INTERGENERIC HYBRIDIZATION BETWEEN *Jatropha curcas* L. and *Ricinus communis* L., THROUGH *IN VIVO* AND *IN VITRO* POLLINATION TECHNIQUES

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(Prof. Dr. Sujin Jinahyon)

…….....MEMBER

(Dr. Saranya Srisuwan)

…….....MEMBER

(Dr. Sirirung Wongsakul)

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Sopian Hadi

Title Intergeneric hybridization between *Jatropha curcas* L. and *Ricinus communis* L., through *in vivo* and *in vitro* pollination techniques

Author Mr. Sopian Hadi

Degree Master of Science (Biotechnology)

Supervisory committee

Dr. Saranya Srisuwan	Chairperson
Dr. Sirirung Wongsakul	Member
Jantraruk Tovaranonte	Member

ABSTRACT

Jatropha curcas (2n= 2x=22) and *R. communis* (2n=2x=20) are becoming commercially important plant species as their oil rich seeds can be processed into a substitute for diesel. Hybridization between *R. communis* and *J. curcas* could potentially produces the hybrid with characteristic such as short plant type, early maturity, determinate inflorescence, high oil quality and productivity/year competitive with oil palm. The pollen grain of *J. curcas* (96 µm) was 3 times bigger than *R. communis* (32 µm). *Jatropha curcas* was introduced as the female parent plant as determined by pollen grains and ovule size. The *in vivo* pollination test resulted 100% of flowers in the field being aborted within an average of 10 days after pollination. Pollen germination and normal growth of the pollen tube were observed in 48 h after pollination but failed to set the seed more than 2 weeks. This indicates that the *in vivo* pollination was due to a lack of pre-zygotic barrier. *In vitro* pollination with cultures in MS medium and the addition of 0.125 mg L-1 benzyladenine (BA), singly or combined with 0.125 mg L-1 1-naphthaleneaceticacid (NAA) can prolong the zygote life for up to 57 days. This study showed the *in vitro* techniques may provide a feasible method in order to obtain new intergeneric plants.

Additionally, the oil content of *J. curcas* showed the strong correlation with the seed weight. However, negative correlation was observed in *R. communis*. The commercial *R. communis* with a small seed size has higher oil content than the wild-type. The percentage of oil content of *J. curcas* toxic, *J. curcas* nontoxic, *R. communis* commercial and *R. communis* wild-type (A) was 53.03%, 39.47%, 27.03% and 23.25%, respectively. Oleic acid (39-40%) and linoleic acid (35-37%) were predominant of the fatty acid composition in *J. curcas*. *Ricinus communis* wild-type (A) showed higher ricinoleic acid (89%) than the commercial type (79%).

Keywords: *Jatropha curcas*, *Ricinus communis,* pollen grain, intergeneric hybridization, post fertilization barriers, pre fertilization barriers, oil content.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

CHAPTER 1

INTRODUCTION

1.1 Introduction

The world's energy needs would be well over 50% higher in 2030 than today (International Energy Agency, 2007). Energy consumption, economic growth, population growth and industrialization lead to a higher energy demand. There are concern that the energy demands in Asia especially in China may accelerate the global warming and the depletion of fossil fuel resource such as oil (Hoshino, 2004). In an age where concerns about rising fuel prices and dwindling energy resources are making headlines around the world, the value of using renewable and eco-friendly fuels has gained wide prominence.

