scars due to the fallen leaves (Figure 1-4b). Branches are glabrous, stout, and contain latex (Heller, 1996). *J. curcas* leaves (Figure 1-4c) are green to pale-green, 5–7 lobed, smooth, alternate to sub-opposite with a spiral phyllotaxis, and hypoamphistomatic, with length and width of 6–15 cm, petiole of 5–20 cm long, and paracytic stomata. In climates characterized by a dry season, it is a deciduous plant and sheds its leaves during the dry season. *J. curcas* develops a deep taproot and four main secondary order roots. These four roots, symmetrically distributed in the horizontal plane, originate at the same depth along the main root and have an inclination of −45°. Adult trees present four clearly dominant secondary order roots, with an inclination of −20° and −40°, and develop a more branched root system, with a dense net of finer horizontal lateral root in the topsoil.

J. curcas, a diploid species with 2n = 22 chromosomes, is monoecious with unisexual male (Figure 1-4e) and female (Figure 1-4d) flowers on the same inflorescence. Inflorescences, formed terminally on specific branches (Figure 1-4a), are dichasial cymes and possess main and co-florescences with paracladi. They contain unisexual, occasionally hermaphroditic, greenish yellowed flowers. Normally, the inflorescences produce a terminal individual female flower surrounded by a group of male flowers (Figure 1-5B). Numerically, 1–5 female flowers and 25–93 male flowers are produced per inflorescence. Male flowers have 10 stamens arranged in 2 distinct whorls in close proximity to each other in the androecium. Female flowers, syncarpous with trilocular ovary, have three slender styles, connate to about two-thirds of their length, and bifurcate stigmas each.

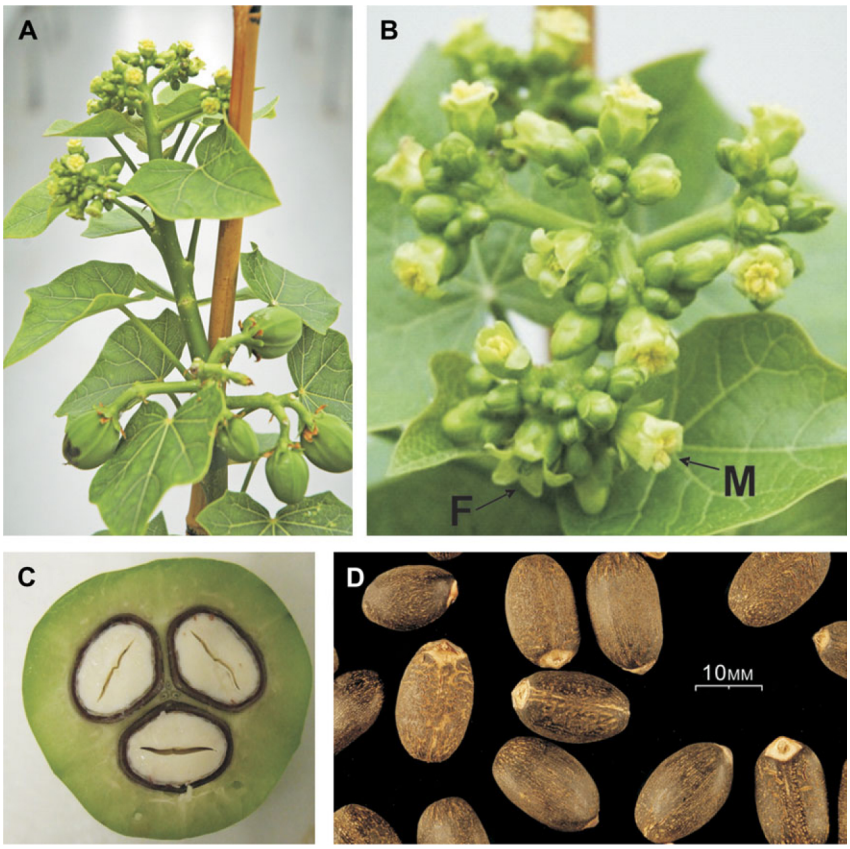

Figure 1-5. Images of *J. curcas*. A: young *J. curcas* plant with both flowers and developing seed pods; B: *J. curcas* inflorescence containing both male staminate flowers (M) and female pistillate flowers (F); C: cross-section of a *J. curcas* seed pod containing three developing seeds; D: mature seeds of *J. curcas* (King *et al*., 2009).

J. curcas male flowers start to open from the first or second day of the inflorescence life for a period of 8–10 days, with pollen viability of 2 days, while the 60% of total female flowers open between the third and the fifth day, with a life span of about 4 days. The flowering season and the number of flowering events, as well as the male and female flower ratio, depend from: temperature, available soil moisture, and soil fertility. Usually, flowering occurs during the wet season but *J. curcas* can flowers throughout the year (Achten *et al*., 2010). The flowers are mainly pollinated by insects, especially by honey bees (Divakara et al., 2010).

Fruits (Figure 1-4g, Figure 1-5A) are green-brown ovoid capsules, 2.0–3.5 g weight, 4 cm long, 3 cm diameter, and generally tri-halved (Figure 1-4f, Figure 1-5C), each comprised of one seed (Heller, 1996). The pericarp remains fleshy, indehiscent, and green until the seeds are mature. After

2/3 months from fruit formation, when fruits are developed and have reached maturity, the exocarp dries, forming the husk (sometimes named fruit coat or fruit shell) of the fruit, and its color changes from green to yellow, brown, and finally black. The husk partially opens, but seeds do not fall out. In a mature fruit, husk is the 30–40% of the total fruit weight and seeds are the remaining 60–70%. Seeds, 2–3 per capsule, are black, ellipsoid, triangular-convex, 0.5–0.7 g weight, about 2 cm long and 1 cm thick (Figure 1-4 i, Figure 1-5 D). The 30–40% of the total seed weight consists of an external brown-black shell (sometimes named hull or seed husk), whereas the remaining 60–70% consists of kernel, the white oil-containing compact nucleolus of seed (Table 1-4).

	Reported ranges
Average seed mass	450-860 mg
Testa (shell) (%)	30-40%
Kernel (%)	60-70%
Average oil content	
Whole seed	39-37%
Kernel	44-62%
Protein content	
Kernel	22-35%
Seed meal after oil extraction	48-64%

Table 1-4. Range of average seed mass, oil content, and protein content reported for *J. curcas* seeds (King *et al.*, 2009).

J. curcas seed contains about 30–35% of oil per dry mass, stored at 99% in the kernel.

The protein composition of *J. curcas* seed meal has been analyzed, and it has been shown to compare favourably with soybean, containing a good balance of essential amino acids, with the exception of lysine. The seeds of most tested varieties of *J. curcas* are inedible, and heat-inactivation treatments used in seed-meal processing are useless (Heller, 1996). As a consequence, the protein rich seed meal of *J. curcas* is not used as animal feed. In fact, the seeds of *J. curcas* contain also a wide range of constituents toxic to humans and animals. Phorbol esters (Figure 1-6)

have been identified as the main agent responsible for *J. curcas* toxicity. Phorbol esters are tetracyclic diterpenoid able to interact with protein kinase C affecting the activities of several enzymes, protein biosynthesis, DNA, polyamines, cell differentiation processes, and gene expression.

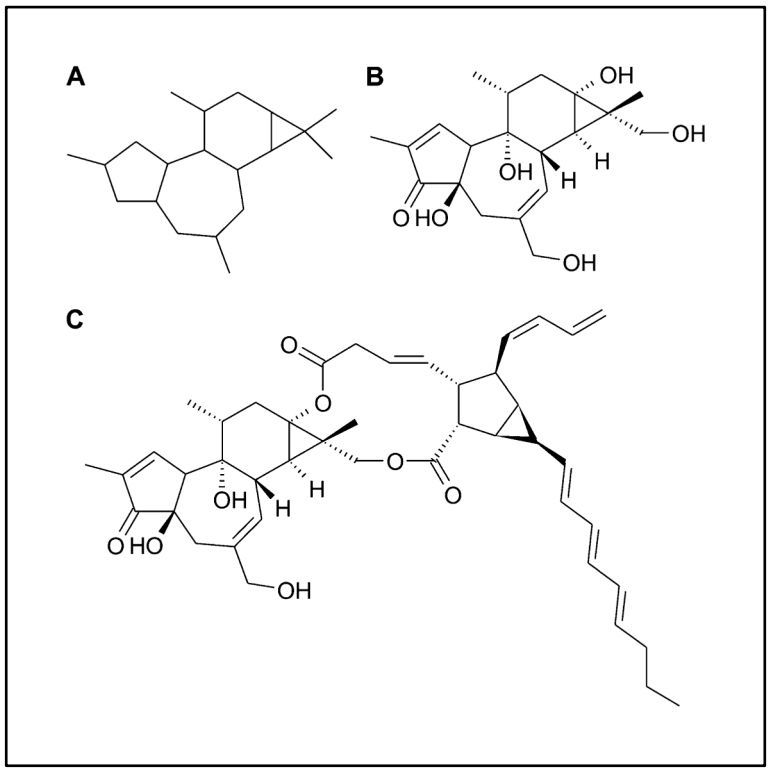

Figure 1-6. A: 5-7-6-3 tigliane ring structure common to all phorbols; B: 12-hydroxy-16-deoxylphorbol structure common to all phorbol-esters from *J. curcas;* C: *J. curcas* factor C1, one of the six phorbol-esters identified in the seeds of *J. curcas* (King *et al*., 2009).

Edible/non-toxic genotypes, characterized by free or low phorbol ester seeds, have been found in Mexico. The phytotoxin (toxalbumin) curcin, a ribosome inactivating protein similar to ricin (phytotoxin of *Ricinus cummunis* L.) isolated from *J. curcas* seed, is able to inhibit protein synthesis. Further toxic components include phytates, saponins, and trypsine inhibitors. So, the press cake cannot be used in animal feed because of its toxic properties, but it is valuable as organic compost since it has a nitrogen content similar to that of the seed cake of castor bean and chicken manure. The nitrogen content ranges from 3.2 to 3.8%, depending on the source (Heller, 1996).

J. curcas is widely cultivated in the tropics as a living fence and preparations of all parts of the plant, including seeds, leaves and bark are used in traditional medicine and for veterinary purposes fresh or as a decoction. The oil has a strong purgative action and is also widely used for skin diseases and to soothe pain such as that caused by rheumatism. A decoction of leaves is used against cough and as an antiseptic after birth. Branches are used as a chewing stick in Nigeria. The lymph flowing from the stem is used to arrest bleeding of wounds. The wound-healing properties of curcain, a proteolytic enzyme isolated from latex, have been demonstrated. Latex has antimicrobial properties against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Candida albicans*. The coagulating effects on blood plasma have been demonstrated too. Extracts from physic nut fruits showed pregnancy-terminating effects in rats and a methanol extract of physic nut leaves provided moderate protection for cultured human lymphoblastoid cells against the cytopathic effects of human immunodeficiency virus. Extract of the leaves showed potent cardiovascular action in guinea pigs and might be a possible source of beta-blocker agent. The seed oil, extracts of physic nut seeds and phorbol esters from the oil were used to control various pests with, in many cases, successful result. In laboratory experiments, ground physic nut showed molluscicidal activity against the host of liver fluke (*Lymnaea auricularia rubiginosa*) and also against the hosts of *Fasciola gigantea* and *Schistosoma* in Senegal. Extracts from crushed whole seeds showed molluscicidal activity against several schistosome vector snails. Phorbol esters were probably the active agents in the different extracts used (Heller, 1996).

Moreover, the primary conservation benefits to be derived from production of jatropha relate to improved soil restoration and management. Heavy metal contaminated soil can be restored by using combination of industrial wastes and suitable bioinoculants strain (*Azotobacter*). Jatropha reduces wind erosion and pressure on timber resources and increases soil moisture retention, in addition to protecting crops from livestock. Nevertheless, jatropha does mine soil nutrients. Jatropha oil projects are

expected to provide income and organic fertilizer to increase crop yields, as well as being an ecologically friendly source of alternative energy to rural farmers (Kumar and Sharma, 2008).

As early as 1911, Rudolf Diesel, who invented the diesel engine, made the following statement: “It is generally forgotten, that vegetable and animal oils can be used directly in diesel engines. A small diesel engine ran with peanut oil during the world exhibition of Paris in 1900, and which worked so exceptionally well, that the change of fuel was realized by only a few visitors”. In experiments carried out until 1950, vegetable oils were used without problem in common engines with pre-chamber injection. Activities involving the use of physic nut oil in engines have been described in Segou, Mali during World War II. Since the oil crisis of the 1970s and recognition of the limitations of world oil resources, this technology has received special attention. Most of the research was carried out in temperate regions with the aim of making available possibilities for diversifying to farmers, in view of the increasing subsidy-driven surpluses in traditional commodities. Another topic for the cultivation of oil crops for energy purposes is the increasing global warming/greenhouse effect. When these fuels are burned, the atmosphere is not polluted by carbon dioxide, since this has already been assimilated during the growth of these crops. The CO_2 balance, therefore, remains equable. Special interest has been shown in the cultivation of physic nut for this purpose, especially since it is drought resistant and can potentially be used to produce oil from marginal semiarid lands, without competing with food production. In addition, these fuels can be used to substitute costly oil imports for landlocked countries. The use of physic nut seed oil in car engines is reported in the literature and in unpublished research reports (Heller, 1996).

The oil from the seeds has valuable properties such as low viscosity as compared to castor oil, a low acidity, good stability as compared to soybean oil, and better cold properties as compared to palm oil. Besides, jatropha oil has a higher cetane number compared to diesel, which makes it a good alternative fuel with no modifications required in the engine.

However, most non-edible oils carry a high level of free fatty acids (FFAs), which is not desirable as it lowers the yield of biodiesel. This is because a high FFA (>1% w/w) will promote soap formation and the separation of products will be difficult during alkali-catalysed trans-esterification. Jatropha oil contains about 14% of FFAs, which is far beyond the acceptable limit of a 1% FFAs level. Thus, pretreatment step to minimize the FFAs of feedstock is required for a better biodiesel yield. Since jatropha oils consist of mainly oleic (C18:1) and linoleic (C18:2) acids, which are unsaturated fatty acids, the biodiesel produced has eligible good low temperature properties. Although jatropha oil has high free fatty acid content too, methods to overcome this high FFA are well developed. Thus, *Jatropha curcas* oil has been highlighted as a potential biodiesel feedstock among the non-edible oils. Table 1-4 shows the composition and characteristics of *J. curcas* oil.

Characteristic	***J. curcas* oil**	***J. curcas* methyl ester**
Calorific value (MJ kg^{-1})	37.83–42.05	39.65–41.63
Density at 15°C (g cm^{-3})	0.92-0.95	0.86–0.88
Viscosity at 30°C (cSt)	37.0–54.8	4.8–5.6
Specific gravity (g cm^{-3})	0.860–0.933	0.86–0.88
Cetane value	38.0–51.0	60.74–63.27
Saponification number (mg g^{-1})	102.9–209.0	-
Pour point (°C)	−3	−6to2
Cloud point (°C)	2	-
Flash point (°C)	201–240	170–192
Acid value (mg KOH^{-1} g^{-1})	0.92–6.16	0.06–0.5
Free fatty acids % (kg kg^{-1} × 100)	0.18–3.40	-
Iodine number (mg L g^{-1})	92.0–112.0	-

Table 1-5. Composition and characteristics of *J. curcas* oil (Koh *et al*., 2011).

So, *J. curcas* biodiesel has comparable properties with those of fossil fuel and conforms to the latest standards for biodiesel, such as European (EN 14214:2003) and USA (ASTM D 6751). Standardization according to Country defined standards for biodiesel is a prerequisite for successful market introduction and penetration of *J. curcas* biodiesel.

The use of *J. curcas* oil as biodiesel source has several controversial aspects, such as economic, social and environmental risks, which combine all the aspects of the wider current debate on the biofuel production. Additionally, the current knowledge gap about the impacts and potentials of *J. curcas* plantations make its cultivation potentially unsustainable (Achten *et al*., 2010). The debate about *J. curcas* is heated and several authors claimed that the careless cultivation of this tree could lead to significant risks in the economies of several countries and to the impoverishment of local populations. Some of the countries with highest economic growth rates, such as India and China, have strongly sustained the production of *J. curcas* biodiesel within their energy policies. In 2003, a two-phase governmental project was launched in India for widespread cultivation of *J. curcas* on wastelands. The project aims at planting 12.5 million hectares on government land across the country and then privatizing the production of *J. curcas* biodiesel. In 2006, China government decided to meet 15% of transportation energy needs with biofuel, leaning on the ambitious plan to raise 11 million hectares of *J. curcas* plantation on marginal lands. In this context, a massive planting program of unprecedented scale encouraged millions of farmers and landless people to plant *J. curcas.* But the results achieved so far are not encouraging. In India seed production does not reach the expectation. In China, until today, the production of biodiesel from *J. curcas* oil is quite low. Nevertheless, according to the current scientific thought, a wise and correct *J. curcas* use at local level, supported by additional studies, might be a good solution in terms of energy services improvement, environmental problem mitigation, and income-generating activities creation in developing countries. Its special characteristics encourage an easy integration of *J. curcas* plantations into the rural economy at village level. Community-based initiatives on *J. curcas* plantation could positively contribute to the rural livelihoods, if based on small plantations in marginal lands or on intercropped agro-forestry systems with the aim at producing oil and by-products. Jatropha can still be considered a wild, undomesticated plant showing considerable performance variability. In

order to reduce the risk of future unsustainable practices and to improve crop performance, further selection, breeding and domestication of jatropha are key topic to optimize. However, substantial pre-breeding knowledge is important to facilitate and guide an effective and robust route towards its domestication. A very significant issue is the yield: jatropha plant bears fruits from the 2nd year of its plantation and the economic yield stabilizes from 4th or 5th year onwards. The plant has an average life with effective yield up to 50 years. Jatropha gives about 2 kg of seed per plant in relatively poor soils. The seed yields have been reported as 0.75–1.00 kg per plants thus the economic yield can be considered to range between 0.75 and 2.00kg/plant and 4.00 and 6.00MT per hectare per year depending on agro-climatic zone and agriculture practices. One hectare of plantation on average soil will give 1.6 MT oil (Achten *et al.*, 2010; Contran *et al.*, 2012).

There is need of new varieties, with a better and uniform quality, that can be developed with new techniques. The characteristics of the plant to handle are numerous: yield of seeds, oil content, toxicity, male/female flowers ratio, synchronous maturation and adaptation to biotic and abiotic stress. The vegetative reproduction can be obtained both *in vivo* and *in vitro* by cuttings and organogenesis.

Plant cell cultures are a suitable tool to overcome the limits imposed by plant cultivation. Plant cells can be maintained in simple and synthetic media to produce the elected molecules. Moreover, bioreactors can be installed on lands not intendend for crops, avoiding the conflict with species grown for human nutrition.

1.4 DGAT

The importance of *J. curcas* in the biodiesel production is due to its capability to synthesize and store TAG into seeds; for this reason the genes involved in the TAG biosynthetic pathway are of great interest and the ability to control their expression for improving their amount and quality is a fundamental aim to reach.

Fatty acid synthesis begins into plastids, continues into the cytosol and into the endoplasmic reticulum (ER) where the so-called Kennedy pathway

(Figure 1-7) occurs. In four sequential reactions, glycerol 3 phosphate is acilated to *lyso*-phosphatidic acid, which in turn is acilated by specific acyltransferase; then, phosphatidic acid is dephosphorylated to diacylglycerol (DAG) that is converted to triacylglycerol by the enzyme diacylglycerol acyltransferase (DGAT).

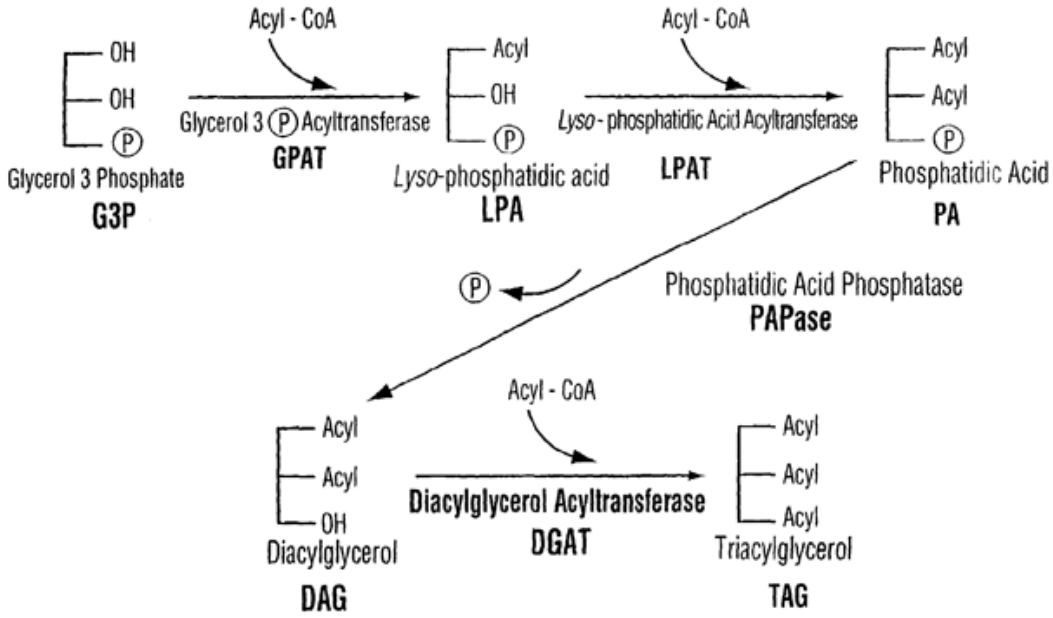

Figure 1-7. Representation of the Kennedy pathway (Zou *et al*., 2009).

The enzyme DGAT is codified by two classes of genes: the type 1 class (DGAT1) and the type 2 class (DGAT2) that have different properties and different characteristics: DGAT 1 proteins are composed of 500 aminoacids and present 10 transmembrane domains while DGAT 2 proteins are 320 aminoacids-long with 2 transmembrane domains. Probably the two enzymes have different roles and DGAT1 catalyzes the integration of common fatty acids, while DGAT2 is responsible for the incorporation of unusual fatty acids. Immunolocalization studies showed that both the enzymes have the C-terminal and N-terminal in the cytosol (Figure 1-8), confirming the even number of transmembrane predicted domains (Shockey *et al*, 2006).

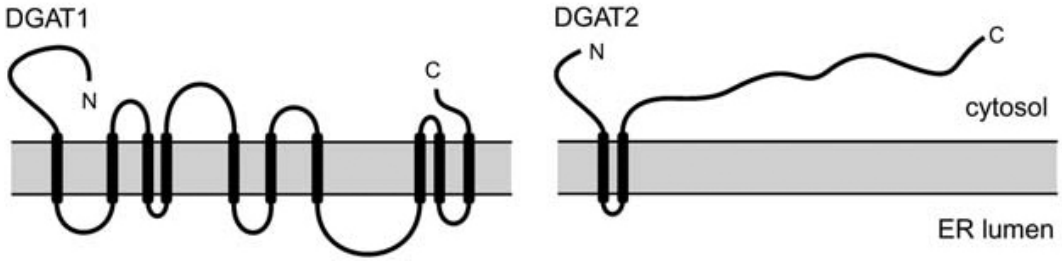

Figure 1-8. Topological structure predicted for the enzymes DGAT1 and DGAT2 (Shockey *et al*., 2006).

Many studies obtained changes in the levels of expression of the enzyme DGAT inserting the sequence of the gene DGAT1 in some plant species: the overexpression led to an increase of TAG accumulation in *A. thaliana* seeds (Jako *et al*., 2001) and *N. tabacum* leaves (Bouvier-Navé *et al.*, 2000; Andrianov *et al*., 2010). These results suggest that the regulation of TAG accumulation in oil seeds is a promising strategy to be studied and to be exploited to increase plant oil productivity.

2 Materials and methods

2.1 Media composition

The set-up of plant cell cultures and the cultivation of bacteria to obtain transformed plant cell cultures required many cultivation media that are listed in the next section.

2.1.1 Plant media

The establishment of plant cell cultures was performed on different media, depending on the plant species and the kind of explant used.

Solid media were prepared adding 8 g/L agar (Duchefa Plant Agar) and pH was adjusted with NaOH (Carlo Erba) before autoclaving at 121°C and 1 atm for 10 minutes.

Cultures were grown in Petri dishes on 20 mL of solidified medium while suspension cultures were grown in 50 mL of medium and kept in constant agitation of 80 rpm; temperature was set up at 24°C and the daylight period was 16 hours of light and 8 hours of dark.

MSB5J		**MSB5JR**	
MS salts + B5 vitamins	4.4 g/L	MS salts + B5 vitamins	4.4 g/L
sucrose	30 g/L	sucrose	30 g/L
pH	5.8	pH	5.8
IBA	5.0 µM	IBA	0.5 µM
BAP	2.2 µM	BAP	13.2 µM
MSB5$^{-1}/_{2}$		**MS$^{-1}/_{2}$**	
MS salts + B5 vitamins	2.2 g/L	MS salts + vitamins	2.2 g/L
sucrose	30 g/L	sucrose	20 g/L
pH	5.8	pH	5.5
MSIB		**MST**	
MS salts + vitamins	3.2 g/L	MS salts + vitamins	3.2 g/L
sucrose	20 g/L	sucrose	20 g/L
pH	5.5	pH	5.5
IBA	5.0 µM	2,4-D	2.2 µM
α-NAA	5.3 µM	BAP	1.1 µM

Table 2-1. Composition of media used for plant cell cultures.

2.1.2 Bacterial media

Escherichia coli and *Agrobacterium tumefaciens* were used for transformation experiments. *E. coli*, strain DH5α, was transformed with the desired plasmids to amplify the DNA amount and recover it by miniprep system (PureYield™ Plasmid Miniprep System by Promega©). *A. tumefaciens* strains LBA4404 and GV3101 were chosen as DNA delivery system for plant cell transformation.

Solid media were prepared adding 16 g/L agar (Bacto™ Agar Oxoid©) and pH was adjusted with NaOH (Carlo Erba) before autoclaving at 121°C and 1 atm for 10 minutes.

Cultures were grown in Petri dishes on 20 mL of solidified medium while suspension cultures were grown in 50-200 mL of medium and kept in constant agitation at 150 rpm; *E. coli* was grown at 37°C while *A. tumefaciens* was grown at 28°C.

LB (Sambrook *et al.,* 1989)		**YEP**	
bacto tryptone (Oxoid©)	10 g/L	peptone (Oxoid©)	10 g/L
bacto yeast extract (Oxoid©)	5 g/L	bacto yeast extract (Oxoid©)	10 g/L
NaCl (Carlo Erba)	10 g/L	NaCl (Carlo Erba)	5 g/L
pH	7.5	pH	7.5

Table 2-2. Composition of media used for bacterial cultures.

2.2 Plant propagation

2.2.1 *Jatropha curcas in vivo*

Different plants from mexican accessions of *J. curcas* were grown in a greenhouse under controlled conditions. Seeds from Huitzilan, Pueblillo, Morelos, Oaxaca and Zapotitlan have been pre-treated to obtain a better germination.

Shells were removed using a scalpel and kernels were soaked in distilled water for 1 hour at room temperature under constant agitation. After rehydration, 8 shells were placed in each Linfabox vessel (RA85, lids RDA145 - Duchefa) on wet filter paper. Boxes were kept in climatic chamber at 24°C and a light-dark cycle of 8 hours of light and 16 hours of dark. When seedlings began to grow endosperms were, if necessary, and

they were planted in plastic pots (ø = 8 cm) in a mix of 70% soil and 30% LECA (light expanded clay aggregate). Pots were then placed in a greenhouse with the temperature between 24° and 30°C and irrigated with tap water daily.

2.2.2 *Jatropha curcas in vivo* propagation

J. curcas in vivo propagation was carried out through flowering and cuttings.

Female flowers were manually pollinated and fruits were collected when mature. Seeds were sowed in soil to assess the vitality.

Cuttings were prepared from the hard parts of the stem (hard cuttings) and from the soft apical meristem (soft cuttings).

15 cm long hard cuttings, containing 3/5 nodes, were diagonally cut to expose the largest contact area with the soil. Explants were let to dry overnight and planted the day after. Leaves were removed or wired to the stem to reduce the photosynthetic area until root formations.

15 cm long soft cuttings were obtained from the apical parts of the plants. The biggest leaves were removed and explants were planted immediately.

Both soft and hard cuttings were planted in plastic pots (ø = 8 cm) containing a mix of 70% of soil and 30% of LECA placing at least 2 nodes under soil level. For the first 7-10 days, the cuttings were kept under a transparent box covered with an obscurant to keep a high humidity and to reduce plant photosynthetic activity. After this acclimation time, cuttings were transferred to *in vivo* standard growth conditions and daily watered.

2.2.3 *Nicotiana tabacum in vivo* propagation

N. tabacum seeds, variety Samsun, were sowed in plug trays and kept in a climatic chamber at 22°C with a light-dark cycle of 16 hours of light and 8 hours of dark.

2.2.4 *Jatropha curcas in vitro* propagation

In order to obtain *in vitro* plants, kernels of *J. curcas* were sterilized and germinated in 2 different media:

- MSB5 ½ solidified with 0.8% (w/V) of agar
- a mixture of 70% soil and 30% LECA

Seeds were soaked in distilled water for 3 hours under constant agitation at room temperature. Shells were then removed with a scalpel and kernels were sterilized for 10 minutes in 75% V/V ethanol + 0.1% V/V TWEEN®20 (P2287 - SIGMA-ALDRICH®). Sterilization was continued in sodium hypochlorite 5% V/V + 0.1% V/V TWEEN®20 for 15 minutes. Kernels were then washed 5 times for 10 minute in sterile distilled water and were maintained under constant agitation at 80 rpm at room temperature through all steps.

Kernels were then placed to germinate in:

- 50 mL test tubes with 25 mL of solidified MSB5 ½
- Linfabox vessel with sterile filter-paper wetted with distilled water

The obtained seedlings were then transferred from 50 mL test tubes to Linfabox with the same media or from Linfabox with filter paper to Linfabox with 70% of soil and 30% of LECA (previously sterilized at 121°C, 1 atm for 10 minutes).

All Linfabox vessels were kept in a climatic chamber at 24°C with the light-dark cycle of 16 hours of dark and 8 hours of light.

2.2.5 *Nicotiana tabacum in vitro* germination

N. tabacum seeds were surface sterilized by vapor-phase sterilization. Microcentrifuge tubes containing the seeds have been placed in a desiccator jar in a fume hood. A 250 mL beaker containing 100 mL of sodium hypochlorite 15% has been put into the desiccator and, immediately prior to sealing the jar, 3 mL of hydrochloric acid 12 M was carefully added to the beaker. Seeds have been sterilized for three hours and then placed in petri dishes with MS ½ agarized with 0.8% w/V of plant agar and kept in the dark at 24°C. After 7/10 days, the seedlings were then transferred to Linfabox vessels with 100 mL MS ½ agarized with 0.8% w/V of agar and kept in a climatic chamber at 24°C with light-dark cycle of 16 hours of dark and 8 hours of light.

2.2.6 *Nicotiana tabacum in vitro* micropropagation

Plants of *N. tabacum* can be easily propagated by *in vitro* cuttings. Every 40 days meristematic apexes were cut and planted on new 100 mL of MS$^-$ ½ agarized with 0.8% w/V to root and grow.

2.3 Callus induction from *Jatropha curcas* leaf explants

Dedifferentiated callus proliferation can be obtained from different tissues. Induction of cell proliferation was achieved from leaf explants of adult plants or from cotyledon or hypocotyl explants of plantlets.

Leaves from the second or third internode of an adult plant were sterilized to obtain dedifferentiated callus for 30 minutes in sodium ipochloride 0.5% V/V and three washes in deionized sterile water.

In all steps the leaves were kept under constant agitation at 80 rpm and at a temperature of 24°C. Explants of 0.5/1 cm^2 were plated on MSB5J agarized with 0.8% of plant agar with the underside down.

Induction from plantlet explants did not need a sterilization procedure. Every experiment was executed in triple and generated calli were transferred on new media every 30-40 days.

2.4 *Jatropha curcas* suspension cell cultures

Callus proliferated on solid media were transferred in liquid media after at least 2 months of proliferation. Little fragments were inoculated in MSB5J and left to adapt to the suspension growth. Media renewal was executed according to the growth curve.

2.4.1 Determination of the growth curve of plant cell cultures

The growth curves of different plant cell cultures were determined after 3 or 4 months needed to adapt plant cells to suspension growth conditions. Initially about 1 g of callus from a petri dish were inoculated in 10 mL of liquid media. After 3 or 4 media renewals, every 1 or 2 weeks (it depends on the plant cell species), the volume of the suspension culture were increased to 50 mL. After cell culture stabilization the growth curve can be evaluated. Three 50 mL flasks containing 0.4 Packed Cell Volume (PCV) of cells in 10 mL of media were prepared for every point of the curve, and

were growth in a climatic chamber at 24°C with the light-dark cycle of 8 hours of light and 16 hours of dark. Cell suspensions were collected in 15 mL tubes on the scheduled days and centrifuged at 490g for 2 minutes. The media were removed and pellets were let drying in a dry heater at 60°C until their weight become constant. Mean values of each point have been calculated, normalized as the initial weight was 1 g, and plotted on a dry weight/time graph.

2.4.2 Regeneration of *Jatropha curcas* via organogenesis

Shoot induction from cell cultures is an alternative way to clonally propagate a species useful in transformation protocols.

J. curcas shoots were obtained changing the phytohormone concentration added to calli cultured on agarized MSB5J. After 2 months, needed to stabilize the proliferating culture, calli were plated on MSB5JR medium.

Once shoots were well extended they were transferred in Linfabox vessels on MSB5·½ agarized with 0.8% of agar.

2.5 Evans Blue assay on *Jatropha curcas* plant cell culture

Viability of suspension plant cell cultures was estimated by the Evans Blue assay. Aliquots of 500 µL were collected from the suspension culture and the standard sample was dipped for 10 minutes in boiling water. This sample was considered the standard with a death rate of the 100%. 500 µL of Evans Blue were added to the sample that were put in agitation for 15 minutes; samples were then transferred in Bio-Rad columns that were washed until the liquid flowing through the columns became transparent. Once columns were dry, 500 µL of 1% w/V SDS solution and methanol 50% V/V were added to lyse the cells. Then samples were placed in a waterbath at 55°C for 30 minutes. The content of columns was transferred into eppendorf tubes and, once diluted 1/5, the absorbance was read at the wavelength of 600 nm. To calculate the death rate was applied the following equation:

$$A_{control} : 100\ (100\%\ death\ cells) = A_{sample} : X$$

2.6 Polymerase Chain Reactions

All PCR assays were performed with an Eppendorf mastercycler ep gradient S. For 35S amplification and screening PCR, GoTaq® DNA Polymerase (Promega®) were used, while for the amplification of YFP gene and DGAT gene the Clontech Advantage® 2 PCR Enzyme System was chosen.

2.7 Agarose gels

Agarose gels were prepared adding Pronadisa Agarose D-1 Low EO at the desired concentration to TAE buffer. Agar was melted by heating with a microwave and GelRed™ Nucleic Acid Gel Stain (10,000X in water) was added before gel hardening. Electrophoretic run were performed with a BIO-RAD PowerPac HC™ set for 10 minutes at 90V and for the remaining running time at 100V.

Gels pictures were taken with the digital transilluminator GelDoc™ XR imaging system and the software Quantity One, BIO-RAD.

2.8 DNA spectrophotometric quantification

DNA was purified using Promega® kits according to manufacturer's instructions and to determine its concentration a spectrophotometric assay was performed. DNA was diluted with distilled water and its absorption at λ_{260} and at λ_{280} was measured with a Beckman Coulter DU530 spectrophotometer. Concentration was calculated with the equation:

$$DNA\ concentration\ \left(\frac{ng}{\mu L}\right) = A_{260\text{nm}} \times 50 \times dilution\ factor$$

2.9 *Escherichia coli* transformation

E. coli strain DH5α was transformed by the heat-shock method (Sambrook, 1989). The bacteria conserved at -80°C was inoculated in 5 mL of LB medium (see 2.1.2) and grown overnight at 37°C in constant agitation. The overnight culture (500 µL) was reinoculated in 50 mL of LB in a 250 mL flask and let grow in constant agitation at 37°C until an OD_{600} of 0.4-0.6 was reached. Then the culture was kept in ice for 10 minutes and centrifuged at 4000g for 10 minutes at 4°C. Cells were resuspended

in 25 mL of sterile ice-cold $CaCl_2$ 0.1 M, kept in ice for 10 minutes and centrifuged at 4000g for 10 minutes at 4°C. Cells were diluted in 1.7 mL of sterile ice-cold $CaCl_2$ 0.1 M and 300 µL of sterile glycerol were added. Stocks (100 µL) of competent cells were prepared in 1.5 mL eppendorfs and conserved at -80°C until use. Competent *E. coli* was transformed adding 150/200 ng of plasmidic DNA (purified with PureYield™ Plasmid Miniprep System by Promega©). Cells were kept in ice for 30 minutes, putted at 42°C for 90 s and again in ice for other 1-2 minutes. Then, 900 µL of LB medium were added and the culture was transferred in a bacteriological tube and let grow in agitation at 37°C for 45 minutes. Different volumes of the culture were plated on LB agarized with 1.6% w/V of Bacto™ agar (BD - 214030) added with the appropriate antibiotics and, if necessary, IPTG (MICROPOLI) and X-Gal (MICROPOLI). The obtained positive colonies were screened by PCR, DNA extraction and control digestions.

All transformed strains were grown overnight at 37°C in 5 mL of LB medium with the appropriate antibiotic, centrifuged and resuspended in 1 mL of LB + antibiotic + 15% glycerol and kept at -80°C until use.

2.10 *Agrobacterium tumefaciens* transformation

Once a desired plasmid was constructed and amplified in *E. coli* it can be transferred into *A. tumefaciens* by the freeze-thaw method (An, 1988). Strains GV3101 and LBA4404 of *A. tumefaciens* were grown overnight at 28°C in 5 mL of YEP medium (see 2.1.2). 50 µL of the overnight cultures were inoculated in 50 mL of YEP medium in 250 mL flasks and kept in vigour agitation at 28°C until an OD_{600} of 0.5-1.0 was reached (usually overnight). Cultures were chilled on ice and centrifuged at 3000g for 5 minutes at 4°C. Cells were resuspended in 1 mL of 20 mM $CaCl_2$ sterile and ice-cold solution. Aliquots of 100 µL of competent *A. tumefaciens* cells were prepared and stocked at -80°C.

The transformation was carried on adding 1 µg of pSoup (see Figure 2-1), that provides replication functions *in trans* for pGreen, and 1 µg of the chosen pGreen, freezing the cells with liquid nitrogen. Cells were thawed by incubating the test tubes at 37°C in a water bath and after 5 minutes 1

mL of YEP medium was added and the tubes were incubated at 28°C for 2-4 hours in gentle shaking. The tubes were centrifuged at 15000g for 30 s and the cells were resuspended in 0.1 mL of YEP medium. The cells were spread on YEP medium agarized with 1.6% of Bacto™ agar containing the appropriate selective antibiotic. Plates were incubated at 28°C and colonies appeared after 4-5 days. Positive colonies were screened by PCR.

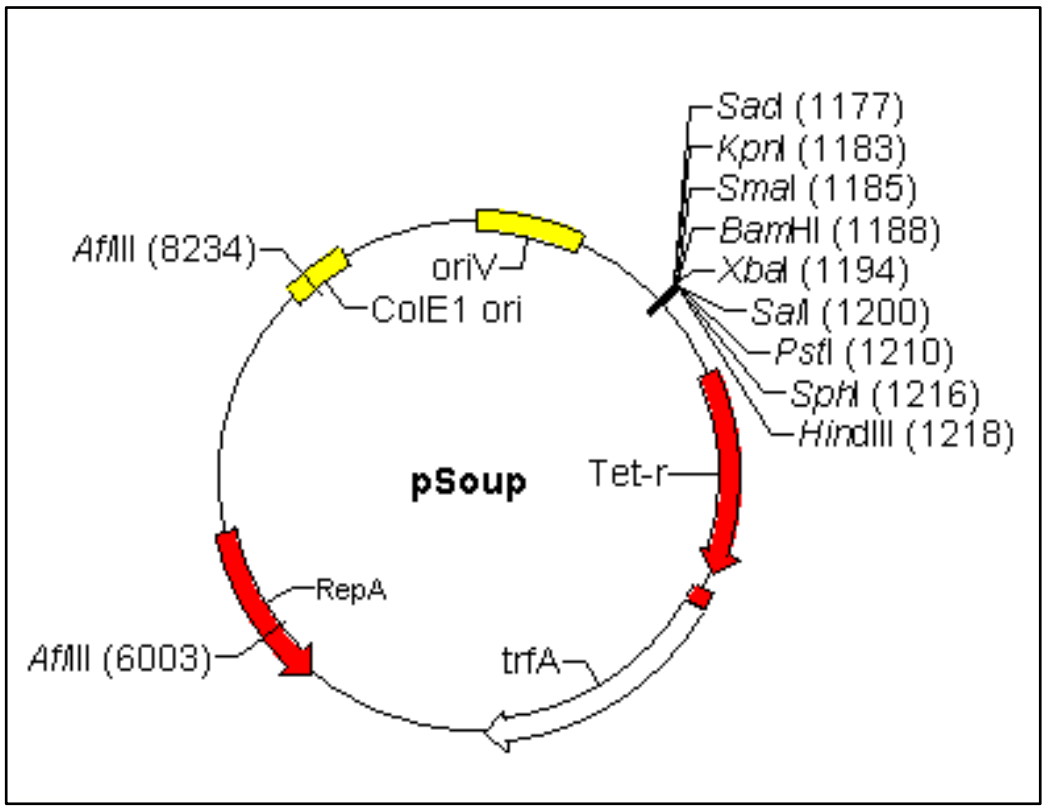

Figure 2-1. Representation of the plasmid pSoup. It provides replication functions *in trans* for pGreen (http://www.pgreen.ac.uk).

All transformed strains were grown overnight at 28°C in 5 mL of YEP medium with the appropriate antibiotic, precipitated by centrifugation and resuspended in 1 mL of YEP + antibiotic + 15% glycerol and kept at -80°C until use.

2.11 Plant transformation set-up

The binary vector pGreen (http://www.pgreen.ac.uk) was chosen to deliver plasmid the constructs of interest. The experimental design provided the insertion of the CaMV 35S promoter in the *lacZ* multicloning site between the restriction sequences cut by *XhoI* and *NotI* of the pGreen0029 (Figure

2-2), that confers kanamycin resistance (Neomycin phosphotransferase gene sequence, Npt) when its T-DNA is expressed in plant. The T-DNA is the portion of the plasmid between the right border (RB) and the left border (LB) that it is translocated and integrated to the plant cell genome after *A. tumefaciens* infection. Outside this sequence there is another Npt sequence useful for the selection of transformed bacteria.

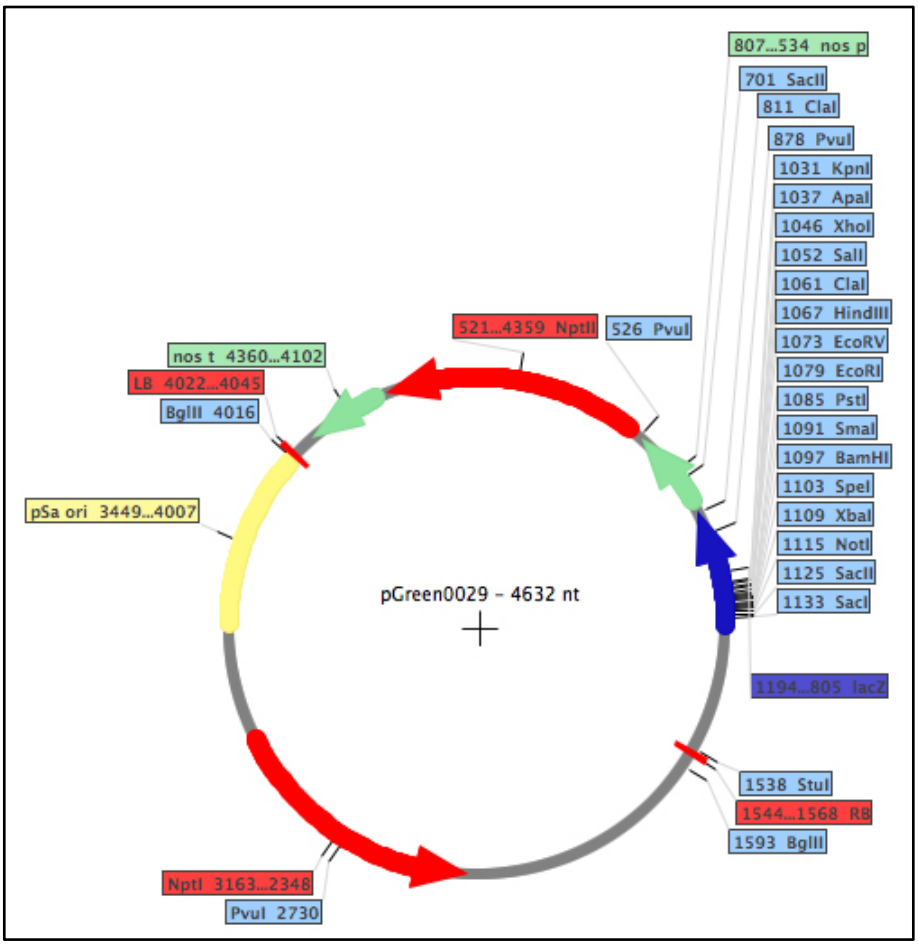

Figure 2-2. Representation of the pGreen0029 backbone. The two sequences coding for the Npt gene are in red, the lacZ coding sequence is in blue (http://www.pgreen.ac.uk).