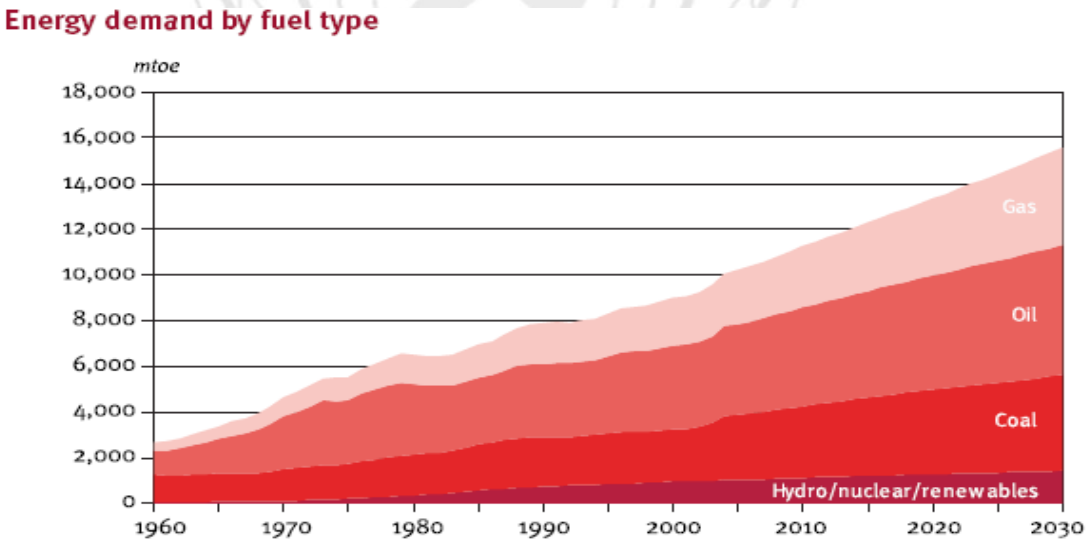


Figure 1.1 Estimation of energy demand next 20 years (International Energy Agency, 2007)

The burning of fossil fuels has been the main cause of increased levels of greenhouse gas emissions in the atmosphere. In the context of climate change and global dependence on fossil fuels, the importance of biofuels as a sustainable alternative has been increasing in recent years. In the short to medium term biofuels there is seen a promising option in reducing greenhouse gas emissions while improving the security of energy supplies (European Commission, 2006). According to the agreements such as UN Framework Convention on Climate Change (UN FCCC) and the Kyoto Protocol, ratifying industrialized nations have to reduce their emissions. Consequently, targets for biofuels in fuel blends have been introduced in national policies all over the world (Soyka, *et al*., 2007).

The energy crisis and climate change also call for policies that remove constrain on the expansion of biotechnology, allowing the technology to grow, and investing in improving biofuel technologies (Sexton *et al*., 2006). The demand for biofuels in the USA, Europe and developing nation is partly driven by the need to replace fossil fuels and lessen dependency on high-priced oil (Boddiger, 2007). The demand of biofuel as renewable energy has revealed oil seed crops as plant oil sources.

The *Euphorbiaceae*, or spurge family, contains about 280 genera, in which there are vast numbers of imprecisely related plants mostly native to the tropics zone. *Jatropha curcas,* or physic nut (English); sabu-dam (Thai); jarak pagar (Indonesia), is widely distributed in tropics with a promise for use as an oil crop for biodiesel. Countries with tropical and subtropical climates can benefit from renewable energy resources as *Jatropha* cultivation (Lozano, 2007).

Non-edible oil bearing trees like *Jatropha* can be utilized either as biofuel or with processing (Kaushik, *et al*., 2007) and it grows on medium and low fertility soils, and in low and high rainfall areas (Lazano, 2007). Production of each hectares reach 4.5-5 tons per year (Prihandana, *et al*., 2007) and Benge (2006) noted that a plantation project in South Africa very optimistically projects, a yield of 12 tons per hectare after 6 year of growth (this plant may still exist 35-45 years) . The oil from *Jatropha* is regarded as a potential fuel substitute (Openshaw, 2000) because it contains more oxygen, with a higher cetane value increasing the combustion quality, it is clean, non-toxic, eco-friendly and economic due to its low cost (Jha, *et al*., 2007). The oil is not edible due to presence of a toxic substance, ‘curcascine’; it is conventionally used in making soap, candles, paints, lubricant and medicinally as a purgative (Sujatha and Mukta, 1996).

Ricinus comunnis, or castor bean (English); la-hung (Thai); jarak kepyar (Indonesia), having unique oil chemical properties, suitable for a wide variety of uses make it of great industrial importance and medicinal purposes. *Ricinus communis* or castor plants have been a well-known plant to produce castor oil since a long time ago as biofuel (Wahyu and Sri, 2007). It will grow and produce a crop in almost any soil, with the exception of very heavy clay and poorly drained soil (Weiss, 2000). Each hectares of castor oil bean plants planted in arid and semi arid regions produces 30-650 kg of oil, which in turn produces 350-650 kg of biodiesel per hectare. Castor oil is the best substance for producing biodiesel because it is the only one that is soluble in alcohol and does not require heat and the consequent energy requirement of other vegetable oils in transforming them into fuel. Another advantages its derivative is indispensable for preventing fuels and lubricants utilized in aircraft and space rockets from freezing at extremely low temperatures (Weiss, 2000), by the high level of ricinoleic acid which is over 85%, unsaturated bond, with a high molecular weight of 298, a low melting point of 5ºC and very low solidification point of between -12ºC to -18ºC it is quite suitable for industrial use and it has the highest and most stable viscosity of any vegetable oil (Chinohonga, 2008). Wahyu and Sri (2007) also mentioned its use for human and veterinary medicine used, soap, wax and polishes, insulator, coating, plastic and nylon.

Figure 1.2 *Jatropha curcas* (a) and *R. communis* (b) plant performance during winter and summer.

Jatropha curcas is reported to be well adapted in low fertility soils (Lazano, 2007) and inarid and semi-arid conditions (Sirimboson, *et al*., 2007). However, its leaves tend to fall during the dry season or cool winter (Gressel, 2008). Openshaw (2000) noticed that flower and seed production of *J. curcas* responded to rainfall and nutrient. It is notable that the production of the seed and content of the oil are strongly influenced by the environment (Sujhata, 2008). This plant is known to have a low average of male to female flower ratio, 29:1 (Raju and Ezradanam, 2002) or 1-5 of the female flowers and 29-93 of the male produced per inflorescence. It is also reported to have low genetically diversity from 40 clonal lines in Thailand (Sakaguchi and Somabhi, 1987), 42 germplasm in India (Basha and Sujatha, 2007), and 58 accessions in China (Sun et al., 2008).

The castor plant is well adapted to the tropical regions and the temperate regions (Weiss, 2000). In the northern part of Thailand the wild *R. communis* has ability to constantly produce seeds during the cool or dry season in November – March. The castor plant produces about 95% female flowers per raceme with an average of 30-50% (Widodo and Sumarsih, 2007). High yielding in castor plants was revealed in the 1990s as a result of extensive genetic improvement programs in India. But 40 accessions from five continents and 35 countries, revealed that castor also has relatively low genetic diversity (Allan, *et al*., 2008). Combining both genera *Jatropha* and *Ricinus* probably will overcome the low diversity through efforts such as intergeneric hybridization.

Intergeneric hybridization has allowed the creation of new forms of plants (Kobayashi, 2003) and upgrading the traditional breeding methods (Dantas, *et al*., 2006). The possibility of obtaining interspesific or intergeneric hybrids is unpredictable and frequently has been completely unsuccessful, particularly in the case of distantly related species (Kobayashi, 2003). Several studies of *in vivo* pollination such as pollen tubes of *Populus tremula, P. tomentosa* and *P. lasiocarpa* penetrated the style of *Salix vim*inalis but did not enter into the placentae, this study was indicated the pollination inhibited by the pre-zygotic barrier and post-fertilization barriers (Zenkteler, 2003). However, it is been reported that success in intergeneric hybrids was obtained in *Franklinia alatamaha* x *Schima argenthea* (Ranney and Eaker, 2003), *Helianthus annuus* x *Verbisinia helianthoides* (Encheva and Christov, 2005) and *Psilanthus ebracteolatus* Hiern (2*n*=22) x *Coffea Arabica* (2*n*=44) (Couturon, *et al*., 1998). Another study revealed that *in vivo*