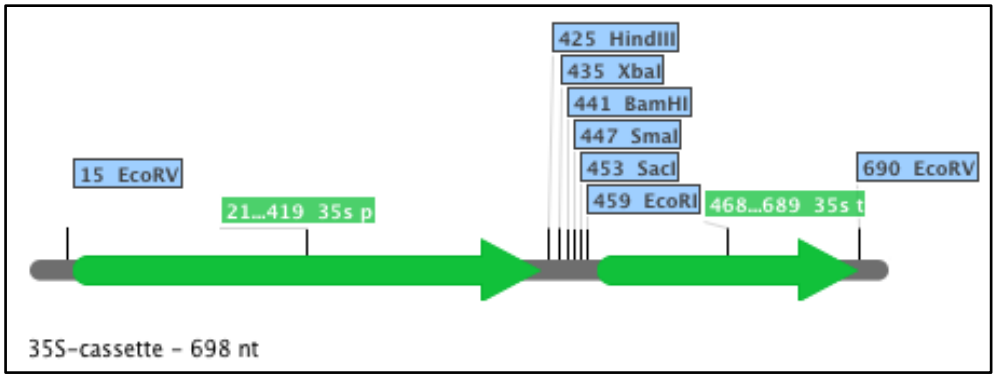

Figure 2-3. Representation of the 35S-cassette.

Then the constructs were cloned between the *BamHI* and *EcoRI* restriction sites present in 35S cassette (see Figure 2-3).

2.11.1 35S-cassette cloning into pGreen0029 multicloning site

35S-cassette was amplified by PCR adding, with pre-designed primers, the chosen restriction enzyme sequences at the edges of the cassette: *XhoI* and *NotI*. The primers were purchased from SIGMA-ALDRICH® and the sequences are:

35S-2_fw

5'-ACTCTCGAGATCGTACCCCTACTCCAA-3'

XhoI 35S

35S-2_rev

5'-TGAGCGGCCGCATCGATCTGGATTTTAGTA-3'

NotI 35S

The PCR amplification was carried out setting different annealing temperatures, from 50°C to 68°C, The program set-up was the following:

2 min at 95°C	initial denaturation
30 sec at 95°C	30 cycles
30 sec at T_{ann}	
60 sec at 72°C	
5 min at 72°C	final extension

After the amplification, the PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega®). Then both pG0029 and 35S-cassette were digested with *XhoI* and *NotI* (New England Biolabs® *Inc.*). In particular, 1µg of DNA was digested with 5 units of each enzyme in NEB buffer 3 adding BSA at a final concentration of 100 µg/mL. Digestions were carried out for 90 min at 37°C (pG0029 single digestion was performed as control). The correct bands were excised from 1% agarose gel and purified with Wizard® SV Gel and PCR Clean-Up System

kit (Promega®) and then ligation was performed with T4 DNA Ligase (Promega®). 162 ng of vector (pG0029) and 74 ng of insert (35S-cassette), with a molar ratio of 3/1, were incubated with 1 unit of T4 DNA ligase at 4°C overnight.
Transformed colony selection was performed with white/blue colony screening and the strain was called pG2935.

2.11.2 eYFP and DGAT genes amplification

The experimental design has implied the amplification of the chosen genes, adding to theirs edges the base sequences recognized by the desired restriction enzymes needed for cloning experiments.
The restriction enzymes, able to cut in the 35S-cassette multicloning site, chosen are *BamHI* and *EcoRI*. So, the sequences recognized by these two enzymes were added by the correct design of the primers used for the amplification. Moreover, the sequence coding for the Influenza Hemagglutinin (HA) peptide has been added at the 5' terminal of the DGAT; this peptide is a common flag that can be detected by antibodies in immuno blot experiments.

2.11.3 eYFP

The coding sequence for the eYFP was amplified by a plasmid already present in the lab. The primers purchased from SIGMA-ALDRICH® are:

YFP_fw_BamNde

5'-ACTGGATCCTGACATATGCTGAGCAAGGGCGAGGAGCTG-3'

BamHI *NdeI* YFP

YFP_rev_Eco

5'-TGAGAATTCTTACTTGTACAGCTCGTCCATGCC-3'

EcoRI YFP

The correct annealing temperature was chosen after a screening test and the final amplification was performed with Clontech Advantage® 2 PCR Enzyme System.

2 min at 95°C	initial denaturation
30 sec at 95°C	30 cycles
30 sec at T_{ann}	
45 sec at 72°C	
5 min at 72°C	final extension

The amplicone was called bnYFPe.

2.11.4 DGAT

The diacylglycerol acyltransferase (DGAT) coding sequence was requested to "The Arabidopsis Information Resource" (TAIR - www.arabidopsis.org) and received in the clone U16373. The *E. coli* strain PIR1, transformed with the vector pUNI51 carrying the full length cDNA (GeneBank Accession Sequence n. BT008883), was plated on 20 mL of LB solidified with 1.6% w/V of Bacto™ agar and added with 50 mg/L of kanamycin and put overnight at 37°C. Single colonies were then amplified in 5 mL of LB added with kanamycin 50 mg/L overnight at 37°C and plasmid DNA was purified by the Promega© miniprep system. The DGAT sequence was amplified by PCR and two different constructs were designed, one coding for the DGAT (called HD) and one lacking the stop codon needed for the construct of the fusion product HA::DGAT::YFP (called HD5).

The primers purchased from SIGMA-ALDRICH® are:

HDGAT*_fw_Bam

5'-ACTGGATCC**ATG**TACCCATACGATGTTCCAGATTACGCTGCGATTTTGGATTCTGCTGGC-3'

BamHI HA flag

HDGAT*_rev_NdeMfe

5'-AGTCAATTGCATATGTCATGACATCGATCCTTTTCGGTTCATCAGGTC-3'

MfeI *NdeI* DGAT

HDGAT_rev_NdeMfe

5'-AGTCAATTGCATATGTGACATCGATCCTTTTCGGTTCATCAGGTC-3'

MfeI *NdeI* DGAT

HDGAT*_fw_Bam and HDGAT*_revNdeMfe were used to amplify the DGAT gene with the HA flag at 5' terminal, while HDGAT*_fw_Bam and HDGAT_rev_NdeMfe amplified the 5' portion of the sequence coding for the fusion protein HA-DGAT-YFP; in fact it lacks the stop codon at the end of the DGAT coding sequence.
The correct annealing temperature was chosen after a screening test and the final amplification was performed with Clontech Advantage® 2 PCR Enzyme System.

2 min at 95°C	initial denaturation
30 sec at 95°C	30 cycles
30 sec at T_{ann}	
90 sec at 72°C	
5 min at 72°C	final extension

The amplicones were called bHDGATnm and 5'bHDGATnm respectively.

2.11.5 Cloning of eYFP and DGAT amplified sequences into the vector pG2935

bnYFPe, bHDGATnm and 5'bHDGATnm were cloned into the pGEM®-T Easy Vector (Figure 2-4) supplied by the Promega® pGEM®-T Easy Vector System. Ligation between vector and inserts was performed following the protocol supplied with the kit.

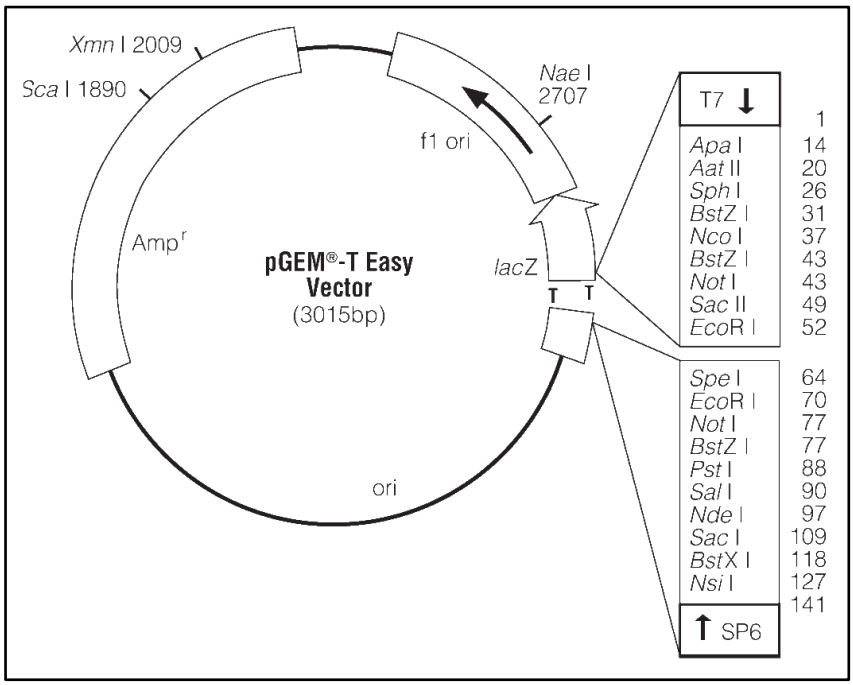

Figure 2-4. Representation of the pGEM®-T Easy Vector (http://www.promega.com)

Competent cells of *E. coli* strain DH5α were transformed with the ligations and positive colonies were selected with ampicillin 100 mg/L.

Transformed *E. coli* DH5α was called pGEMY when transformed with bnYFPe cloned in pGEM®-T Easy Vector, pGEMHD when transformed with bHDGATnm cloned in pGEM®-T Easy Vector and pGEMHD5 when transformed with 5'bHDGATnm clone in pGEM®-T Easy Vector.

The screening was possible by the white/blue colonies, but PCR screenings with the appropriate primers were performed to select colonies that correctly integrated the insert; strain amplification by inoculum in liquid LB added with ampicillin was carried out overnight at 37°C in continuous shaking. pG2935 was amplified in LB added with kanamycin 50 mg/L in the same conditions.

Then plasmids were purified with the miniprep kit by Promega® and digestions were performed as follow:

- 1 µg of pG2935 and 500 µg of pGEMYFP were digested with 5 units of *BamHI* and *EcoRI* each (New England Biolabs® *Inc.*)
- 500 µg of pGEMHD and 500 µg of pGEMHD5 were digested with 5 units of *BamHI* and *MfeI* each (New England Biolabs® *Inc.*)

Digestions were carried out for 90 min at 37°C. The samples were excised from 1% agarose gel and purified with Wizard® SV Gel and PCR Clean-Up System kit (Promega®) and then ligations were performed with T4 DNA Ligase (Promega®).

100/200 ng of vector pG2935 and the appropriate amount of insert, calculated according to the molar ratio of 3/1, was incubated with 1 unit of T4 DNA ligase at 4°C overnight.

E. coli competent cells were transformed with the ligations and selection of transformed colonies was performed adding 50 mg/L of kanamycin to LB media solidified with Bacto™ agar. PCR screenings were carried on to correctly select colonies harbouring the plasmid with the insert.

Positive strains were called *E. coli* pG29Y, pG29HD and pG29HD5 respectively. All plasmids were collected by miniprep of an overnight culture and sequenced to verify the accuracy of the base sequences.

In order to obtain the gene expressing for the fusion product HA::DGAT::YFP, plasmids pG29Y and pG29HD5 were collected by miniprep Promega® of an overnight culture of the appropriate *E. coli* strain. 500 µg of pG29Y and 1 µg of pG29HD5 were digested with 5 units of *BamHI* and *NdeI* (New England Biolabs® *Inc.*).

The correct bands were excised from 1% agarose gel and purified with Wizard® SV Gel and PCR Clean-Up System kit (Promega®) and then ligation was performed with T4 DNA Ligase (Promega®). 100 ng of vector (pG29HD5) and 80 ng of insert (YFP band), with a molar ratio of 3/1, were incubated with 1 unit of T4 DNA ligase at 4°C overnight.

Competent *E. coli* DH5α cells were transformed with the ligation product and positive colonies have been screened by PCR. The strain harbouring the correct plasmid was called pG29HDY.

2.12 Transient and stable transformations of *Jatropha curcas* and *Nicotiana tabacum*

Agroinfiltration to express the protein YFP were performed to verify susceptibility of *J. curcas* to *Agrobacterium*-mediated transformation. Once

confirmed the capability of *A. tumefaciens* to transfer the DNA portion of interest to *J. curcas* cells, stable transformations were executed.

2.12.1 Transient *Agrobacterium*-mediated transformation of *Jatropha curcas* and *Nicotiana tobacco* leaves with pG29Y and pG29HDY

Single colonies of the two strains were inoculated in 20 mL of YEP added with 25 mg/L gentamycin, 100 mg/L rifampicin, 50 mg/L kanamycin, 20 µM acetosyringone and were left to grow overnight until an OD_{600}= 0.6-08. Bacteria were sedimented by centrifugation at 1600g for 15 minutes and resuspended in 20 mL of MSB5J without phytohormones. Bacterial solution was injected in *J. curcas* leaves with a 1 mL serological syringe deprived of the needle. The tip of the syringe was pressed against the underside of a leaf applying a gentle pressure on the other side of the leaf. The solution was then injected into the airspaces inside the leaf through the stomata.

2.12.2 Stable *Agrobacterium*-mediated transformation of *Jatropha curcas* explants

J. curcas leaves were sterilized as RIF and explants of 0.5-1 cm^2 were placed on MSB5J agarized with 0.8% w/V of plant agar for 4 days (Kumar *et al.*, 2010). Then explants were co-cultured with a suspension of agrobacteria grown overnight as 2.13.1 for 30 minutes, adding acetosyringone 200 µM at the moment of bacteria resuspension. Acetosyringone is needed to induce the activity of *vir* genes in *A. tumefaciens*. Thus explants were replated on MSB5J for two days to allow agrobacteria infection. Explants were transferred on the same solid media added with the appropriate antibiotics to select transformed cells and to stop *A. tumefaciens* proliferation.

2.12.3 Stable *Agrobacterium*-mediated transformation of *Nicotiana tabacum* explants

Tobacco transformation was carried out with a yet known protocol for leaf explant transformation (Horsch *et al*., 1985). Leaves were cut from *in vitro* cultured plants and explants of 0.5-1 cm^2 were co-cultured with a

suspension of agrobacteria grown overnight as 2.13.1 for 30 minutes, adding acetosyringone 200 µM at the moment of bacteria resuspension. Explants were then plated on MSIB agarized with 0.8% of plant agar with the underside down and left for 48 hours at 24°C in the dark. After this time frame explants were transferred on the same media added with the antibiotics 50 mg/L kanamycin and 500 mg/L cefotaxime and media was renewed every 14 days until shoots appeared.

Shoots were then transferred in Linfabox vessels on MSIB added with 0.8% agar and the antibiotics until rooting. From the leaves of transformed plants were obtained calli, placing leaf explants on the media MST with antibiotics. Media were renewed every month.

2.13 Extraction of genomic DNA from transformed plant cells and tissues and detection of constructs by PCR

Extraction of genomic DNA was performed on transformed plant cells and tissues to check, by PCR, the successful integration of our constructs.

Sample were collected in 1.5 mL eppendorfs, tissues were frozen with liquid nitrogen and homogenized with a motor pestle. (Z359971 by SIGMA-ALDRICH®). 500 µL of Extraction Buffer (100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl) and 35 µL of 20% w/V of SDS were added. Samples were incubate at 65°C for 5 minutes. 130 µL of KOAc 5 M were added and incubation for 5 minutes in ice was carried out. Samples were centrifuged at 15000g for 10 minutes and supernatants were transferred in new eppendorfs. 640 µL of isopropanol and 60 µL of NaOAc 3 M were added and incubation at -20°C was performed for 10 minutes. Samples were centrifuged at 15000 g for 10 minutes, supernatants were discarded and washed with 300 µL of 70% ethanol. After another centrifugation at 15000 g for 5 minutes, samples were resuspended in water.

PCR was performed with the primers 35S-2_fw and 35S-2_rev and the set-up was:

2 min at 95°C	initial denaturation
30 sec at 95°C	30 cycles
30 sec at 60°C	
180 sec at 72°C	
5 min at 72°C	final extension

2.14 Triglyceride detection assay

A colourimetric assay was optimized (from Conkey and Feirer, 1988) for the quantification of intracellular triglycerides. It is possible to assess the lipid amount in any kind of plant explants, from seeds to cell cultures. The protocol laid three steps: extraction of triglycerides, purification and quantification with spectrophotometer.

TAG extraction. Samples were collected in 1.5 mL eppendorfs and 100 µl of isopropanol were added. Tissues were frozen with liquid nitrogen and homogenized with a motor pestle (Z359971 by SIGMA-ALDRICH®). Then 900 µL of isopropanol were added and the extraction continued on an orbital shaker for 15 minutes. Cell debris were pelleted at 14200 g for 5 minutes.

TAG purification. 15 mL tubes were prepared in advance with 0.8 g of aluminium oxide (A1522 - SIGMA-ALDRICH®) and 800 µl of the supernatant were added. Tubes were kept in continuous agitation on an orbital shaker for at least 5 minutes. Aluminium oxide was precipitated by centrifugation at 3000 g for 5 minutes.

Colour development. 800 µL of the supernatant were transferred to a new 15 mL tube and were added 200 µL of KOH (Carlo Erba) 1 M. The samples were kept in a water bath at 60°C for 5 minutes. Samples were left to cool at room temperature, then 200 µL of sodium (meta) periodate (S1878 - SIGMA-ALDRICH®) 11.7 mM in acetic acid 2 N were added. After 10 minutes, 1.2 mL of the colour development solution were added and samples were kept in a water bath at 60°C for 30 minutes. Colour development solution is composed of 2-propanol (278475 - SIGMA-ALDRICH®) + ammonium acetate (A1542 - SIGMA-ALDRICH®) 1 M + 0.12% V/V acetylacetone (P7754 - SIGMA-ALDRICH®).

Triglyceride quantification was performed reading sample absorbance at 410 nm. The standard curve was obtained from a 2.5 mg/mL triolein (glyceryl trioleate, T7140 - SIGMA-ALDRICH®) solution. Standards of 0, 125, 250, 500 and 750 µg/mL were prepared in triple and data were interpolated to calculate the linear equation correlating absorbance to concentration.

If the absorbance of an unknown sample exceeded that of the highest standard, it was diluted; to avoid precipitate, a diluent solution was prepared: 2-propanol + 0.33 M ammonium acetate + 83 mM KOH + 1 mM sodium (meta) periodate in acetic acid 2 N + 0.12% V/V acetylacetone.

As chlorophylls absorb at the λ used, it was necessary to perform parallel assays avoiding the reaction with KOH when green samples were tested. The absorbance values obtained, given only by chlorophylls, were subtracted to samples values.

It is very important that solutions are mixed well in any step. The resulting colour is quite stable, but it is better to measure the absorbance within 20 minutes.

2.15 Light and fluorescence microscopy

Explants for the agroinfiltrated leaves were observed with a Leica DM5000 B microscope and images were acquired with a DFC 425 camera controlled by the software LAS 3.8.

YFP was excited at 470±40 nm and observed at 525±50 nm.

3 Results and discussion

Jatropha curcas is a tropical species that accumulates oil in the seeds at high levels. By conventional propagation techniques the yield is uncertain and the oil content is not constant because it is difficult to have homogenous genetic material to plant. The development of *in vitro* regeneration system is an important tool to avoid, at least partially, these problems, exploiting the flexibility and the totipotence of plant cells. Moreover the possibility to obtain genetically transformed varieties, mainly to increase TAG accumulation, has focused the attention on this plant for producing biofuel.

In this work the principal aims is: a) to transform *J. curcas* inserting the gene coding for the diacylglycerol acyltransferase (DGAT), an important enzyme involved in the last step of TAG biosynthesis, b) to set up the best condition to propagate and to produce biomasses from both the wild type and the transformed plant lines.

3.1 Domestication of *Jatropha curcas* is easily obtained in greenhouse and *in vitro*

Seeds of *J. curcas* were available from 5 different mexican accessions (see Figure 3-1).

Figure 3-1. Mexican *J. curcas* accessions studied.

3.1.1 Germination of *Jatropha curcas in vivo* and *in vitro*

Seeds from Oaxaca (MXOA) had a germination efficiency of about 80%; the other varieties had no great difference among them and the germination obtained, through the 3 years of experiments, was of 6/7 over to 10 seeds. The only exceptions were the seeds from Zapotitlan (MXZA), which were not able to germinate in all the assayed conditions from the end of the second year of conservation.

All seeds were treated for the shell removal and soaked, then placed on wet filter paper for germination. Sterilization after soaking was performed in case of in vitro germination and seeds were then placed on sterile wet filter paper. The two different treatments gave the same rate of seed germination. A plant grown *in vivo* and a plant grown *in vitro* for 20 days are shown in Figure 3-2.

Figure 3-2. Plants of *J. curcas* growing *in vivo* (left) and *in vitro* (right).

After 10 days, germinated plants were placed in a mixture of 70% soil and 30% LECA both *in vivo* and *in vitro* conditions, and also in the medium MSB5^{-} ½ in the case of *in vitro* cultures.

It was clear that in the *in vivo* condition the plant had a greater development in height respect to the *in vitro* condition, where the plants remained small developing continuously new branches. Cotyledons of *in* vivo plants are larger and usually lasted more than two months. root

suffering was further observed when plants were grown *in vitro* in agarized media instead of the mixture of 70% soil and 30% LECA; roots became darker and darker and leaves started to dry. This behaviour showed general unwellness of *J. curcas* grown in agar-added media; this could be due to the high humidity that roots has to bear immersed in agar, while, in natural conditions, this plant prefers more drained soils and with a lower moisture content, like the mixture of soil and LECA.

In any case, the germination of the considered varieties under controlled condition is higher respect to what reported in literature for the germination in natural conditions (Divakara *et al*, 2010).

Plants obtained *in vivo* were kept in a greenhouse and were left to grow simulating the conditions of the subtropical area, mainly characterized by high and constant temperatures (see 2.2.2). These plants had a great development in height, reaching the 3 meters (Figure 3-3 A), and they showed a correct development of branches and leaves. Occasional flowering has also been observed with male and female flowers on the same inflorescence (Figure 3-3 B); both by self or manual pollination the correct fruit formation (Figure 3-3 C) was obtained. Fruits developed as described in literature and the collected seeds (Figure 3-3 D to F) were viable and able to germinate a new individual (Figure 3-3 G).

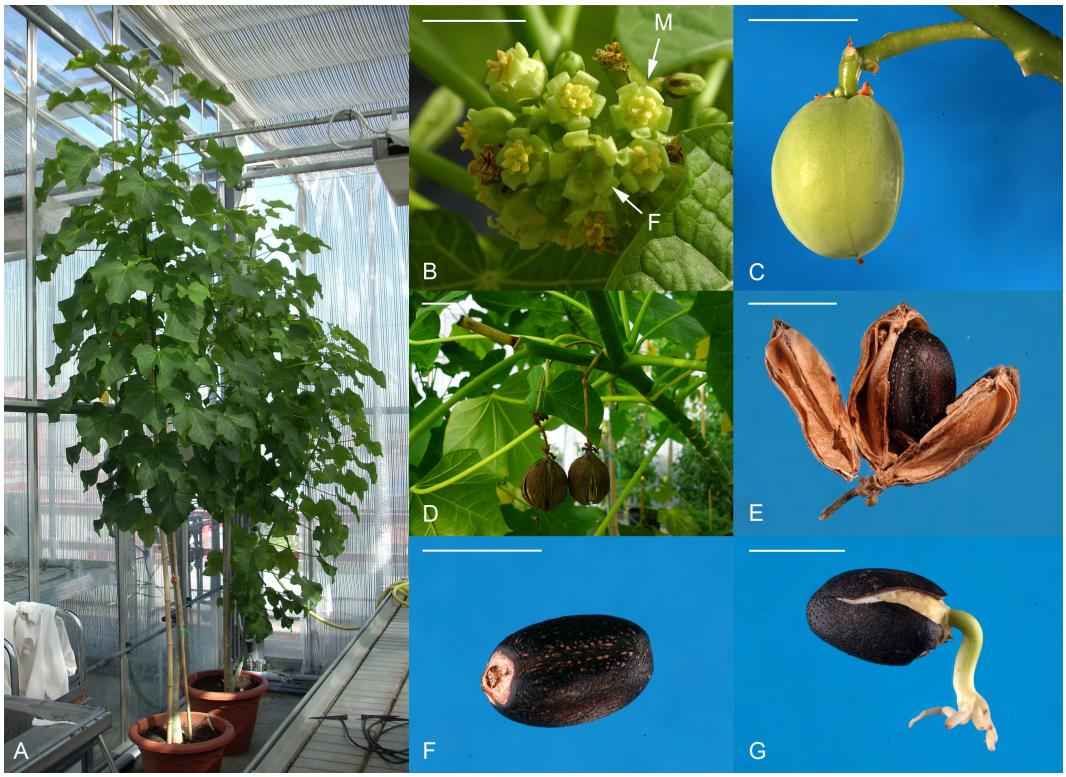

Figure 3-3. A: plants of *J. curcas* in the greenhouse of the Department of Biology, Padua; B: flowers; C: fruit; D: dry fruits; E: fruit with dry exhocarp; F: seed; G: germinated seed (bar = 1 cm)

3.1.2 *Jatropha curcas in vivo* can be propagated by cuttings

Propagation by cuttings is a tool to clonally propagate species of economic interest and to maintain valuable characteristics. Soft and hard cuttings (see 2.2.2) were performed from all the varieties available and the outliving percentage was greater for the hard cuttings respect to the soft cuttings. The latter need a strong control of moisture; in fact, in the same conditions of temperature, light and humidity of hard cuttings, they easily developed rotten areas close to the soil, causing the death of cuttings.

The morphology of the plants obtained from both soft and hard cuttings is correct, with the aerial part identical to normal plants (Figure 3-4) while none formation of the taproot was observed; as reported in literature, cuttings developed only lateral roots (Heller, 1996).

Figure 3-4. *J. curcas* cuttings. First row: hard cuttings after 2 weeks (left), after 1 month (right). Second row: soft cuttings after 1 month (left), after 2 months (right).

Rooting trials by addition of phytohormones, as auxin, worsen the rooting and the outliving of cuttings (data not shown). Cuttings from all the *J. curcas* available varieties, that survived the first 3 weeks, had a good capability of rooting and outliving. Over the 80% of the hard cuttings outlived while about the 50-60% of the soft cuttings were able to survive. These results reflect the weakness of soft cuttings: they need greater care then hard cuttings.

This tool have been proved to be useful and efficient to clonally propagate jatropha which has low percentage of germination in natural conditions and hardly guarantees the quality and quantity of the needed material to the large scale cultivation.

3.1.3 *Jatropha curcas* can be micropropagated *in vitro*

In vitro plants of *J. curcas* have been micropropagated by cuttings with a low rooting efficiency. The difficulty to obtain rooting explants could be reconducted to media moisture as described above in the case of root suffering. Cuttings were prepared from *in vitro* germinated seeds after at least one month of culture

Explant rooting was obtained on MSB5^{-}½ and, after 4 weeks and emergence of roots (Figure 3-5), the plants could be transferred to *in vivo* conditions. Roots were washed from agar with tap water and plants were transferred in pots with the mixture of 70% soil and of 30% LECA for the acclimatization period that lasted for 3 weeks. Plants were kept under controlled humidity to avoid leaf dehydration and, when acclimatized, transferred in standard greenhouse conditions.

Figure 3-5. Rooting of *J. curcas in vitro* cuttings

3.2 Binary vectors construction

Transformation with a gene involved in the biosynthesis of triglycerides was planned in order to try to increase the lipid accumulation in the green biomass and in the cell cultures of *J. curcas*. The chosen gene catalyzes the final rate-limiting step of triglycerides condensation: diacylglycerol acyltransferase (DGAT).

The binary vector pGreen was chosen for the *Agrobacterium*-mediated transformation and the first step was the cloning of the 35S-cassette of the Cauliflower Mosaic Virus (CaMV) into the empty pGreen0029 kanamycin resistant. *E. coli* strain DH5α was transformed by the heat-shock method (see 2.9) with the plasmid pGreen0029 and grown overnight at 37°C. Plasmidic DNA was extracted by miniprep Promega® and digestion with *XhoI* and *NotI* was performed. Results of the digestion are shown in Figure 3-6. As control, single digestions were performed with both enzymes and the expected band was observed between 4000 and 5000 base pairs (pGreen is 4632 bp long). The double digested pG0029 was purified from gel.

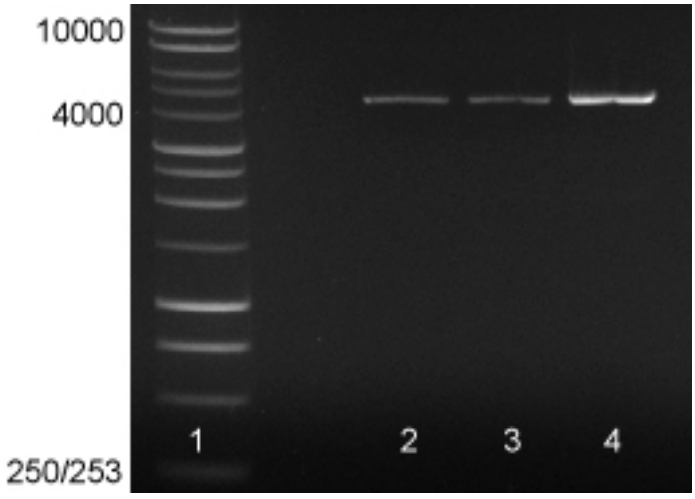

Figure 3-6. Electrophoretic run in 1% agarose gel. Lane 1: ladder; 2: plasmid pG0029 digested with *XhoI*; 3: plasmid pG0029 digested with *NotI*; 4: plasmid pG0029 double digested with *XhoI* and *NotI*.

The 35S-cassette was amplified by PCR. The PCR assay was performed with different annealing temperatures. In Figure 3-7 is shown the result of the electrophoretic run. The best primer annealing was obtained at 56°C (lane 3) and the cassette had a length of 691 bp.

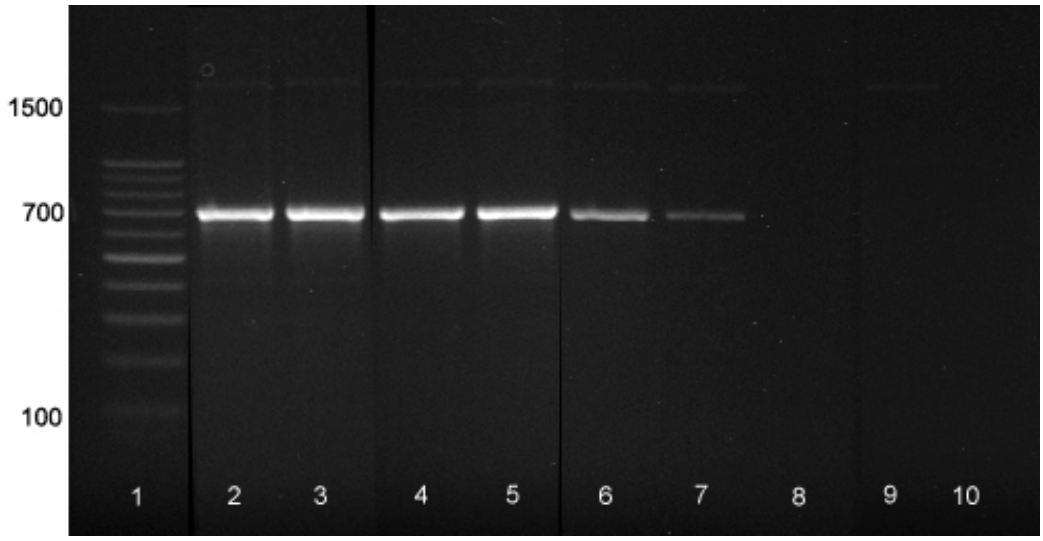

Figure 3-7. Electrophoretic run in 2% agarose gel. Lane 1: ladder; 2: T_{ann}= 50°C; 3: T_{ann}= 56°C; 4: T_{ann}= 60°C; 5: T_{ann}= 62°C; 6: T_{ann}= 65°C; 7: T_{ann}= 68°C; 8: no 35S-2_fw; 9: no 35S-2_rev; 10: template = water.

PCR amplicone was purified from gel and digested with *XhoI* and *NotI*. After another purification the 35S-cassette with cohesive end was cloned into the double digested pG0029 by an overnight ligation at 4°C.

E. coli DH5α competent cells were then transformed with the ligation product (see 2.9) and plated on LB agar added with IPTG 0.2 mM, X-gal 40 mg/L and kanamycin 50 mg/L. Positive colonies were picked up by the white/blue screening (Figure 3-8).

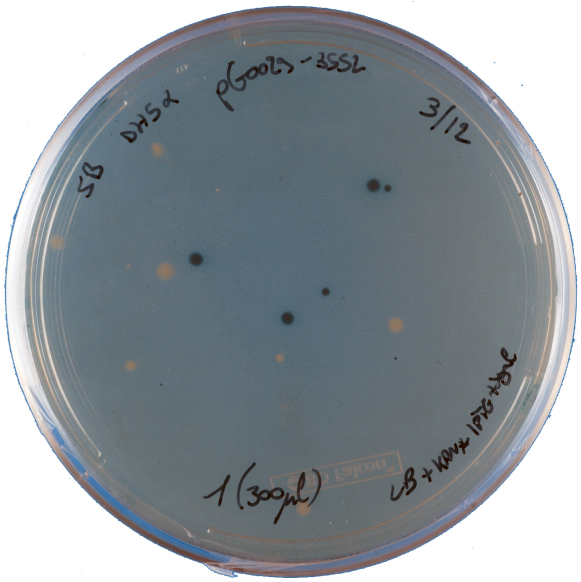

Figure 3-8. *E. coli* DH5α transformed with the plasmid pG2935 and plated on agarized LB added with 0.2 mM of IPGT, 40 mg/L of X-gal and 50 mg/L of kanamycin.

White colonies were inoculated in LB added with kanamycin and left to grow overnight. The plasmid, purified by miniprep, was long about 5200 bp and it was called **pG2935** (Figure 3-9).

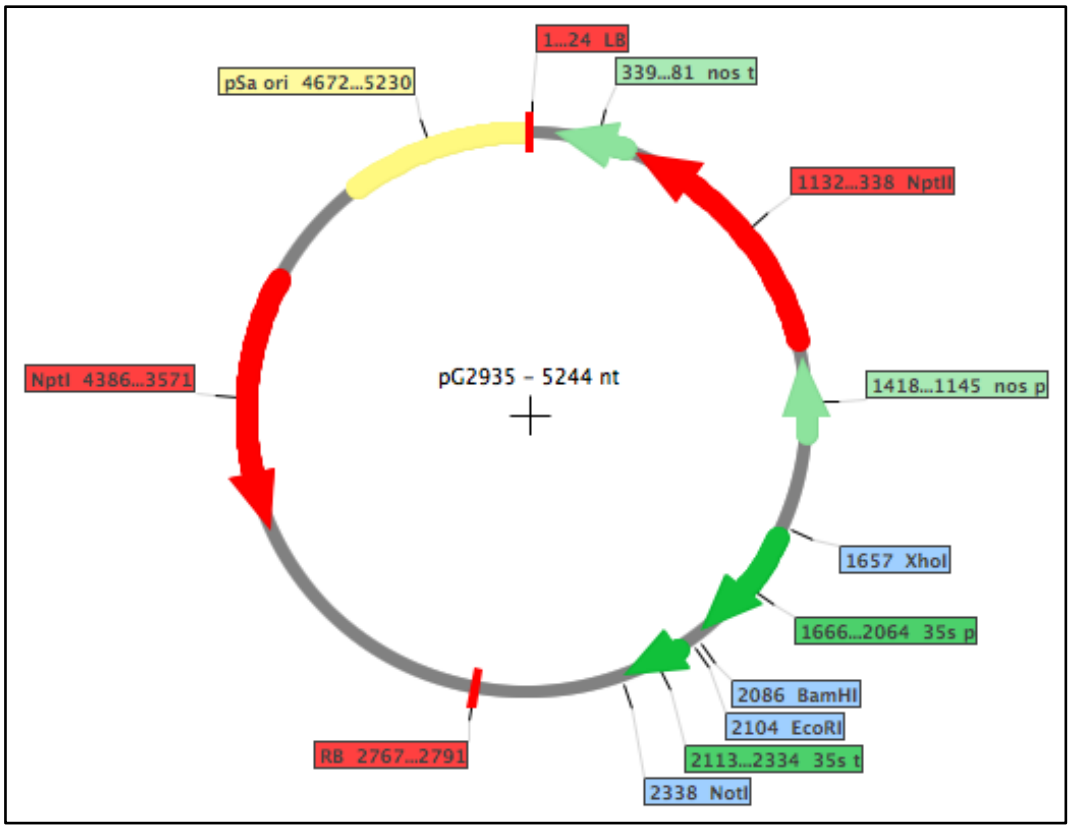

Figure 3-9. Representation of the pG2935. 35S-cassette is dark green. Important restriction enzyme sites are highlighted.

In parallel, the amplification of the genes eYFP and HA::DGAT was carried out. Primers were designed for cloning the two sequences between the restriction enzyme sites for *BamHI* and *EcoRI* present in the 35S cassette. As the DGAT has a restriction site for the enzyme *EcoRI* inside the sequence at the position 1170-1180, the experimental design gave place to the addiction to the 3'-terminal of this gene of the sequence for the enzyme *MfeI* instead of the sequence recognized by *EcoRI*: *EcoRI* and *MfeI* recognize different sequences (-GAATTC- the first, -CAATTG- the latter), but generate the same cohesive ends, permitting the cloning into the 35S-cassette.

The restriction sequence recognized by the enzyme *NdeI* was added at the 5'-terminal of the YFP sequence and at the 3'-terminal of the DGAT,

before the sequence recognized by *MfeI*, to generate the sequence coding for the fusion product HA::DGAT::YFP by another cloning.

The amplicone for the YFP was obtained from a plasmid already present in the laboratory, while the amplicone for the HA::DGAT was amplified from the vector pENTR223 received in the clone U16373 from TAIR. A preliminary PCR test was performed to choose the annealing temperature of the desired primers, considering that the Clontech Advantage® taq polymerase kit required an annealing and extension temperature of 68°C. As shown in Figure 3-10 primers gave good amplicones at all the checked temperatures.

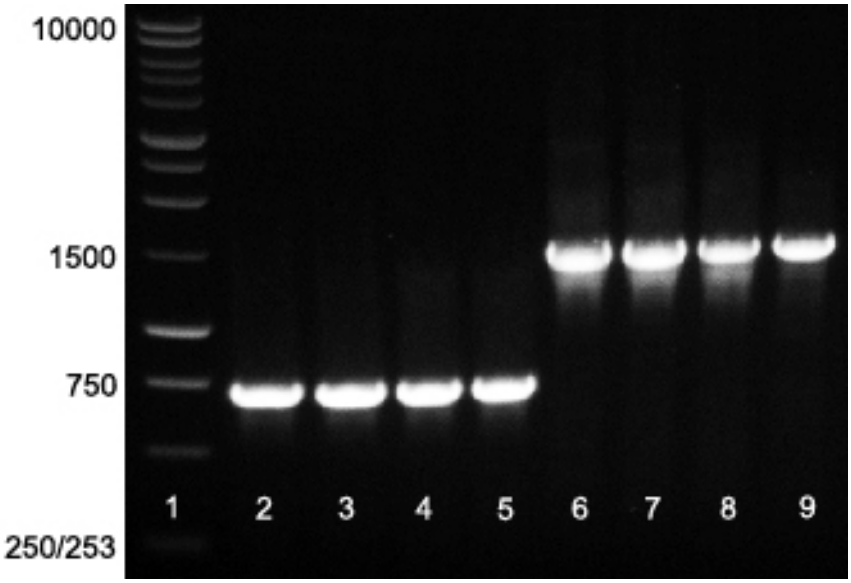

Figure 3-10. Electrophoretic run in 1% agarose gel. Lane 1: ladder; 2: YFP amplification at T_{ann}= 50°C; 3: YFP amplification at T_{ann}= 56°C; 4: YFP amplification at T_{ann}= 61°C; 5: YFP amplification at T_{ann}= 66°C; 6: DGAT amplification at T_{ann}= 50°C; 7: DGAT amplification at T_{ann}= 54°C; 8: DGAT amplification at T_{ann}= 61°C; 9: DGAT amplification at T_{ann}= 68°C.

Then the PCR was executed with the Clontech Advantage® according to the protocol provided by the producer, with the temperatures of annealing and extension set at 68°C. So, the amplicone of the YFP and the two amplicones of the HA::DGAT were obtained (Figure 3-11).

Electrophoretic run confirmed the expected length of the amplicones.

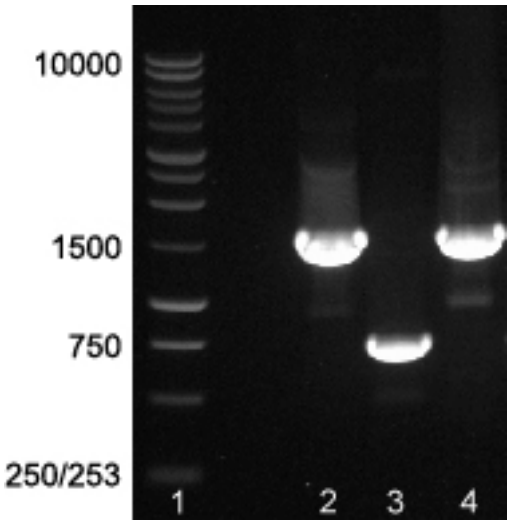

Figure 3-11. Electrophoretic run in 1% agarose gel. Lane 1: ladder; 2: DGAT amplicone obtained with the primers HDGAT*_fw_Bam and HDGAT*_rev_NdeMfe; 3: YFP amplicone obtained with the primers YFP_fw_BamNde and YFP_rev_Eco; 4: DGAT amplicone obtained with the primers HDGAT*_fw_Bam and HDGAT_rev_NdeMfe.

PCR products were purified with the Promega® miniprep system and, before cloning them into the pG2935, they were cloned into the Promega® pGEM®-T Easy Vector System. This step was necessary in order to optimize the successive reaction with the chosen restriction enzymes. Promega® pGEM®-T Easy Vector System is designed to permit a rapid selection of positive colonies by white/blue screening. White colonies were inoculated in LB added with 100 mg/L ampicillin and were left to grow overnight. pGEM®-T vectors were then collected by Promega® miniprep system and named: pGEMY, pGEMHD and pGEMHDY (see 2.11.5 for details).

Digestions were performed on these three plasmids to extract the inserts that had to be cloned into the pG2935. In particular, pGEMYFP was digested with *BamHI* and *EcoRI* while pGEMHD and pGEMHDY were digested with *BamHI* and *MfeI*. Bands corresponding to the gene sequences were purified from gel with the Promega® Wizard® SV Gel and PCR Clean-Up System kit and finally cloned into the pG2935 double digested with *BamHI* and *EcoRI*. The amount of DNA used in the ligation assays were:

- 130 ng of pG2935
- 96 ng of the insert bnYFPe

- 208 ng of the insert bHDGATnm
- 208 ng of the insert 5'bHDGATnm

Ligations were performed at 4°C overnight and *E. coli* strain DH5α was then transformed with the three different ligation products. Positive colonies were chosen after PCR screening.

Diagnostic digestions were then executed on the three plasmid isolated by an overnight culture of the three *E. coli* strains called pG29Y, pG29HD and pG29HD5, to have a first confirmation of the accuracy of the insert cloning into the backbone of the pG2935.

Concerning this, Figure 3-12 show diagnostic digestions for the **pG29Y** (35S**::**YFP construct). In particular, plasmid DNA was digested with *EcoRI*, *EcoRI* and *BamHI*, *EcoRI* and *NdeI*.

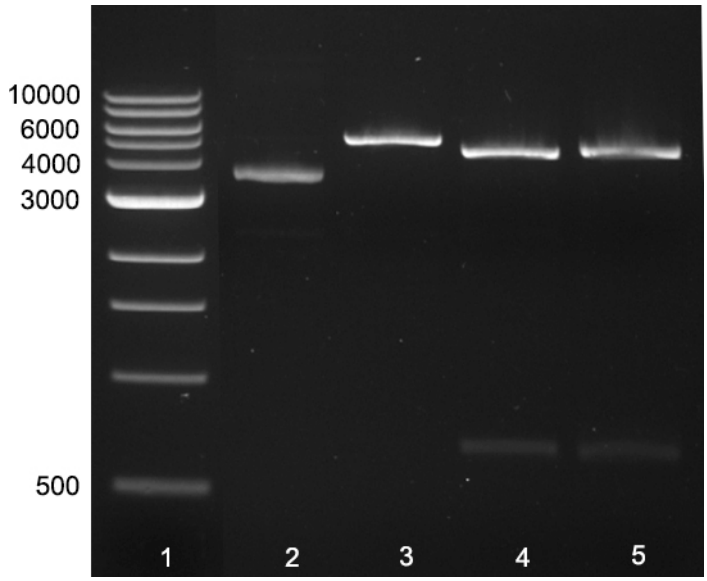

Figure 3-12. Diagnostic digestions of the plasmid pG29Y in a 1% agarose gel. Lane 1: ladder; 2: plasmidic DNA; 3: plasmid digested with *EcoRI*; 4: plasmid digested with *EcoRI* and *BamHI*; 5: plasmid digested with *EcoRI* and *NdeI*.

As expected, plasmidic DNA gave many bands (usually three bands) and the most brighten had a low molecular weight in comparison with the linearized one (lane 3); this is due to the supercoiled form of a closed plasmid. Linearization with *EcoRI* confirmed the expected dimensions, about 5900 bp.

Lane 4 and 5 confirmed that the YFP coding sequence is located between the correct sites for the chosen restriction enzymes (Figure 3-13).