intergeneric hybridization followed by *in vitro* pollination was successful in obtaining new intergeneric hybrids between *Rhapanus sativus* and *Brassica* wild species (Bang, *et al*., 1997) and the stigmatic *in vitro* technique between *Festuca* and *Lolium* genus (Lee and Han, 1989). These results bring new perspective techniques to obtaining hybrid plants between *Jatropha* and *Ricinus*. Since the interspesific hybridization has been successful between two economically important species of *J. curcas* x *J. integerrima* (Dehgan, 1984) the breeders were stimulated to hybridize *Jatropha* and the monotypic genus, *Ricinus*. Furthermore, intergeneric hybrid plant between *J. curcas* x *R. communis* may potentially create higher genetic diversity and desire ornamental characteristics, expectedly. However, an attempt has been made by Sujatha (1996) to hybridize between these two genera and the result was not successful (Sujhata and Reddy, 1998).

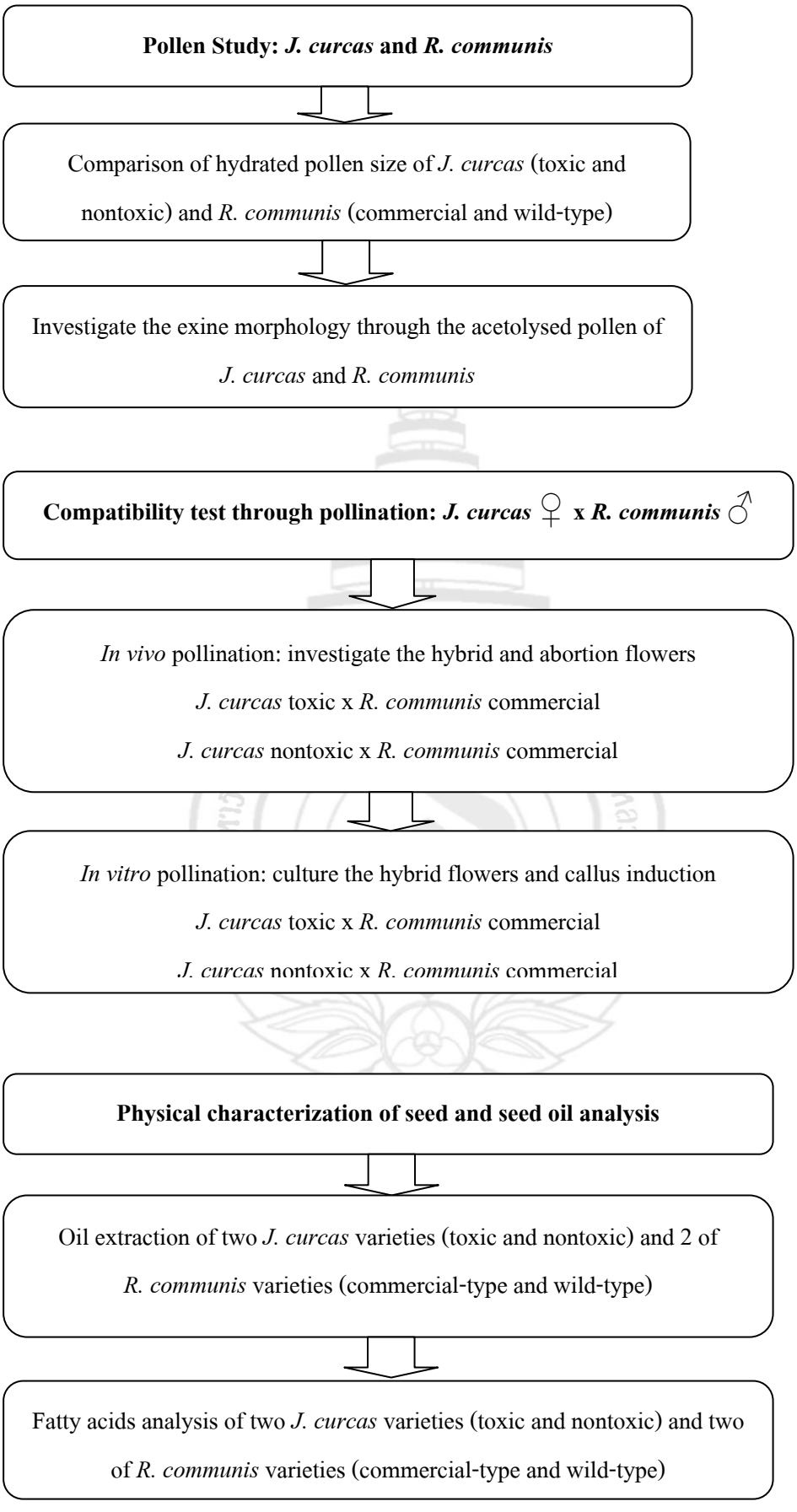
The major goals of the present studies were 1) to study the pollen size of jatropha and castor, 2) to investigate their compatibility through *in vivo* pollination, and 3) to reveal whether the application of the *in vitro* technique would be able to cross jatropha and castor.