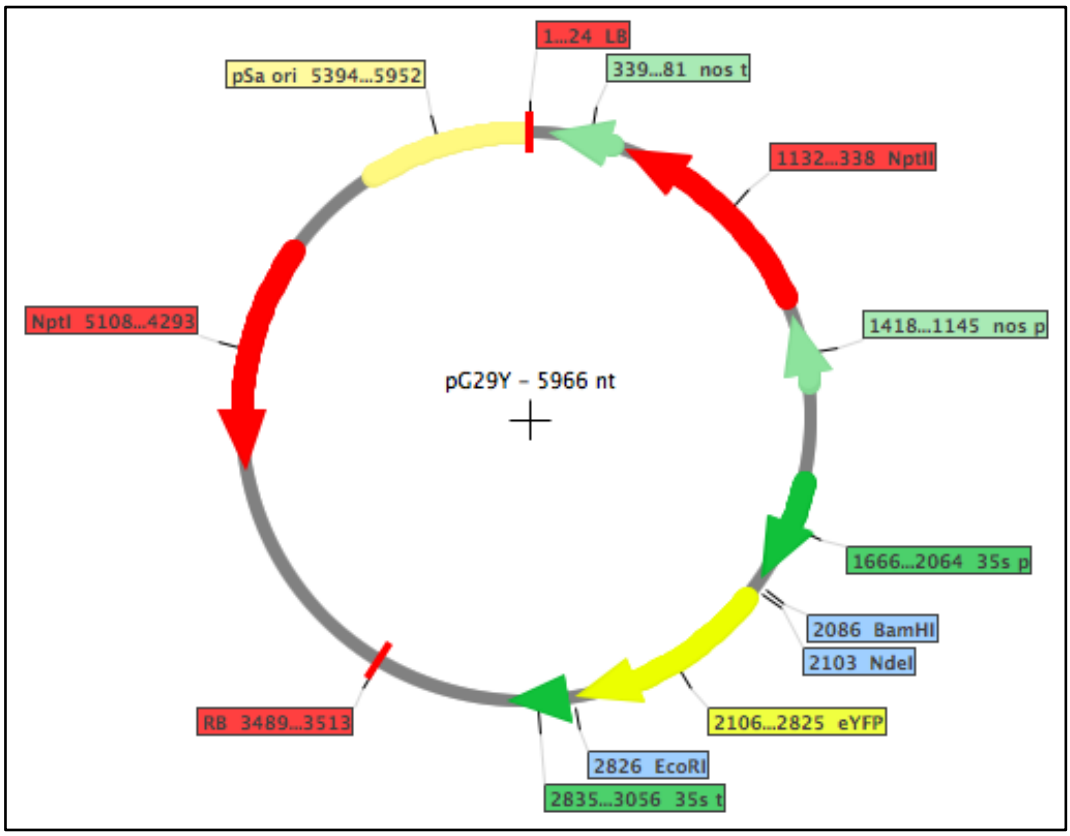

Figure 3-13. Representation of the plasmid pG29Y. The correct insertion of the YFP coding sequence into the multiple cloning site of the 35S-cassette is showed, together with the restriction sites useful for the cloning and the future insertion of the 5'bHDGAT.

Diagnostic digestions on the **pG29HD** and **pG29HD5** (35S::HA::DGAT construct) were performed with the enzymes *BamHI*, *NdeI* and *EcoRI* and results are shown in Figure 3-14 and Figure 3-16 respectively. Plasmidic DNA was digested with *BamHI* and *NdeI* to extract the coding sequence of the HA::DGAT, while the digestion with *EcoRI*, cutting into the sequence coding for the DGAT, should give a linearized plasmid (about 7000 bp).

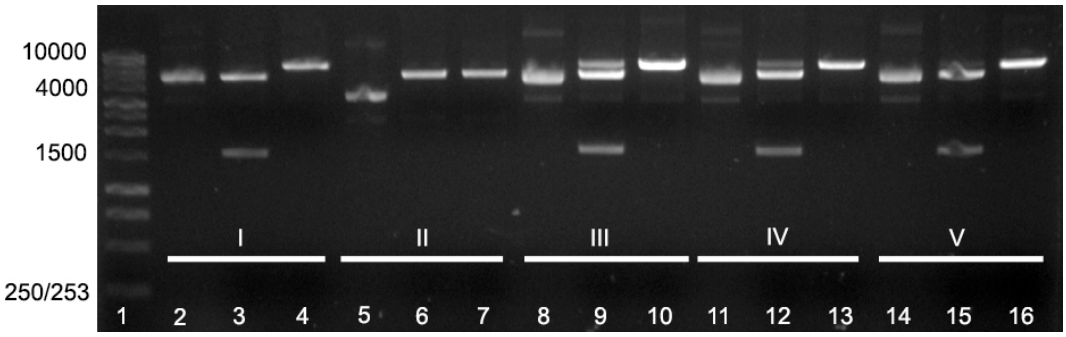

Figure 3-14. Diagnostic digestions of five plasmids pG29HD in a 1% agarose gel. Lane 1: ladder; 2-5-8-11-14: plasmidic DNA; 3-6-9-12-15: digestion with *BamHI* and *NdeI*; 4-7-10-13-16: digestion with *EcoRI*.

The screening revealed that the insert is present in all the samples, except the second colony. As expected, the linearized plasmid is about 7000 bp while the insert is a little bit longer than 1500 bp. In Figure 3-15 is represented pG29HD. In the samples III and IV is evident a low efficiency of one of the restriction enzymes: in fact, it can be observed the band corresponding to the whole linearized plasmid.

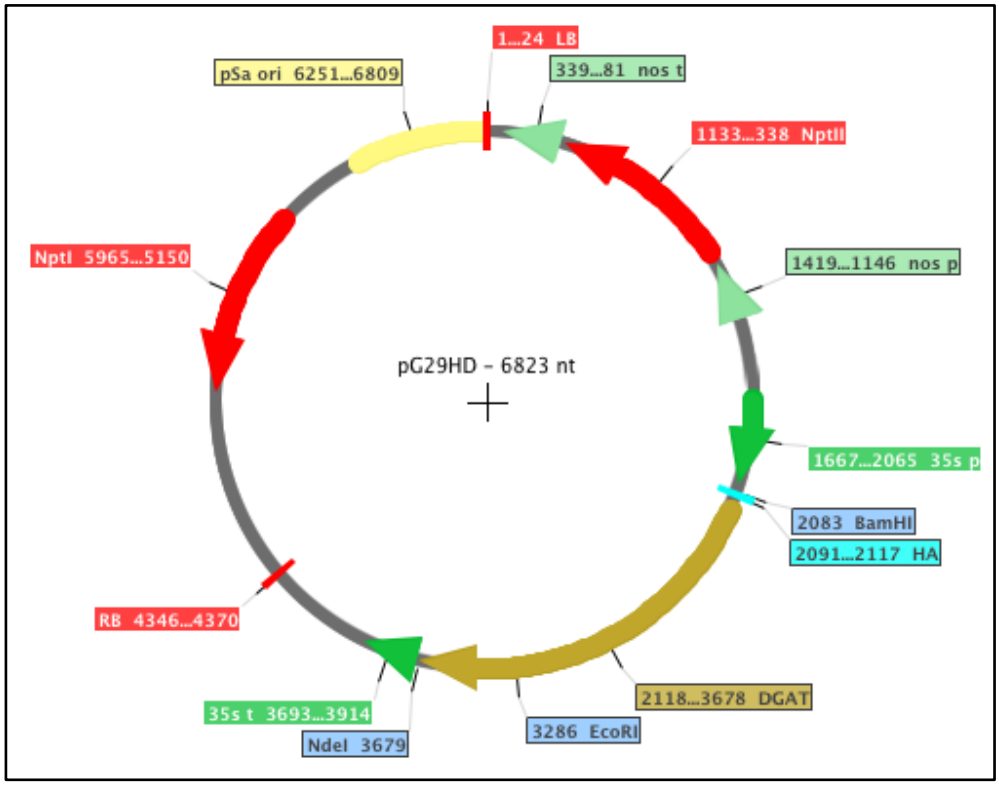

Figure 3-15. Representation of the plasmid pG29HD. The correct insertion of the DGAT coding sequence into the multiple cloning site of the 35S-cassette is showed, together with the restriction sites useful for the cloning and the diagnostic digestions.

The same considerations made for the diagnostic digestions of the plasmid pG29HD are true for the plasmid pG29HD5 (Figure 3-16).

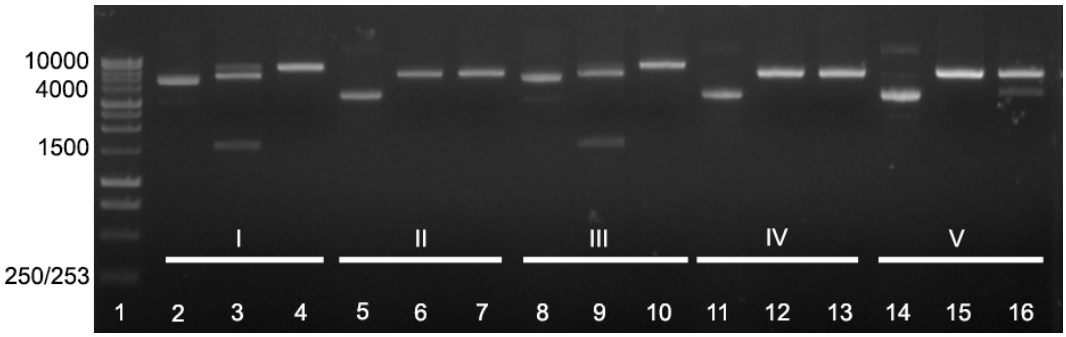

Figure 3-16. Diagnostic digestions of five plasmids pG29HD5 runned in a 1% agarose gel. Lane 1: ladder; 2-5-8-11-14: plasmid DNA; 3-6-9-12-15: digestion with *BamHI* and *NdeI*; 4-7-10-13-16: digestion with *EcoRI*.

In this case the first and the third colonies were considered positive as they integrated the sequence of interest. Low efficiency of one restriction enzyme is evident also in these digestions. Figure 3-17 is a representation of the plasmid pG29HD5.

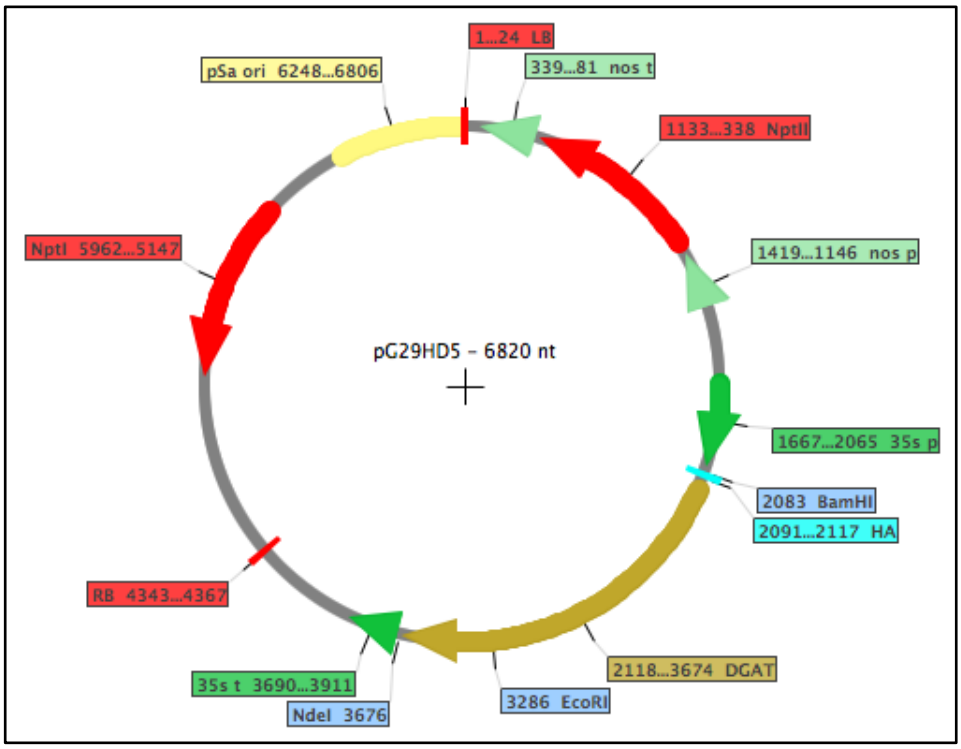

Figure 3-17. Representation of the plasmid pG29HD5. The correct insertion of the DGAT coding sequence into the multiple cloning site of the 35S-cassette is showed, together with the restriction sites useful for the cloning and the diagnostic digestions.

Once established the accuracy of digestion profiles, samples were sequenced at BMR-Genomics (Padova) to verify the absence of point mutations in the sequences due to the initial amplification by PCR.

The results of the sequencing are the following:

YFP

```
BamHI       NdeI
|           |
gga tcc tga cat atg ctg agc aag ggc gag gag ctg ttc acc ggg gtg gt  < 50
        *   H   M   L   S   K   G   E   E   L   F   T   G   V   V

g ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc g < 100
  P   I   L   V   E   L   D   G   D   V   N   G   H   K   F   S   V

tg tcc ggc gag ggc gaG GGC GAT GCC ACC TAC GGC AAG CTG ACC CTG AAG  < 150
   S   G   E   G   E   G   D   A   T   Y   G   K   L   T   L   K

TTC ATc TGC ACC ACC GGC AAG CTG CCC GTG CCC TGG CCC ACC CTC GTG AC  < 200
F   I   C   T   T   G   K   L   P   V   P   W   P   T   L   V   T

C ACC TTC GGc TAC GGC CTG CAG TGC TTC GCC CGC TAC CCC GAC CAC ATG A < 250
  T   F   G   Y   G   L   Q   C   F   A   R   Y   P   D   H   M   K

AG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG  < 300
   Q   H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E

CGC ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GT  < 350
R   T   I   F   F   K   D   D   G   N   Y   K   T   R   A   E   V

G AAG TTC GAG GGC GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC G < 400
  K   F   E   G   D   T   L   V   N   R   I   E   L   K   G   I   D

AC TTC AAG GAG GAC GGC AAC ATC CTG GGG CAC AAG CTG GAG TAC AAC TAC  < 450
   F   K   E   D   G   N   I   L   G   H   K   L   E   Y   N   Y

AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC AAG CAG AAG AAC GGC ATC AA  < 500
N   S   H   N   V   Y   I   M   A   D   K   Q   K   N   G   I   K

G GTG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC GTG CAG CTC G < 550
  V   N   F   K   I   R   H   N   I   E   D   G   S   V   Q   L   A

CC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG  < 600
   D   H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L

CCC GAC AAC CAC TAC CTG AGC TAC CAG TCc gcc ctg agc AAA GAC CCC AA  < 650
P   D   N   H   Y   L   S   Y   Q   S   A   L   S   K   D   P   N

C GAG AAG CGC GAT CAC Atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg a < 700
  E   K   R   D   H   M   V   L   L   E   F   V   T   A   A   G   I
                                          EcoRI
                                          |
tc act ctc ggc atg gac gag ctg tac aag taa gaa ttc  < 738
   T   L   G   M   D   E   L   Y   K   *
```

HA::DGAT

```
BamHI
|
GGA TCC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT GCG ATT TTG GAT TC  < 50
        M   Y   P   Y   D   V   P   D   Y   A   A   I   L   D   S

T GCT GGC GTT ACT ACG GTG ACG GAG AAC GGT GGC GGA GAG TTC GTC GAT C < 100
  A   G   V   T   T   V   T   E   N   G   G   G   E   F   V   D   L

TT GAT AGG CTT CGT CGA CGG AAA TCG AGA TCG GAT TCT TCT AAC GGA CTT  < 150
   D   R   L   R   R   R   K   S   R   S   D   S   S   N   G   L

CTT CTC TCT GGT TCC GAT AAT AAT TCT CCT TCG GAT GAT GTT GGA GCT CC  < 200
L   L   S   G   S   D   N   N   S   P   S   D   D   V   G   A   P

C GCC GAC GTT AGG GAT CGG ATT GAT TCC GTT GTT AAC GAT GAC GCT CAG G < 250
  A   D   V   R   D   R   I   D   S   V   V   N   D   D   A   Q   G

GA ACA GCC AAT TTG GCC GGA GAT AAT AAC GGT GGT GGC GAT AAT AAC GGT  < 300
   T   A   N   L   A   G   D   N   N   G   G   G   D   N   N   G

GGT GGA AGA GGC GGC GGA GAA GGA AGA GGA AAC GCC GAT GCT ACG TTT AC  < 350
G   G   R   G   G   G   E   G   R   G   N   A   D   A   T   F   T

G TAT CGA CCG TCG GTT CCA GCT CAT CGG AGG GCG AGA GAG AGT CCA CTT A < 400
  Y   R   P   S   V   P   A   H   R   R   A   R   E   S   P   L   S

GC TCC GAC GCA ATC TTC AAA CAG AGC CAT GCC GGA TTA TTC AAC CTC TGT  < 450
   S   D   A   I   F   K   Q   S   H   A   G   L   F   N   L   C

GTA GTA GTT CTT ATT GCT GTA AAC AGT AGA CTC ATC ATC GAA AAT CTT AT  < 500
V   V   V   L   I   A   V   N   S   R   L   I   I   E   N   L   M

G AAG TAT GGT TGG TTG ATC AGA ACG GAT TTC TGG TTT AGT TCA AGA TCG C < 550
  K   Y   G   W   L   I   R   T   D   F   W   F   S   S   R   S   L

TG CGA GAT TGG CCG CTT TTC ATG TGT TGT ATA TCC CTT TCG ATC TTT CCT  < 600
   R   D   W   P   L   F   M   C   C   I   S   L   S   I   F   P

TTG GCT GCC TTT ACG GTT GAG AAA TTG GTA CTT CAG AAA TAC ATA TCA GA  < 650
L   A   A   F   T   V   E   K   L   V   L   Q   K   Y   I   S   E

A CCT GTT GTC ATC TTT CTT CAT ATT ATT ATC ACC ATG ACA GAG GTT TTG T < 700
  P   V   V   I   F   L   H   I   I   I   T   M   T   E   V   L   Y

AT CCA GTT TAC GTC ACC CTA AGG TGT GAT TCT GCT TTT TTA TCA GGT GTC  < 750
   P   V   Y   V   T   L   R   C   D   S   A   F   L   S   G   V

ACT TTG ATG CTC CTC ACT TGC ATT GTG TGG CTA AAG TTG GTT TCT TAT GC  < 800
T   L   M   L   L   T   C   I   V   W   L   K   L   V   S   Y   A

T CAT ACT AGC TAT GAC ATA AGA TCC CTA GCC AAT GCA GCT GAT AAG GCC A < 850
  H   T   S   Y   D   I   R   S   L   A   N   A   A   D   K   A   N

AT CCT GAA GTC TCC TAC TAC GTT AGC TTG AAG AGC TTG GCA TAT TTC ATG  < 900
   P   E   V   S   Y   Y   V   S   L   K   S   L   A   Y   F   M

GTC GCT CCC ACA TTG TGT TAT CAG CCA AGT TAT CCA CGT TCT GCA TGT AT  < 950
V   A   P   T   L   C   Y   Q   P   S   Y   P   R   S   A   C   I

A CGG AAG GGT TGG GTG GCT CGT CAA TTT GCA AAA CTG GTC ATA TTC ACC G < 1000
  R   K   G   W   V   A   R   Q   F   A   K   L   V   I   F   T   G

GA TTC ATG GGA TTT ATA ATA GAA CAA TAT ATA AAT CCT ATT GTC AGG AAC  < 1050
   F   M   G   F   I   I   E   Q   Y   I   N   P   I   V   R   N

TCA AAG CAT CCT TTG AAA GGC GAT CTT CTA TAT GCT ATT GAA AGA GTG TT  < 1100
S   K   H   P   L   K   G   D   L   L   Y   A   I   E   R   V   L

G AAG CTT TCA GTT CCA AAT TTA TAT GTG TGG CTC TGC ATG TTC TAC TGC T < 1150
  K   L   S   V   P   N   L   Y   V   W   L   C   M   F   Y   C   F

TC TTC CAC CTT TGG TTA AAC ATA TTG GCA GAG CTT CTC TGC TTC GGG GAT  < 1200
   F   H   L   W   L   N   I   L   A   E   L   L   C   F   G   D
    EcoRI
    |
CGT GAA TTC TAC AAA GAT TGG TGG AAT GCA AAA AGT GTG GGA GAT TAC TG  < 1250
R   E   F   Y   K   D   W   W   N   A   K   S   V   G   D   Y   W

G AGA ATG TGG AAT ATG CCT GTT CAT AAA TGG ATG GTT CGA CAT ATA TAC T < 1300
  R   M   W   N   M   P   V   H   K   W   M   V   R   H   I   Y   F

TC CCG TGC TTG CGC AGC AAG ATA CCA AAG ACA CTC GCC ATT ATC ATT GCT  < 1350
   P   C   L   R   S   K   I   P   K   T   L   A   I   I   I   A

TTC CTA GTC TCT GCA GTC TTT CAT GAG CTA TGC ATC GCA GTT CCT TGT CG  < 1400
F   L   V   S   A   V   F   H   E   L   C   I   A   V   P   C   R

T CTC TTC AAG CTA TGG GCT TTT CTT GGG ATT ATG TTT CAG GTG CCT TTG G < 1450
  L   F   K   L   W   A   F   L   G   I   M   F   Q   V   P   L   V

TC TTC ATC ACA AAC TAT CTA CAG GAA AGG TTT GGC TCA ACG GTG GGG AAC  < 1500
   F   I   T   N   Y   L   Q   E   R   F   G   S   T   V   G   N

ATG ATC TTC TGG TTC ATC TTC TGC ATT TTC GGA CAA CCG ATG TGT GTG CT  < 1550
M   I   F   W   F   I   F   C   I   F   G   Q   P   M   C   V   L
                                                        NdeI
                                                        |
T CTT TAT TAC CAC GAC CTG ATG AAC CGA AAA GGA TCG ATG TCA TGA CAT A < 1600
  L   Y   Y   H   D   L   M   N   R   K   G   S   M   S   *

TG CAA TTC  < 1608
```