1.2 Objectives

1. To study the compatibility between *J. curcas* and *R. communis* through their pollen size and stigma morphology.
2. To hybridize and analyze the intergeneric barriers through *in vivo* pollination.
3. To establish the intergeneric *in vitro* pollination method between two genus.
4. To investigate diversity of seed size, oil contents and fatty acids.

1.3 Scope of work

1. *Jatropha curcas* toxic (Thailand) and toxic (Mexico); *R. communis* wild-type A (Thailand) and commercial-type (Thailand).
2. Pollen investigation through light and scanning electron microscopes.
3. Intergeneric hybridization through *in vivo* and *in vitro* methods.
4. Seed physical measurement and oilseed analysis.


Pollen Study: *J. curcas* and *R. communis*
Comparison of hydrated pollen size of *J. curcas* (toxic and nontoxic) and *R. communis* (commercial and wild-type)
Investigate the exine morphology through the acetolysed pollen of *J. curcas* and *R. communis*
Compatibility test through pollination: *J. curcas* ♀ x *R. communis* ♂
In vivo pollination: investigate the hybrid and abortion flowers
J. curcas toxic x *R. communis* commercial
J. curcas nontoxic x *R. communis* commercial
In vitro pollination: culture the hybrid flowers and callus induction
J. curcas toxic x *R. communis* commercial
J. curcas nontoxic x *R. communis* commercial
Physical characterization of seed and seed oil analysis
Oil extraction of two *J. curcas* varieties (toxic and nontoxic) and 2 of *R. communis* varieties (commercial-type and wild-type)
Fatty acids analysis of two *J. curcas* varieties (toxic and nontoxic) and two of *R. communis* varieties (commercial-type and wild-type)

CHAPTER 2

LITERATURE REVIEW

2.1 *Jatropha curcas* L.

Jatropha curcas is a well known oil seed crop which is wildly cultivated in the tropics as a living fence. It is a plant with many attributes, multiple uses and considerable potential (Openshaw, 2000). Many parts of the plant are used in traditional medicines. The seeds are used for oil extraction and soap production. *Jatropha curcas* is belongs to the Euphorbiaceae and it has approximately more than 200 species (Ram, *et al.*, 2007).

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Malpighiales
Family	:	Euphorbiaceae
Subfamily	:	Crotonoideae
Tribe	:	Jatrophaeae
Genus	:	*Jatropha*
Species	:	*Jatropha curcas*

Dehgan and Webster (1979) *cit.* Wilbur (1954) said as follows: “it was without doubt part of the flora of Mexico and probably of northern Central America before the arrival of Cortez, and it most likely originated there…the subsection, hence, appears to be one which originally was nearly or completely restricted to Mexico. According to other sources, the physic nut seems to be native to Central America as well as to Mexico where it occurs naturally in the forests of coastal

regions (Aponte, 1978). Many records also exist for the Caribbean regions (Bahama, Cuba, Puerto Rico, etc.) and West Indian countries. From the Caribbean, this species was probably distributed by Portuguese seafarers via Cape Verde Islands and former Portuguese Guinea to other countries in Africa and Asia. Today it is cultivated in many countries.

Jatropha is almost tropical now, and although toxic, is widely planted as a fence plant. A non-toxic variety is reported to exist in Mexico and Central America.

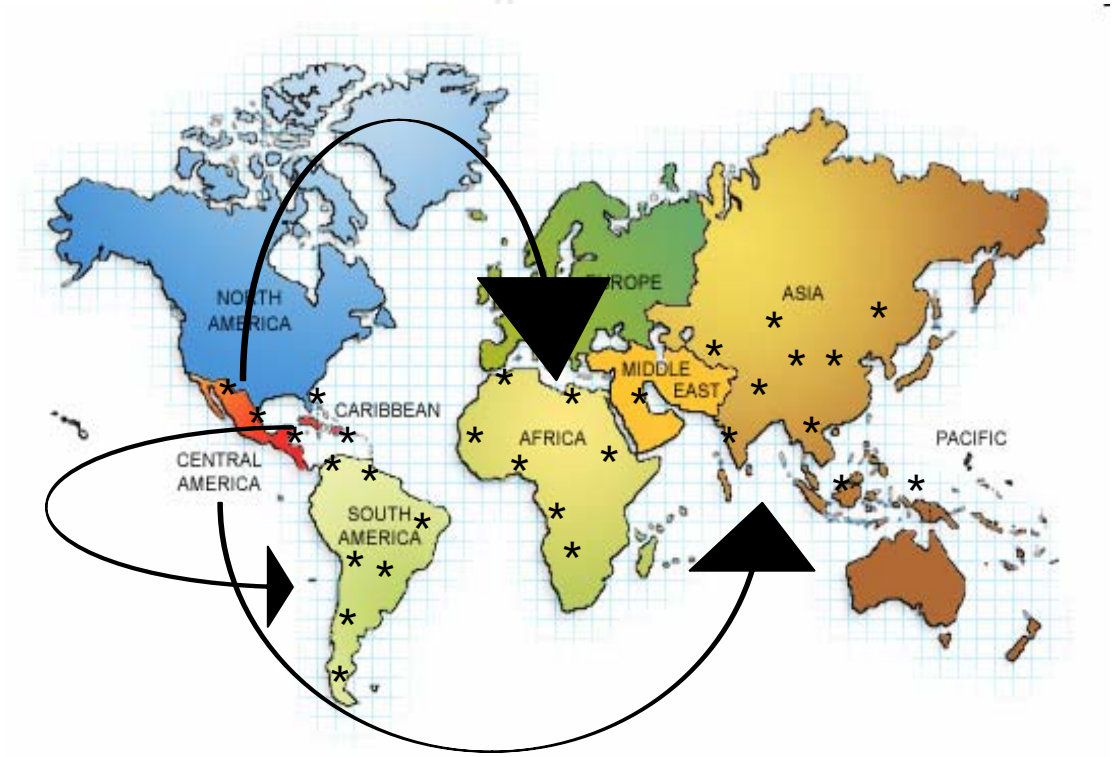


Figure 2.1 Geographical distribution maps of *J. curcas*. Central America and Caribbean proposed as centers of origin (Heller, 1996).

The physic nut is a small tree or shrub which can reach up to five meter, has a shallow lobed leaves with a length and width of six to 15 centimeters, which are arranged alternately. Inflorescences are formed terminally on branches and are complex. The plant is monoceous and the flowers are unisexual; occasionally hermaprodhite flowers occur (Dehgan and Webster, 1979). Raju and Ezradanam (2002) explain that *Jatropha* plant produces flowers in racemose

inflorescence, with a dichasial cyme pattern. The flowers of *Jatropha* are unisexual, and male and female flowers are produced in the same inflorescence. Numerically, 1-5 female flowers and 25-29 male flowers are produced per inflorescence. The average male and female flower ratio is 29:1. The pollen grains are yellow, globular and inaperturate; the exine is semitacte and verrucate. While Gupta (1985) in Heller (1996) investigated its chromosomes, the physic nut is a diploid species with $2n = 2x = 22$ chromosomes.