HA::DGAT (lacking the stop codon)

```
BamHI
|
GGA TCC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT GCG ATT TTG GAT TC  < 50
        M   Y   P   Y   D   V   P   D   Y   A   A   I   L   D   S

T GCT GGC GTT ACT ACG GTG ACG GAG AAC GGT GGC GGA GAG TTC GTC GAT C < 100
  A   G   V   T   T   V   T   E   N   G   G   G   E   F   V   D   L

TT GAT AGG CTT CGT CGA CGG AAA TCG AGA TCG GAT TCT TCT AAC GGA CTT  < 150
   D   R   L   R   R   R   K   S   R   S   D   S   S   N   G   L

CTT CTC TCT GGT TCC GAT AAT AAT TCT CCT TCG GAT GAT GTT GGA GCT CC  < 200
L   L   S   G   S   D   N   N   S   P   S   D   D   V   G   A   P

C GCC GAC GTT AGG GAT CGG ATT GAT TCC GTT GTT AAC GAT GAC GCT CAG G < 250
  A   D   V   R   D   R   I   D   S   V   V   N   D   D   A   Q   G

GA ACA GCC AAT TTG GCC GGA GAT AAT AAC GGT GGT GGC GAT AAT AAC GGT  < 300
   T   A   N   L   A   G   D   N   N   G   G   G   D   N   N   G

GGT GGA AGA GGC GGC GGA GAA GGA AGA GGA AAC GCC GAT GCT ACG TTT AC  < 350
G   G   R   G   G   G   E   G   R   G   N   A   D   A   T   F   T

G TAT CGA CCG TCG GTT CCA GCT CAT CGG AGG GCG AGA GAG AGT CCA CTT A < 400
  Y   R   P   S   V   P   A   H   R   R   A   R   E   S   P   L   S

GC TCC GAC GCA ATC TTC AAA CAG AGC CAT GCC GGA TTA TTC AAC CTC TGT  < 450
   S   D   A   I   F   K   Q   S   H   A   G   L   F   N   L   C

GTA GTA GTT CTT ATT GCT GTA AAC AGT AGA CTC ATC ATC GAA AAT CTT AT  < 500
V   V   V   L   I   A   V   N   S   R   L   I   I   E   N   L   M

G AAG TAT GGT TGG TTG ATC AGA ACG GAT TTC TGG TTT AGT TCA AGA TCG C < 550
  K   Y   G   W   L   I   R   T   D   F   W   F   S   S   R   S   L

TG CGA GAT TGG CCG CTT TTC ATG TGT TGT ATA TCC CTT TCG ATC TTT CCT  < 600
   R   D   W   P   L   F   M   C   C   I   S   L   S   I   F   P

TTG GCT GCC TTT ACG GTT GAG AAA TTG GTA CTT CAG AAA TAC ATA TCA GA  < 650
L   A   A   F   T   V   E   K   L   V   L   Q   K   Y   I   S   E

A CCT GTT GTC ATC TTT CTT CAT ATT ATT ATC ACC ATG ACA GAG GTT TTG T < 700
  P   V   V   I   F   L   H   I   I   I   T   M   T   E   V   L   Y

AT CCA GTT TAC GTC ACC CTA AGG TGT GAT TCT GCT TTT TTA TCA GGT GTC  < 750
   P   V   Y   V   T   L   R   C   D   S   A   F   L   S   G   V

ACT TTG ATG CTC CTC ACT TGC ATT GTG TGG CTA AAG TTG GTT TCT TAT GC  < 800
T   L   M   L   L   T   C   I   V   W   L   K   L   V   S   Y   A

T CAT ACT AGC TAT GAC ATA AGA TCC CTA GCC AAT GCA GCT GAT AAG GCC A < 850
  H   T   S   Y   D   I   R   S   L   A   N   A   A   D   K   A   N

AT CCT GAA GTC TCC TAC TAC GTT AGC TTG AAG AGC TTG GCA TAT TTC ATG  < 900
   P   E   V   S   Y   Y   V   S   L   K   S   L   A   Y   F   M

GTC GCT CCC ACA TTG TGT TAT CAG CCA AGT TAT CCA CGT TCT GCA TGT AT  < 950
V   A   P   T   L   C   Y   Q   P   S   Y   P   R   S   A   C   I

A CGG AAG GGT TGG GTG GCT CGT CAA TTT GCA AAA CTG GTC ATA TTC ACC G < 1000
  R   K   G   W   V   A   R   Q   F   A   K   L   V   I   F   T   G

GA TTC ATG GGA TTT ATA ATA GAA CAA TAT ATA AAT CCT ATT GTC AGG AAC  < 1050
   F   M   G   F   I   I   E   Q   Y   I   N   P   I   V   R   N

TCA AAG CAT CCT TTG AAA GGC GAT CTT CTA TAT GCT ATT GAA AGA GTG TT  < 1100
S   K   H   P   L   K   G   D   L   L   Y   A   I   E   R   V   L

G AAG CTT TCA GTT CCA AAT TTA TAT GTG TGG CTC TGC ATG TTC TAC TGC T < 1150
  K   L   S   V   P   N   L   Y   V   W   L   C   M   F   Y   C   F

TC TTC CAC CTT TGG TTA AAC ATA TTG GCA GAG CTT CTC TGC TTC GGG GAT  < 1200
   F   H   L   W   L   N   I   L   A   E   L   L   C   F   G   D
     EcoRI
     |
CGT GAA TTC TAC AAA GAT TGG TGG AAT GCA AAA AGT GTG GGA GAT TAC TG  < 1250
R   E   F   Y   K   D   W   W   N   A   K   S   V   G   D   Y   W

G AGA ATG TGG AAT ATG CCT GTT CAT AAA TGG ATG GTT CGA CAT ATA TAC T < 1300
  R   M   W   N   M   P   V   H   K   W   M   V   R   H   I   Y   F

TC CCG TGC TTG CGC AGC AAG ATA CCA AAG ACA CTC GCC ATT ATC ATT GCT  < 1350
   P   C   L   R   S   K   I   P   K   T   L   A   I   I   I   A

TTC CTA GTC TCT GCA GTC TTT CAT GAG CTA TGC ATC GCA GTT CCT TGT CG  < 1400
F   L   V   S   A   V   F   H   E   L   C   I   A   V   P   C   R

T CTC TTC AAG CTA TGG GCT TTT CTT GGG ATT ATG TTT CAG GTG CCT TTG G < 1450
  L   F   K   L   W   A   F   L   G   I   M   F   Q   V   P   L   V

TC TTC ATC ACA AAC TAT CTA CAG GAA AGG TTT GGC TCA ACG GTG GGG AAC  < 1500
   F   I   T   N   Y   L   Q   E   R   F   G   S   T   V   G   N

ATG ATC TTC TGG TTC ATC TTC TGC ATT TTC GGA CAA CCG ATG TGT GTG CT  < 1550
M   I   F   W   F   I   F   C   I   F   G   Q   P   M   C   V   L
                                                          NdeI
                                                          |
T CTT TAT TAC CAC GAC CTG ATG AAC CGA AAA GGA TCG ATG TCA CAT ATG   < 1600
  L   Y   Y   H   D   L   M   N   R   K   G   S   M   S

CAA TTC  < 1608
```

To obtain the plasmid coding for the fusion product, pG29Y and pG29HD5 were both digested with *BamHI* and *NdeI* (Figure 3-18).

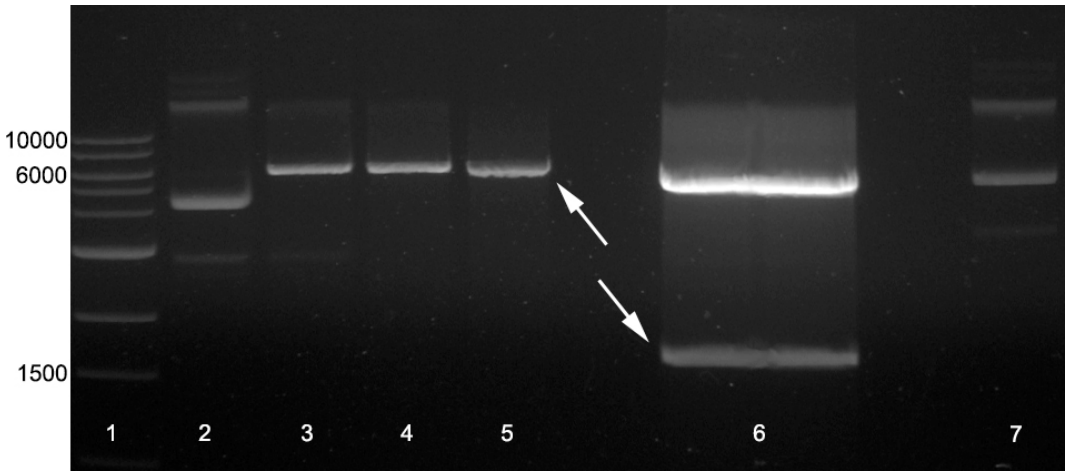

Figure 3-18. Digestions of plasmids pG29Y and pG29HD5 loaded on 1% agarose gel. Lane 1: ladder; 2: plasmid pG29Y; 3: plasmid pG29Y digested with *BamHI*; 4: plasmid pG29Y digested with *NdeI*; 5: plasmid pG29Y digested with both *BamHI* and *NdeI*; 6: plasmid pG29HD5 digested with both *BamHI* and *NdeI*; 7: plasmid pG29HD5.

After the electrophoretic run, DNA from the lane 5, corresponding to the open pG29Y, and from the lower band (1500 bp) of the lane 6, corresponding to the insert HA::DGAT without stop codon, were purified from gel with the Wizard® SV Gel and PCR Clean-Up System kit (Promega®).

Ligation was then carried out with about 100 ng of vector (pG29Y) and 80 ng of insert (HA::DGAT), overnight at 4°C. Then *E. coli* strain DH5α was transformed with the ligation product, plated on agarized LB containing 50 mg/L kanamycin and left to grow overnight at 37°C.

Colonies were screened by PCR (Figure 3-19) using the primers for the 35S-cassette. The presence of the construct 35S::HA::DGAT::YFP was revealed by a band at about 3000 bp, while negative colonies gave an amplicone at about 1400 bp (35S::YFP).

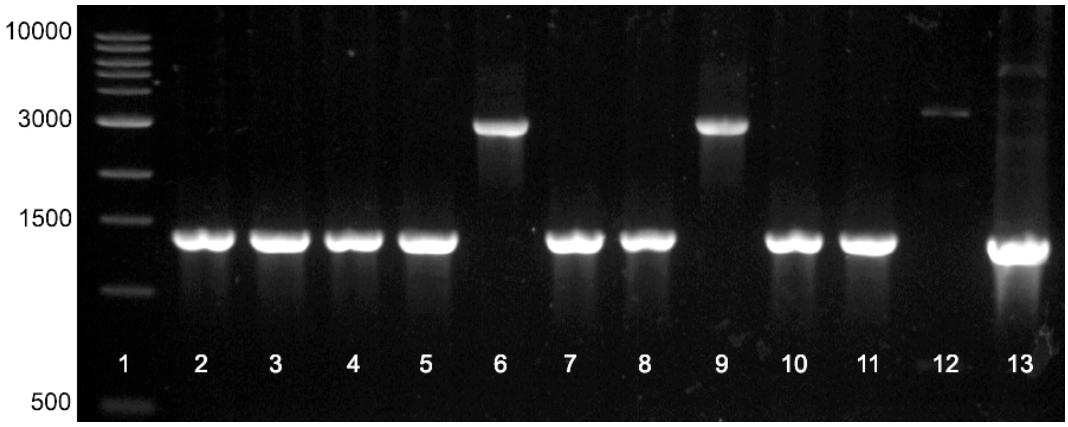

Figure 3-19. Screening PCR in 1% agarose gel. Lane 1: ladder; lanes 2-11: colony PCR; lane 12: PCR on w.t. *E. coli* DH5α; lane 13: PCR on plasmid pG29HD. Colonies 6 and 9 are positive.

The two positive samples were inoculated in LB added with 50 mg/L kanamycin and left to grow overnight. Plasmidic DNA was collected by miniprep and called **pG29HDY** (35S::HA::DGAT::YFP construct); diagnostic digestions were performed again (Figure 3-20).

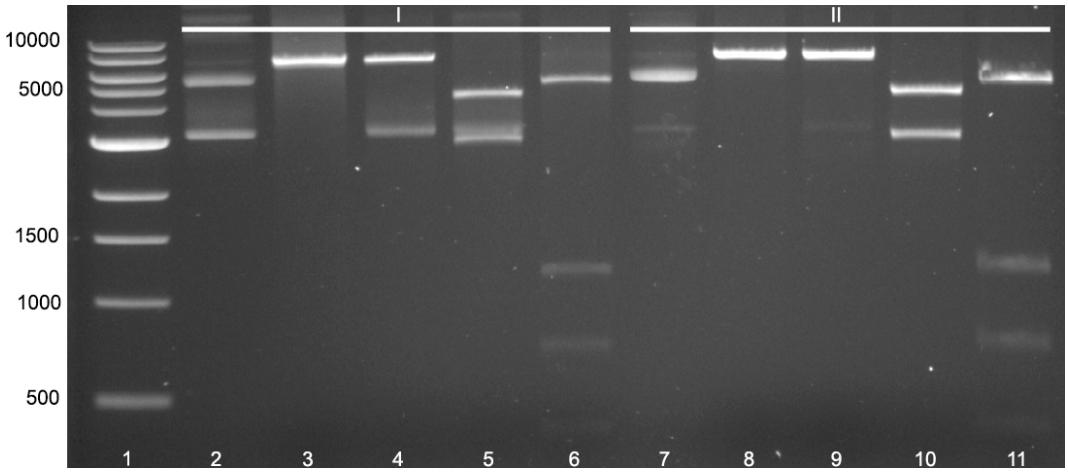

Figure 3-20. Diagnostic digestions on the plasmid pG29HDY of the two positive colonies. Lane 1: ladder; 2-7: plasmid pG29HDY; 3-8: plasmid pG29HDY digested with *NdeI*; 4-9: plasmid pG29HDY digested with *BamHI*; 5-10: plasmid pG29HDY digested with *XhoI* and *NotI*; 6-11: plasmid pG29HDY digested with *BamHI, NdeI* and *EcoRI*.

The two pG29HDY purified from the two positive colonies seems to had a different kind of supercoiling. In fact the band pattern for the intact plasmid is different, as it is different the digestion pattern with *BamHI* and the double digestion with *XhoI* and *NotI*: it can be hypothesized that the first plasmid has a strong supercoiling and restriction sites are partially hidden and inaccessible to the enzymes. Linearization gave a band between 6000

and 8000 bp as expected as pG29HDY should be about 7500 bp long, and the double digestion with *XhoI* and *NotI* showed two bands: the highest (< 5000 bp) is the backbone of the plasmid pG0029 while the lowest (3000 bp) is the cassette expressing for the fusion product. Also the triple digestion gave the correct result: the highest band (> 5000 bp) is the backbone of the plasmid pG2935 while the other three bands are the result of the splicing of the YFP and the DGAT coding sequences and a cut by *EcoRI* into the DGAT sequence. The representation of the plasmid pG29HDY is showed in Figure 3-21.

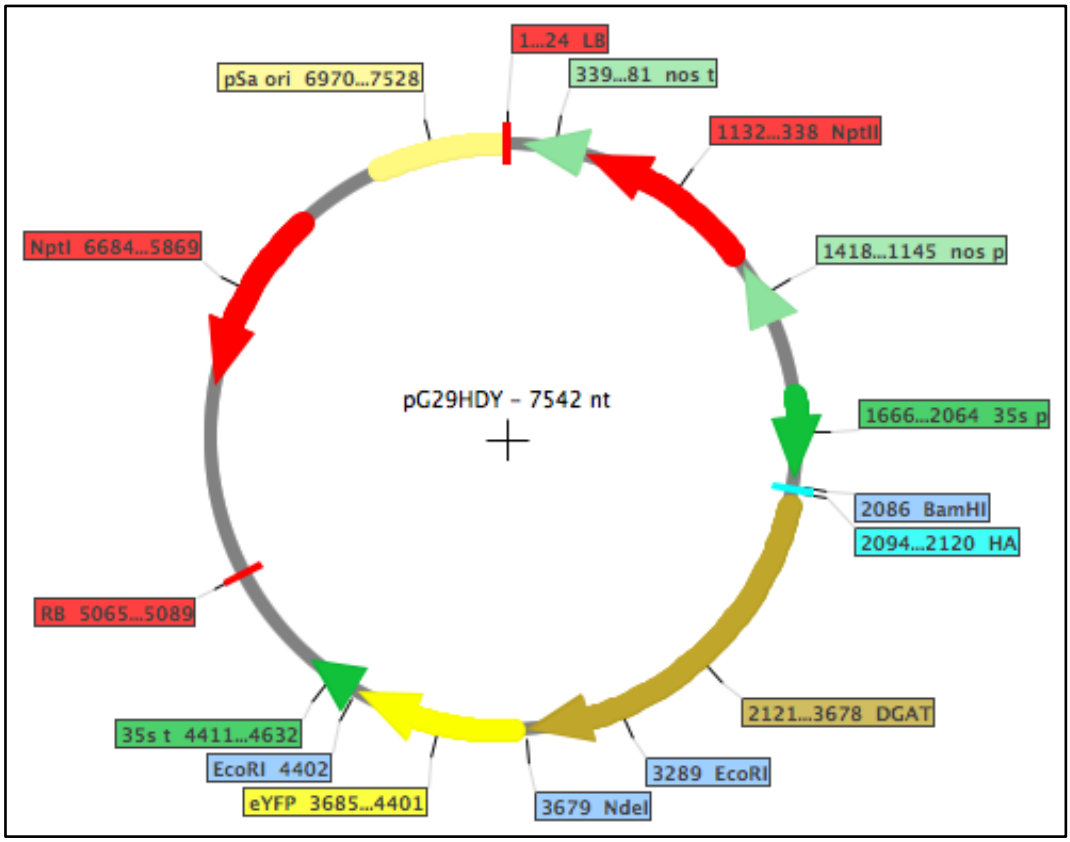

Figure 3-21. Representation of the plasmid pG29HDY. The correct insertion of the DGAT coding sequence before the YFP coding sequence into the pG29Y is showed, together with the restriction sites useful for the cloning and the diagnostic digestions.

Then, the *A. tumefaciens* strain GV3101 was co-transformed with each different plasmid and pSoup (see 2.10) and plated on YEP added with 1.6% Bacto™ agar, 25 mg/L of gentamycin, 100 mg/L of rifampicin and 50 mg/L of kanamycin. Even if pSoup confers resistance to tetracycline this antibiotic is not necessary because pGreen cannot replicate without pSoup.

Colonies appeared after 4-5 days of incubation at 28°C. They were inoculated in the same medium without agar and left to grow overnight. Then were stocked for the next use in transformation experiments.
The strains were called GV3101Y, GV3101HD and GV3101HDY.

3.3 Plant transformations

A. tumefaciens mediated transformation was planned to overexpress the *Arabidopsis thaliana* DGAT gene in plants of *J. curcas*. *N. tabacum* was chosen as transformation control in all the experiments.
N. tabacum is a model species and well-established propagation protocols have already been executed. Plants were grown *in vivo* and *in vitro* as described in 2.2.3, 2.2.5 and 2.2.6 in a controlled climatic chamber. Plants grown *in vitro* have been mainly used for stable transformation with the desired plasmids, while plants grown *in vitro* were mainly intended for transient transformation assay.

3.3.1 *Nicotiana tabacum* propagation *in vivo*

Tobacco plants were obtained *in vivo* by sowing wild type seeds in vessels where germinated plants were left to grow until leaves were ready for agroinfiltration (Figure 3-22). When needed, plants were transferred in larger pots where they can grow until flowering and seed collecting.

Figure 3-22. Plantlets (left) and 1 month-old plants of tobacco (right)

3.3.2 *Nicotiana tabacum* propagation *in vitro*

Tobacco plants were propagated *in vitro* by cutting with a scalpel the apexes or internodal explants and planting in new MS^{-} ½ medium added with 0.8% of plant agar (Duchefa) in Linfabox vessels (Figure 3-23). Root emergence was observed after 1 week from the transfer in the new media and new cuttings were performed every 30-40 days.

Figure 3-23. Rooting of wild type tobacco cuttings *in vitro.*

Before performing the stable transformation on *J. curcas* explants there was the need to know if this species was transformable with *A. tumefaciens* and how it is possible to select transformed explants.

3.3.3 *Jatropha curcas* can be transformed by *Agrobacterium tumefaciens* GV3101

Agroinfiltration was performed on leaves of *J. curcas* to check if *A. tumefaciens* GV3101 was able to transform this species. Transient transformation was performed with the *Agrobacterium* strains GV3101Y and GV3101HDY, as the expression of the YFP produces an easily detectable signal.

Agroinfiltration was executed as described in 2.12.1 and infected plants (Figure 3-24) were kept in a growth chamber at 22°C with a light/dark cycle of 16 hours of light and 8 hours of dark.

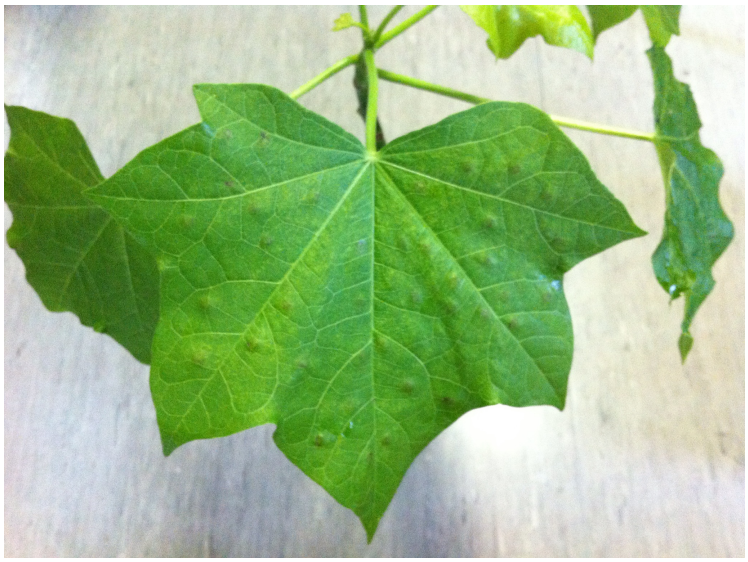

Figure 3-24. Agroinfiltrated leaf of *J. curcas*

Leaves were observed after 3, 5 and 7 days after agroinfiltration. The best fluorescence is recorded at 5 days (Figure 3-25). Agroinfiltration of jatropha leaves was harder respect to tobacco leaves, with a lower area subjected to the injection of the bacterial solution.

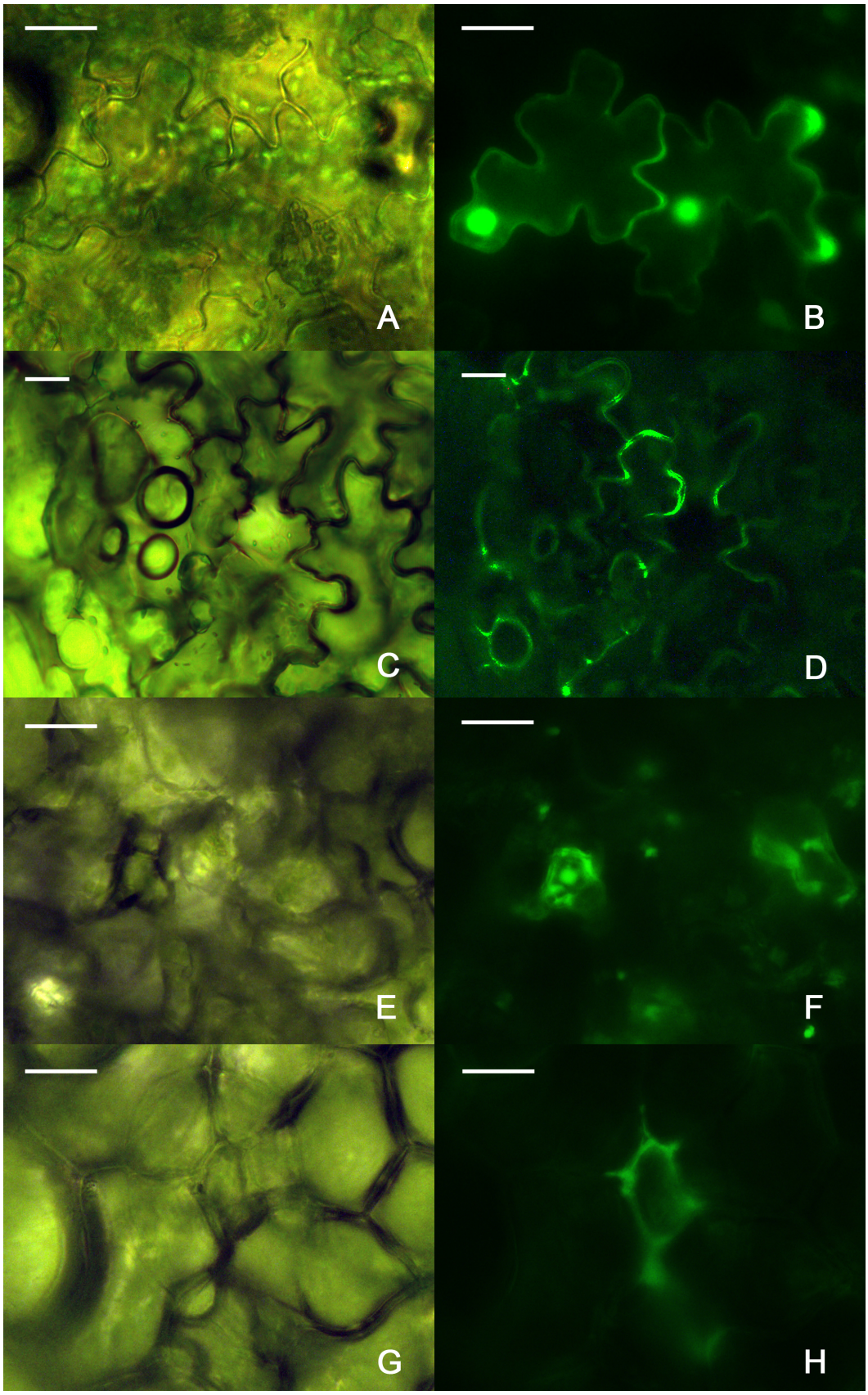

Figure 3-25. Agroinfiltration of jatropha and tobacco leaves. A-D: tobacco leaves; E-H: jatropha leaves; A-B-E-F: leaves agroinfiltrated with GV3101Y; C-D-G-H: leaves agroinfiltrated with GV3101HDY; A-C-E-G: bright field; B-D-F-H: fluorescence field.