Figure 2.2 Old *J. curcas* tree in Sumba Island, Indonesia. The tree is about 63 years old and still produces 163 kg of seed/year. It's diameter of the canopy was more than six meters. (Photo by Mrs. De Ruwe, 8 October, 2008)

Figure 2.3 An inflorescence (a), pollinated female flowers (b) and fruits (c) of *J. curcas*.

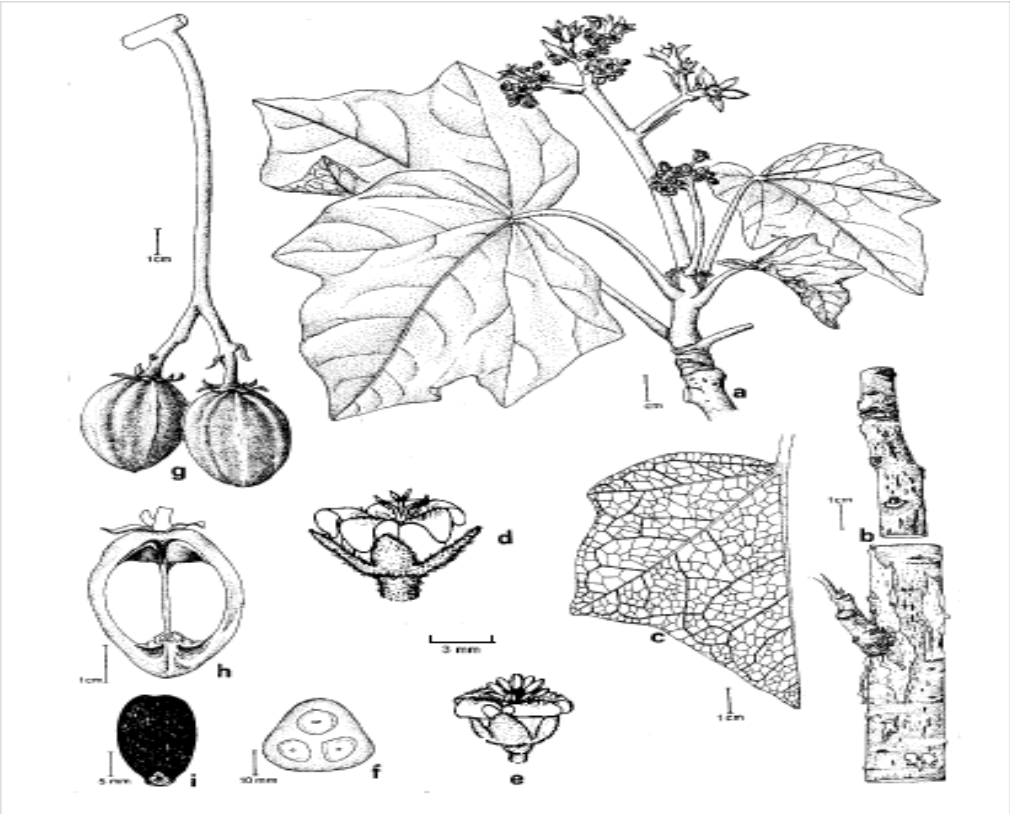


Figure 2.4 Different component of the physic nut (*J. curcas*): a. flowering branch, b. bark, c. leaf veins, d. pistillate (male) flower, e. staminate (female) flower, f. cross-cut of immature fruit, g. fruits, h. longitudinal cuts of fruits (Heller, 1996).