In Figure 3-25 is shown the fluorescence emitted by the YFP alone in *N. tabacum* (B) and *J. curcas* (F), and the fluorescence emitted by the YFP fused to the DGAT in *N. tabacum* (D) and *J. curcas* (H). The leaf structure is characterized, in epidermal and mesophyll cells, by the presence of a wide vacuole that press the cytosolic fraction close to the plasma membrane and the cell wall; this impaired an accurate subcellular localization of the two different expression products. It is possible to observe the signal of the YFP, codified by the plasmid pG29Y, localized in the cytosol and in the nucleus of both plant species (Figure 3-25 B and F); in literature the translocation of this small fluorescent molecule to the nucleus was previously described (Grebenok *et al.*, 1997; Brandizzi *et al.*, 2002). The transient expression of the fusion product codified by the plasmid pG29HDY results in a localized signal that seems to be confined in specific cellular compartments. It is known that the DGAT enzyme could be a transmembrane protein that acts in the endoplasmic reticulum and is able to correctly localize also if fused with a fluorescent protein (Shockey *et al.*, 2006).

The fluorescence recorded in agroinfiltered area of jatropha leaves are localized in limited portion of the tissue while in tobacco agroinfiltered leaves all the cells of the infected area are fluorescent; this could be due to a different efficiency of the agroinfiltration. *J. curcas* leaves in fact are coriaceous and epidermal cells have a thicker wall rich of cutin and waxes respect to tobacco leaves. Another possibility is a different efficiency in the transformation process mediated by *A. tumefaciens*: jatropha could be harder to transform compared to tobacco, a plant that is known for its easily manipulation.

Moreover it is important to underline that *Agrobacterium* proliferation in the apoplast, as its potential of infection, may be very variable; furthermore physiological conditions of the leaf are determinant. Infection and DNA transfer mediate by *A. tumefaciens* does not happen in a specific moment: agrobacteria can infect plant cells in a long lapse of time, meanwhile they are proliferating in the aqueous layer of the apoplast, rich in sugars and salts (Denecke *et al.*, 2012).

However these images demonstrate that *J. curcas* is susceptible to the *A. tumefaciens*-mediated transformation.

3.3.4 Transgenic explants of *Jatropha curcas* can be selected with kanamycin

Once established that *J. curcas* can be transformed and before performing the stable transformation, it was necessary to define the antibiotics necessary to select transformed cells and to eliminate *A. tumefaciens*: if the T-DNA does not integrate in the genetic set of the plant cells, resistance to a particular antibiotic is not observed. pG0029 is resistant to kanamycin so resistance of *J. curcas* explants to this substance was assayed. Moreover both cefotaxime and carbenicillin were chosen as antibiotic against *Agrobacterium*. All the antibiotics were added to the medium at concentrations as follow:

- 50 mg/L or 200 mg/L of kanamycin
- 500 mg/L of cefotaxime
- 500 mg/L of carbenicillin

Leaf explants were placed in agarized MSB5J containing the antibiotics. Kanamycin was able to block cellular proliferation at the two concentrations assayed. Cefotaxime did not affect the normal callus proliferation while carbenicillin induced a stronger proliferation respect to the control without antibiotics (Figure 3-26).

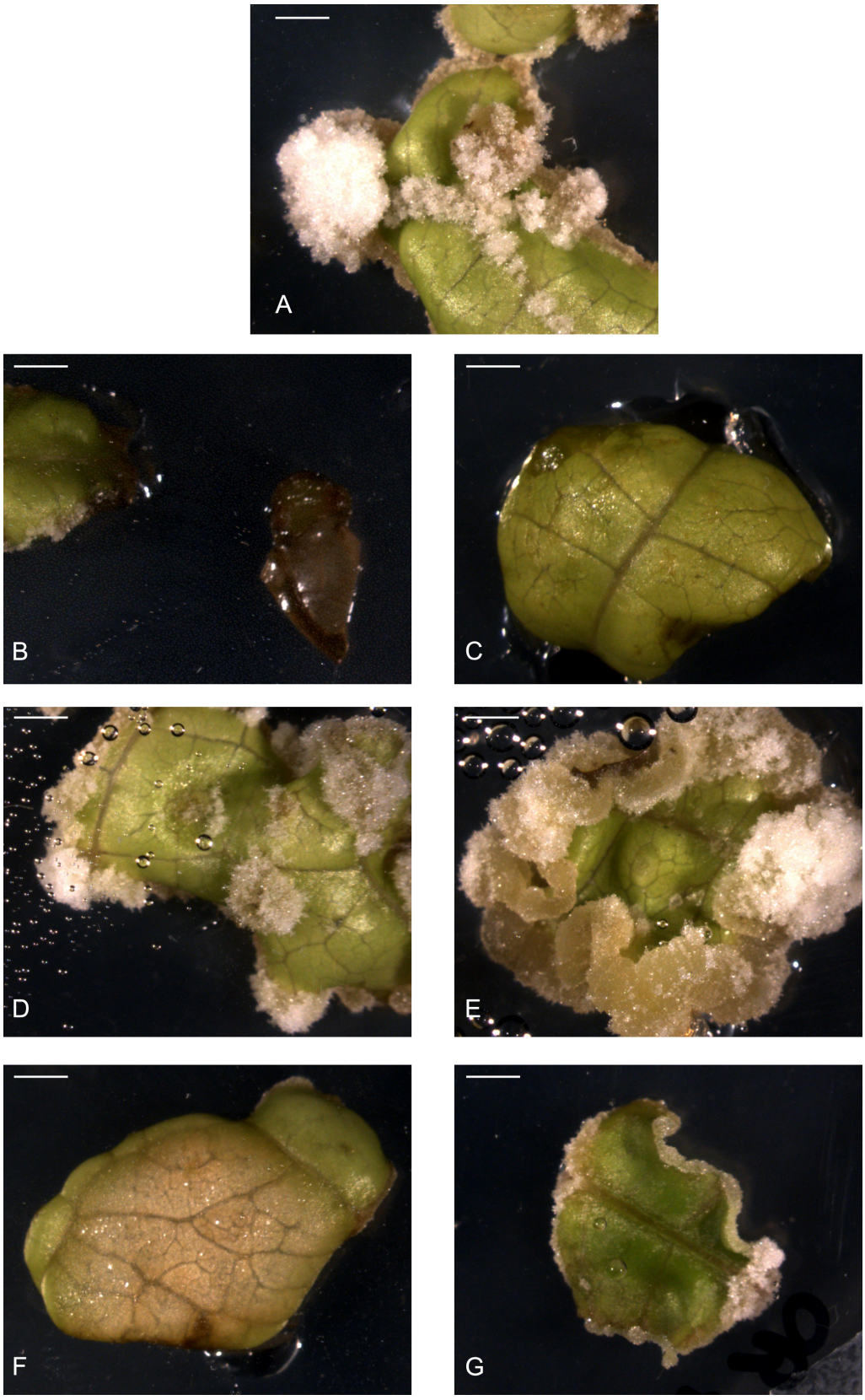

Figure 3-26. Response at antibiotics of *J. curcas* explants after one month of culture. A: control; B: 50 mg/L of kanamycin; C: 200 mg/L of kanamycin; D: 500 mg/L of cefotaxime; E: 500 mg/L of carbenicillin; F: 50 mg/L of kanamycin and 500 mg/L of cefotaxime; G: 50 mg/L of kanamycin and 500 mg/L of carbenicillin.

In Figure 3-26 A is showed the normal proliferation induced from *J. curcas* leaf explants, while Figure 3-26 B and C shows the total inhibition caused by kanamycin at both the concentrations assayed. Cefotaxime (Figure 3-26 D) did not affect callus proliferation otherwise carbenicillin (Figure 3-26 E) induces a stronger cellular proliferation. In combination with kanamycin, no proliferation is observed adding cefotaxime (Figure 3-26 F), while carbenicillin (Figure 3-26 G) induces a slighter callus proliferation, when kanamycin was at the concentration of 50 mg/L (Table 3-1).

		no antibiotics	cefotaxime	carbenicillin
no antibiotics		P	P	P+
kanamycin	50 mg/L	NP	NP	P-
	200 mg/L	NP	NP	NP

Table 3-1. Results of the antibiotic screening on cellular proliferation from *J. curcas* leaf explants. P = proliferation, NP = no proliferation.

Thus the concentrations chosen to select transformed cells were 50 mg/L of kanamycin and 500 mg/L of cefotaxime. Antibiotic concentrations used for tobacco transformation experiments were already known (Horsch *et al*., 1985).

3.3.5 *Jatropha curcas* stable transformation

Stable transformation of *J. curcas* leaf explants from all the accession available was performed following 2.12.2. Explants of 1 cm^2 were sterilized and adapted for 4 days on MSB5J solidified with 0.8% plant agar. After the co-culture in the agrobacteria solution and other two days on the same media, explants were transferred in the selective MSB5J agarized media containing the opportune antibiotics described in 3.3.4 (Figure 3-27).

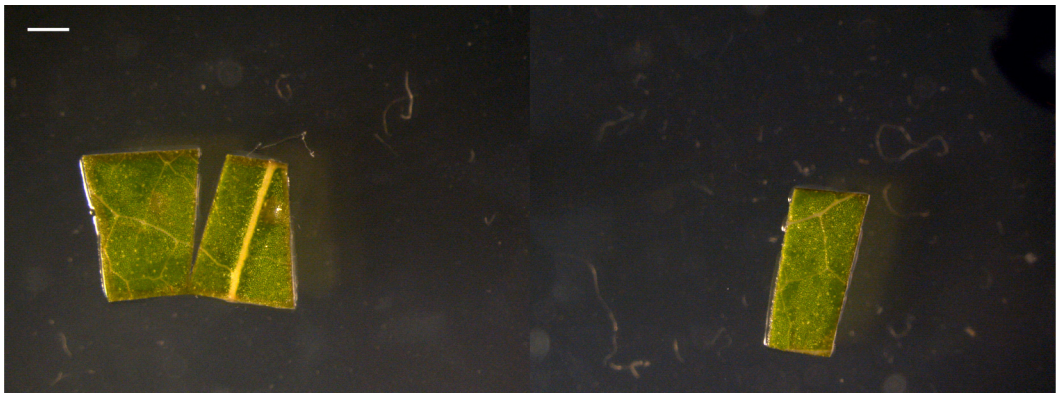

Figure 3-27. *J. curcas* explants plated on MSB5J added with 50 mg/L of kanamycin and 500 mg/L of cefotaxime (bar = 2mm).

Callogenesis was observed in control plates, where explants were plated in absence of antibiotics, after 4 weeks of culture. Cell proliferation was obtained from explants of all the varieties with slight differences in terms of proliferation rate. Little calli were observed from transformed explants after 8-12 weeks of culture with a slow proliferation rate. Best results were observed for explants of the varieties MXHU and MXOA. In Figure 3-28 calli obtained after 4 months of culture are shown: wild type calli proliferation (Figure 3-28 A) is higher respect to transformed calli (Figure 3-28 C, E and G) and there is no evidence of fluorescence in the emission of the YFP (Figure 3-28 B). As expected, calli transformed with the plasmids pG29Y and pG29HDY (Figure 3-28 D and F) showed an emission at the wavelength of the YFP, confirming the integration of the sequence carried by the two vectors. At this stage it is not possible to assure the transformation with the plasmid pG29HD (Figure 3-28 G and H), but the response of explants comparable to the other transformed ones, and resistance to kanamycin suggest that the transformation has occurred correctly. A resume about all the varieties is reported in Table 3-2.

	wild type		transformed	
	% viable explants	% callogenesis	% viable explants	% callogenesis
MXHU	100	100	30±4	100
MXMO	100	100	27±2	80±8
MXZA	100	90±3	10±2	50±6
MXOA	100	100	28±3	100
MXPU	100	92+4	13±2	60±4

Table 3-2. Resume of callogenesis experiments on all the available varieties of *J. curcas*

Wild type explants viability was of the 100% far all the varieties plated on MSB5J, with a little decrease in the percentage of callogenesis for the varieties MXZA and MXPU. More differences were observed when transformations were attempted: the varieties MXHU, MXOA and MXMO showed a percentage of viability of the explants of about 30%, while explants of varieties MXZA and MZPU showed a lower explant outliving. The different behaviour could be due to diverse intra varieties efficiency of transformation or to different physiological conditions of the leaves. Moreover, the reduced percentage of callogenesis from wild type explants of the varieties MXZA and MXPU was also reflected by the lower callogenesis percentage of transformed explants. Media were renewed every 4 weeks waiting to have the minimum amount of calli required to apply the regeneration protocol set up for regeneration of wild type explants.

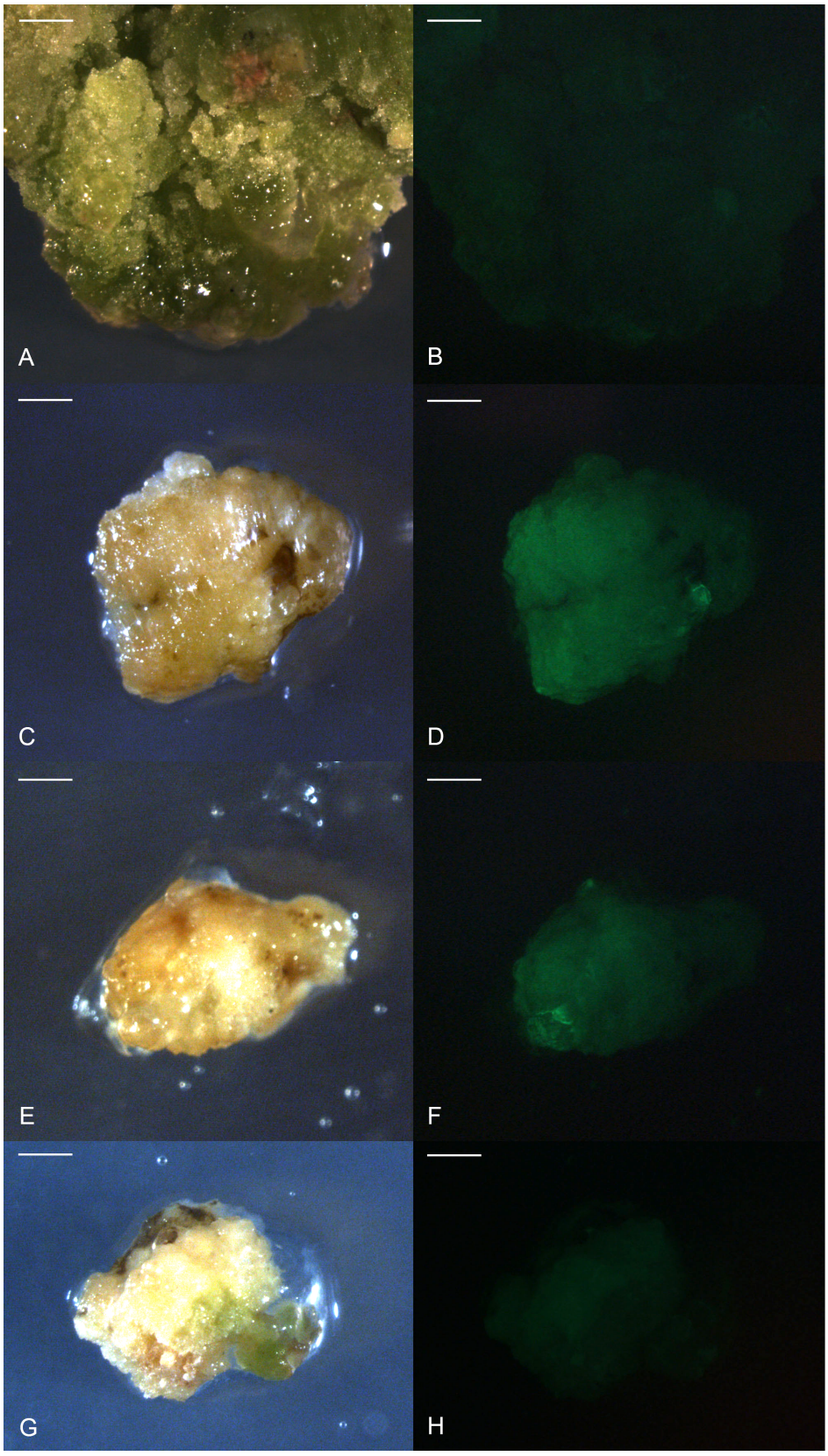

Figure 3-28. Callogenesis from wild type explants and transformed explants of *J. curcas* varieties MXHU. A-C-E-G: bright field; B-D-F-H: fluorescent field; A-B: wild type; C-D: callus transformed with pG29Y; E-F: callus transformed with pG29HDY; G-H: callus transformed with pG29HD.

3.3.6 *In vitro* regeneration of *Jatropha curcas* from proliferating calli

To obtain transformed plants via regeneration a valuable protocol was setup on wild type calli in parallel to realization of constructs, transient and stable transformations.

Regeneration of *J. curcas* through wild type calli was performed as described in 2.5, moving cell culture obtained after at least 2 months of culture in MSB5J (Figure 3-29 A) to MSB5JR. Within 2 months well-extended shoots were observed (Figure 3-29 B).

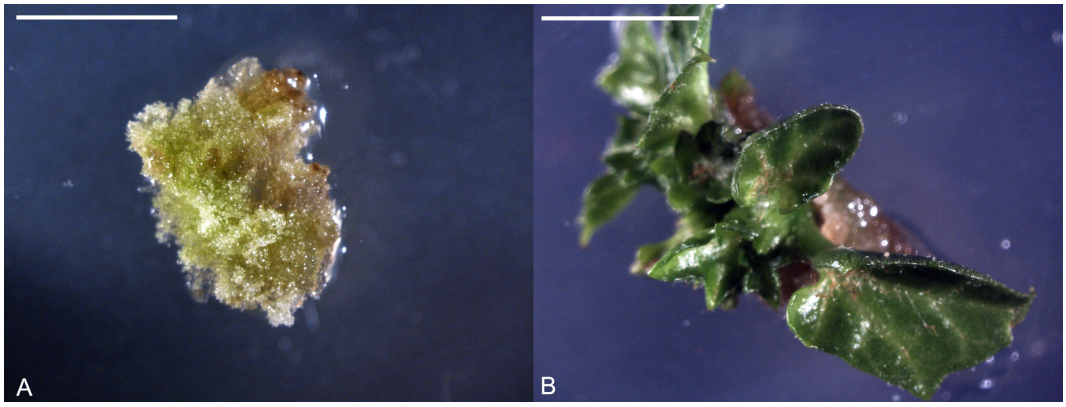

Figure 3-29. Shoot induction from *J. curcas* calli. A: proliferating callus; B: well-extend shoot (bar = 1 cm).

The shoots can be transferred in Linfabox vessels on MSB5^{-}½ in order to achieve rooting and whole plant regeneration. This step is still not optimized in terms of culture conditions and further assays are needed.

In parallel to stable transformation of *J. curcas*, a known protocol (Horsch *et al.*, 1985) was applied to transform tobacco plants, the chosen experimental control.

3.3.7 *Nicotiana tabacum* stable transformation

Leaf explants were taken from *in vitro* plants (see 3.3.2) and transformation through regeneration was carried out. Explants were transformed by co-culture with *A. tumefaciens* harbouring the chosen plasmid and plated on agarized MSIB and containing 50 mg/L kanamycin and 500 mg/L cefotaxime. The wild type explants, plated on media without antibiotics, showed shoots development (Figure 3-30 A) earlier respect to the transformed explants (Figure 3-30 B). After 4-6 weeks the shoots were

transferred in Linfabox vessels, containing MS^{-}½ agarized with 0.8% of plant agar, were rooting was observed after 7 days.

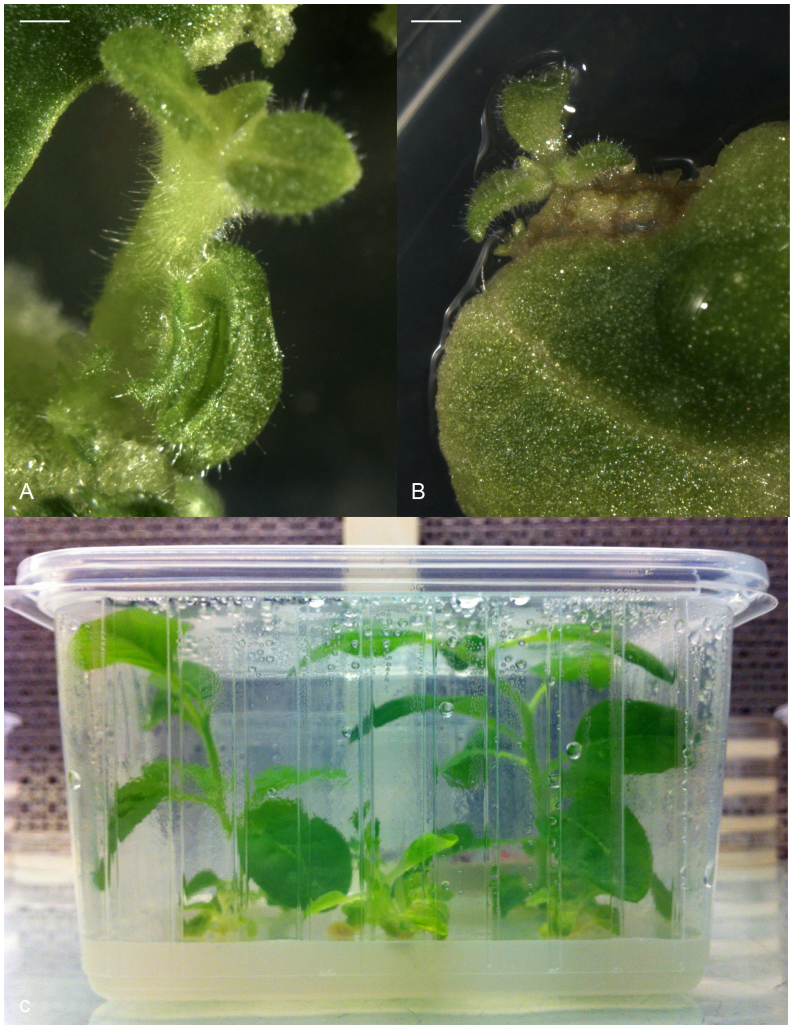

Figure 3-30. Regeneration of tobacco transformed plants from shoots. A: wild type shoot after 4 weeks from induction (bar = 1 mm); B: transformed shoot after 4 weeks from induction (bar = 1 mm); C: transformed plants after 4 weeks from transfer in Linfabox vessel.

Only transformed plants are able to root and outlive when kanamycin was added at 50 mg/L (Figure 3-30 C).

Callus proliferation was obtained from leaves of these tobacco plants grown *in vitro* placing leaf explants on MST. Callogenesis was observed from all the explants and, to confirm transformation, calli expressing the YFP were observed with the fluorescence microscope (Figure 3-31).

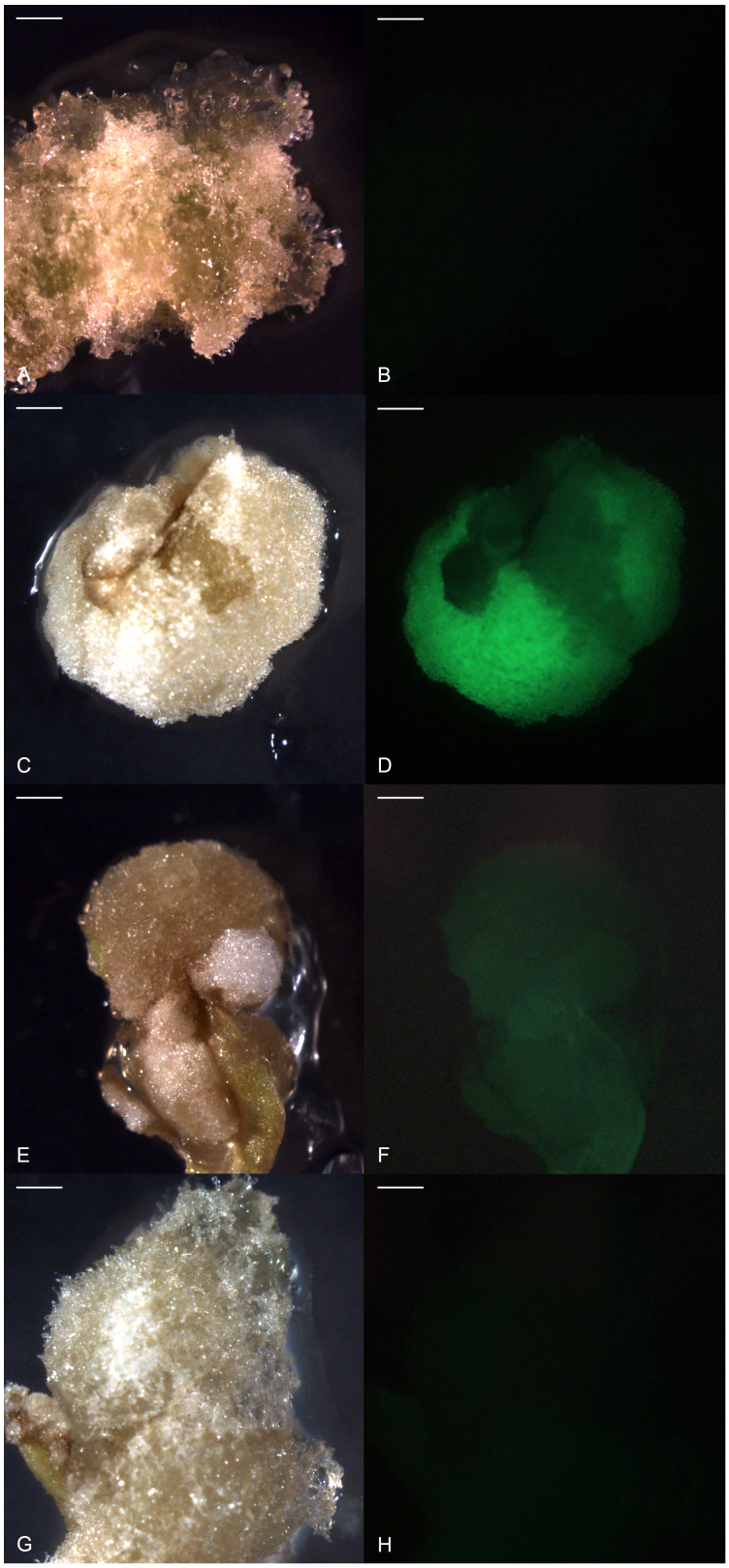

Figure 3-31. Callogenesis from wild type explants and transformed explants of *N. tabacum*. A-C-E-G: bright field; B-D-F-H: fluorescent field. A-B: wild type; C-D: callus transformed with plasmid pG29Y; E-F: callus transformed with plasmid pG29HDY; G-H: callus transformed with pG29HD.

In Figure 3-31 are shown calli obtained after 2 months of culture: wild type calli proliferation (Figure 3-31 A) is slightly higher respect to transformed calli (Figure 3-31 C, E and G) and there is no evidence of fluorescence in the emission of the YFP (Figure 3-31 B). Calli transformed with the plasmids pG29Y and pG29HDY (Figure 3-31 D and F), therefore expressing the constructs, showed green signal after excitation at the wavelength of the YFP (488 nm), confirming the integration of the sequence carried by the two vectors. At this stage it is not possible to assure the transformation with the plasmid pG29HD (Figure 3-31 G and H), but the response of explants and resistance to kanamycin suggest that the transformation has occurred correctly.

Moreover, from both calli and leaves of tobacco, a PCR assay was planned to quickly detect the presence of the constructs in the genomic DNA. Extraction was performed as described in 2.13 and results are shown in Figure 3-32.

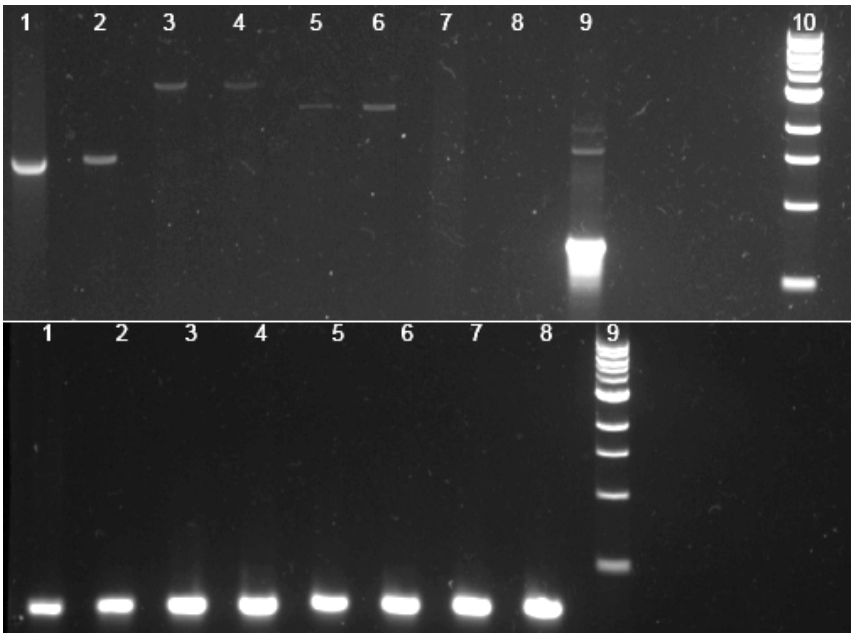

Figure 3-32. Electrophoretic run in 1% agarose gel of the PCRs on genomic DNA of transformed cells and leaves of tobacco. First gel: lane 1: PCR from calli transformed with pG29Y; lane 2: PCR from leaves transformed with pG29Y; lane 3: PCR from calli transformed with pG29HDY; lane 4: PCR from leaves transformed with pG29HDY; lane 5: PCR from calli transformed with pG29HD; lane 6: PCR from w.t. leaves; lane 9: PCR on pG2935; lane 10: ladder. Second gel: lanes 1-8: PCR with primers for 18S on the same samples of the first gel; lane 9: ladder.

In both transformed calli and leaves it was possible to amplify the constructs indicating the integration of the sequences in the genomic DNA, while in wild type calli was not observed any amplification. Amplifications of the 18S were performed as control of the reaction (second gel of Figure 3-32).

Stable transformation of tobacco was successfully achieved and cell cultures were obtained from transformed plants. Also *J. curcas* can be transformed and calli resistant to kanamycin were obtained. More experiments are needed to achieve transformed jatropha plants, from which cell cultures can be obtained.

3.4 *Jatropha curcas* cells were successfully grown in liquid media

In order to simulate growth conditions in bioreactor, suspension cultures of *J. curcas* were obtained inoculating small amounts of calli from solid to liquid MSB5J medium. After an adaptation time to the new cultivation medium, that lasted from 2 to 4 months, acclimatized suspension cultures were obtained (Figure 3-33) and it was possible to assess the growth curve as described in 2.4.1.

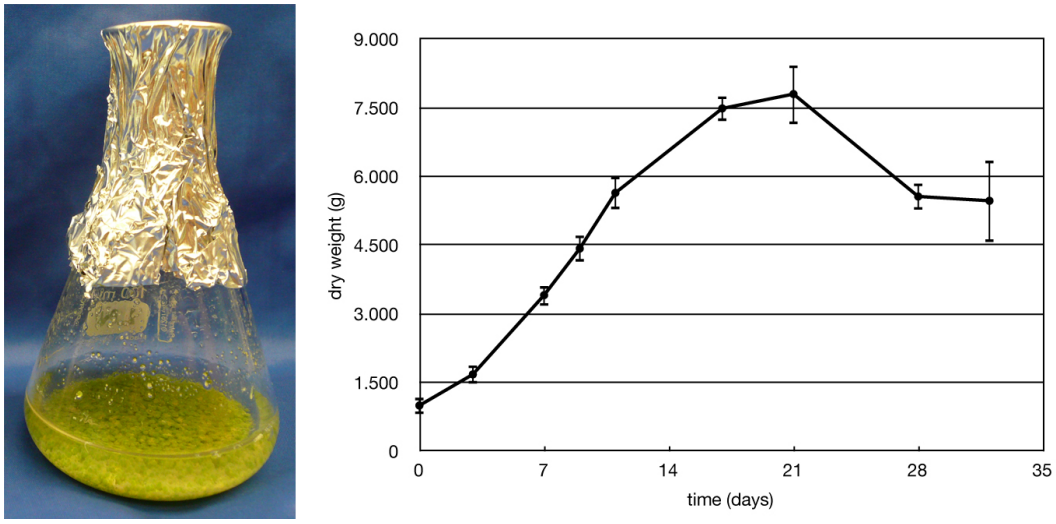

Figure 3-33. Suspension cell culture of *J. curcas* and its calculated growth curve in 50 mL of medium.

Growth curve was prepared measuring the dry weight of the culture at fixed time. The experiment was conducted in triple and every point of the

curve is the mean of the obtained values (Figure 3-33). Jatropha cell growth was slow in the first 4 days of culture: in the lag phase, in fact, cells are in adaptation to the medium renewal. In the next phase the culture grew quickly reaching a biomass production of 8 times respect to the inoculum. The log phase lasts until day 21 when cell biomass entered the death phase. According to this data the medium renewal of cell cultures was executed every 15 days to take advantage of the exponential growth.
Evans blue dye was used to assay the viability of the cell culture in the late log phase: percentage of death cells is about the 19%. This value is greater respect to the common rate of cell culture, that is about the 10%.

3.5 Lipid assay on wild type seeds and plant cell cultures

The success of the planned transformation depends not only on the transformation itself, but also on the comparison of the TAG accumulated by wild type seeds and cell cultures respect to transformed ones.
Triglyceride content in seeds was estimated with the lipid assay (see 2.14). A linear standard curve was obtained and known concentrations of triolein were correlated to the absorbance (Figure 3-34).

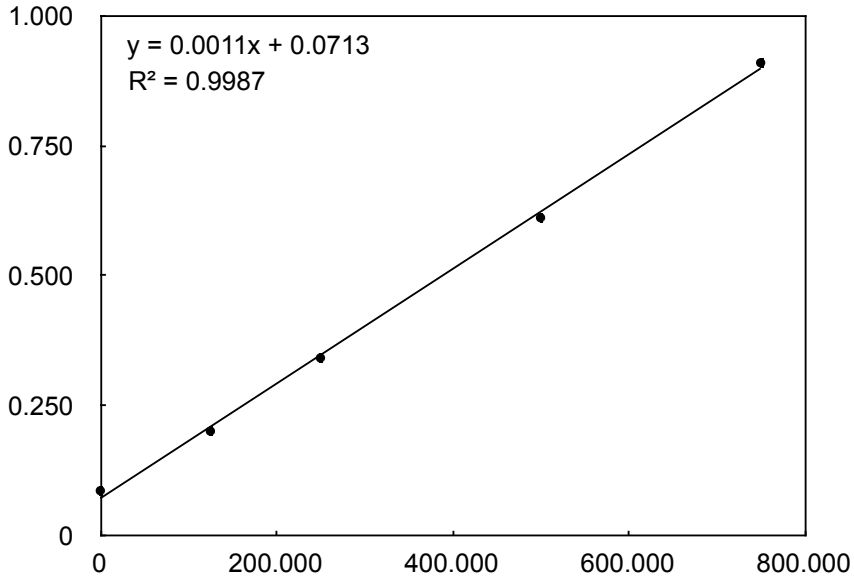

Figure 3-34. Linear standard curve obtained with known amount of triolein.

J. curcas seeds of the five different accessions were deprived of the hull and the lipid accumulation in the kernels was estimated. Seeds showed

various amount of TAG accumulated, with a range from about the 25% to the 50% of the seed dry weight (Figure 3-35). These data are comparable to data available from literature. *J. curcas*, in fact, shows a great variation in the amount of lipid accumulated among the different varieties and the different area of cultivation (Benge, 2006; Achten *et al.*, 2010; Divakara *et al.*, 2010).

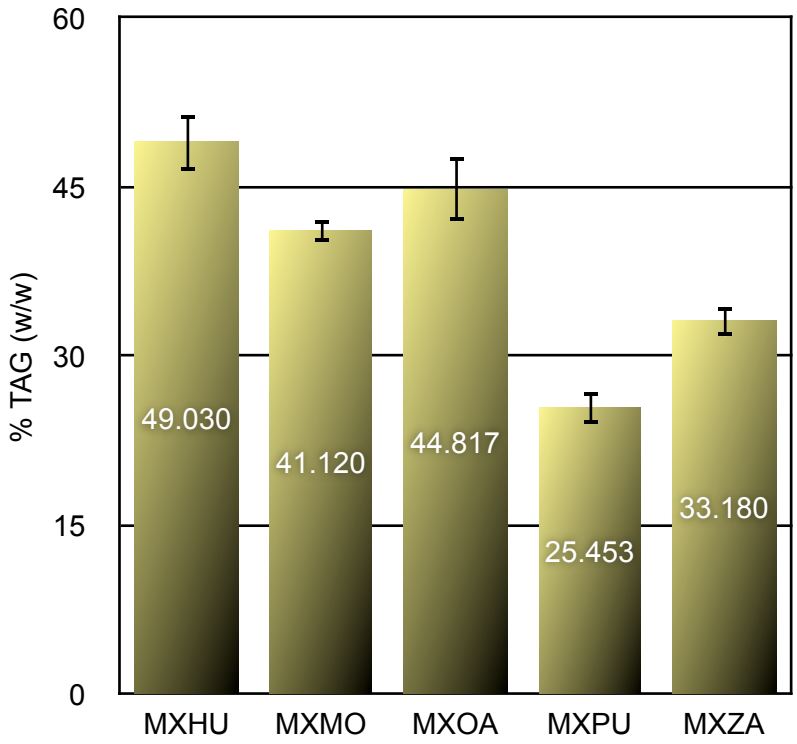

Figure 3-35. Lipid accumulation in the seeds of the five different accessions of *J. curcas*

Preliminary data on TAG accumulated by the suspension cultures showed a percentage of lipids ranging from the 0.05-0.1% weight/dry weight. As expected, wild type suspension cultures in active proliferation does not need to store reserves of energy, and they are composed of more of the 90% of water.

If the transformation with the DGAT enzyme is able to induce a greater lipid accumulation will be established in future assays.

4 Conclusions

Main aims of this research, that can be placed in the biotechnological field, were to investigate the mechanisms underlying plant cell proliferation, to set up methods to increase the production of plant biomass *in vitro* and to propose strategies for the improvement of lipid accumulation in plants and in cell cultures from *Jatropha curcas*. This work, in collaboration with Geneticlab (Noventa Vicentina, VI) took origin from the present discussion about negative consequences that the use of fossil fuel has on the environment. The possible utilization of *Jatropha curcas*, as model plant for its potential application in cultivation of marginal lands, is envisaged in order to have no reduction of food supplies (soybean, corn, rape), currently used to obtain biofuels. Furthermore, the generation of a great amount of *in vitro* plant biomass, by cell suspension cultures, provides a valuable platform for the production of high-value substances which could pave the way for the setup of plant biomass production at industrial level. The increase of triglyceride accumulation in jatropha plant tissues, other than seeds, and in cultured jatropha cells has been the other goal of this study.

Main obtained results and conclusions of this work are:

- domestication of a wild species as *J. curcas*
- induction of cell cultures from different explanted tissues
- *J. curcas* propagation and regeneration via organogenesis
- assessment of suspension cell cultures
- construction of vectors harbouring the sequences coding for YFP and DGAT
- *Agrobacterium*-mediated transformation of *J. curcas* and *N. tabacum*

The proposed project, even being of evident application guidance, was characterized by a strong basic research content, necessary to open new and unexpected possibilities in the renewable energy sources. The collaboration with a private company will lead, in a short time, to the scale-up of the obtained results from laboratory to industrial facilities.

5 References

Achten W, Nielsen L, Aerts R, Lengkeek A (2010). Towards domestication of *Jatropha curcas*. *Biofuels*, 1(1): 91-107.

An G, Ebert PR, Mitra A, Ha SB (1988). Binary vectors. In: S.B. Gelvin and R.A. Schilperoort (Eds.), Plant Molecular Biology Manual. *Kluwer Academic Publishers, Dordrecht, Netherlands*, pp. A3: 1–19.

Brandizzi F, Fricker M, Hawes C (2002). A greener world: the devolution in plant bioimaging. *Nature*, 3: 520-530.

Brittaine R, Lutaladio N (2010). Jatropha: a smallholder bioenergy crop. The potential for pro-poor development. *Integrated Crop Management*, 8:1-114.

Chisti Y (2007). Biodiesel from microalgae *Biotechnology Advances*, 25: 294-306.

Conkey J, Feirer R (1988). Determination of triglycerides in plant tissues. *IPC techincal papers series*, 316: 1-5.

Contran N, Chessa L, Lubino M, Bellavite D, Roggero PP, Enne G (2013). State-of-the-art of the *Jatropha curcas* productive chain: from sowing to biodiesel and by-products. *Industrial Crops and Products*, 42:202–215.

Denecke J, Aniento F, Frigerio L, Hawes C, Hwang I, Mathur J, Neuhaus JM, Robinson DG (2012). Secretory pathway research: the more experimental system the better. *The Plant Cell*, 24: 1316-1326.

Divakara B, Upadhyaya H, Wani S, Gowda C (2010). Biology and genetic improvement of *Jatropha curcas* L.: A review. *Applied Energy*, 87:732-742.

Graham IA (2008). Seed storage oil mobilization. *Annual Review of Plant Biology*, 59: 115–142.

Grebenok RJ, Lambert GM, Galbraith DW (1997). Characterization of the target nuclear accumulation of GFP within the cells of transgenic plants. *The Plant Journal*, 12: 685-696.

Heller J (1996). Physic nut, *Jatropha curcas*. Promoting the conservation and use of underutilized and neglected crops. International Plant Genetic Resources Institute (IPGRI), Rome, Italy.

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Sciences of the United States of America*, 103(30): 11206-10.

Horsch RB, Rogers SG, Fraley RT (1985). Transgenic plants. *Cold Spring Harbor Symposia on Quantitative Biology*, 50: 433-437.

Hsieh K, Huang AHC (2004). Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. *Plant Physiology*, 136(3): 3427–3434.

Kasim NS, Tsai TH, Gunawan S, Ju YH (2009). Biodiesel production from rice bran oil and supercritical methanol. *Bioresource Technology*, 100(8): 2399-2403.

King AJ, He W, Cuevas JA, Freudenberger M, Ramiaramanana D, Graham IA (2009). Potential of *Jatropha curcas* as a source of renewable oil and animal feed. *Journal of Experimental Botany,* 60(10): 2897-2905.

Koh MY, Ghazi TM (2011). A review of biodiesel production from *Jatropha curcas* L. oil. *Renewable and Sustainable Energy Reviews*, 15: 2240-2251.

Kumar A, Sharma S (2008). An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. *Industrial Crops and Products*, 28: 1-10.

Kumar N, Vijay Anand K, Pamidimarri D. (2010). Stable genetic transformation of Jatropha curcas via *Agrobacterium tumefaciens*-mediated gene transfer using leaf explants. *Industrial Crops and Products*, 32:41-47.

Lung SC, Weselake RJ (2006). Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis. *Lipids*, 41(12): 1073–1088.

Martin G, Mayeux A (1984). Reflections on oil crops as sources of energy: 2. Curcas oil (*Jatropha curcas* L.): a possible fuel. *Oleagineux*, 39(5): 283-287.

Metzger JO, Bornscheuer U. (2006). Lipids as renewable resources: current state of chemical and biotechnological conversion and diversification. *Applied Microbiology and Biotechnology*, 71(1): 13–22.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd edition. *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York*.

Shockey JM, Gidda SK, Chapital DC, Kuan JC, Dhanoa PK, Bland JM, Rothstein SJ, Mullen RT, Dyer JK (2006). Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. *The Plant Cell*, 18: 2294-2313.

Zou J, Taylor DC, Wei Y, Jako CC (2009). Diacylglycerol acyltransferase gene from plants. *United States Patent No.: US 7,511,189*.

other sources

Benge M (2006). Assessment of the potential of *Jatropha curcas*, (biodiesel tree) for energy production and other uses in developing countries. http://www.echotech.org

Gunstone FD (2012). Thel AOCS lipid library. Oils and fats in the market place. http://lipidlibrary.aocs.org/market/index.html

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Kayoko *et al.* (2003) Empirical analysis of transcriptional activity in the *Arabidopsis* genome. *Science*, 302:842-846.

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