Pollination is by insects, Heller (1992) *cit*. Heller (1996) observed a number of different insects that visited flowers and could pollinate. In Senegal, he observed that staminate flowers open later than pistilate flowers in the same inflorescene, this mechanism promotes cross-pollination. It seems that the mechanism is influenced by the environment. After pollination, trilocular ellipsoidal fruit is formed. The exocarp remains fleshly until the seeds are mature. The seeds are black, 2 cm long and 1 cm thick. Ophenshaw (2000) found that flower and seed production respond to rainfall and nutrients, a poor nutrient level will lead to increased failure of seed development. Thus, it is important to maintain soil fertility and is contrary to statements made in some publications.

Seed of *Jatropha* contains curcin, phorbol ester and curcasine which are toxic to goats, sheeps and calves. In many countries human poisoning is common; especially in children who mistakenly eat them as edible nuts. It can be the cause of diarrhea, nausea, vomiting and burning sensation in the abdomen (Bakod, 1989) making it unsuitable for animal feed because of toxic properties. The products from fruit -- the exocarp (coat), shell and processed seed-cake—are rich in nitrogen, phosphorus and potassium (NPK) and/or can be used as soil improvers (Openshaw, 2000). The nitrogen content ranges from 3.2 to 3.8 %, depending on the source. The production of oil inevitably produces a press-cake, resulting in *Jatropha* having a high level of protein 58-60 % suggesting that it would be an excellent animal feed (Joachim, 1996). The Binga Trees Project supported a trial in which a 1 kg per square meter application of seed-cake raised the yield of cabbage from 16.8- 35.8 kg per meter square.

Table 2.1 Nutritional analyses of *J. curcas* oil seed-cake and manure

Property	***J.curcas* seed-cake (%)**	**Neem oil cake (%)**	**Cow manure (%)**	**Chicken manure (%)**
Nitrogen	3.2 – 4.4	5.0	0.97	3.04
Phosphorus	1.4 – 2.09	1.0	0.69	6.27
Potassium	1.2 – 1.68	1.5	1.66	2.08

In the oil extraction process, the fruits must be dehulled, the nut must be shelled, the kernels must be dried and then the oil has to be extracted (Sirisomboson, 2007). The oil content in the *Jatropha* seed is around 30-40% (Sarin, *et al*., 2006), in the kernels its 55-60% (Heller, 1996). *Jatropha* oil contains about 14% free fatty acid (FFA), which is far beyond the limit of the 1% FFA level that can be converted into biodiesel by transesterification using an alkaline catalyst. Hence, an integrated optimized procedure for converting *Jatropha* oil, which contains high FFA% into biodiesel, is very much required. Few researchers have worked with feedstock having higher FFA% levels using alternative processes, which include a pretreatment to less than 1% followed by the transesterification reaction with an alkaline catalyst. This procedure yielded more than 95% biodiesel (Tiwari, *et al*., 2007).

The high viscosity of the *J. curcas* oil, which is considered as a potential alternative fuel for the compression ignition (C.I) engine, is decreased by blending it with diesel (Sirisomboson, 2007).

2.2 *Ricinus communis* L.

Castor (*Ricinus communis*), an important crop of the *Euphorbiaceae*, is indigenous to eastern Africa and most probably originated in Abyssinia (Sujatha, *et al*., 2008) but now it is widely grown for its seeds which yield the much value castor oil (Sujatha and Reddy, 1998). Subsequently, castor seed oil and its derivates have become important commodities and an increasing number of uses are being found for them in the industrial world (Sujatha and Sailaja, 2005). Thailand's production of castor steadily decreased from 40,000 t in 1970 and to less 10,000 t by 1990, but since then it has been given official support with a target of 50,000 t annually.

Kingdom	:	Plantae
Division	:	Spermatophyta
Subdivision	:	Angiospermae
Class	:	Dicotyledonae
Order	:	Euphorbiales
Family	:	Euphorbiaceae
Genus	:	*Ricinus*
Species	:	*Ricinus communis*

According Balittas (2000) *cit*. (Heyne, 1987): Castor's contemporary distribution is in the warmer regions worldwide, although its origin is obscured by wide dissemination in ancient times and the ease and rapidity in which it becomes established. Africa, South America, and Asia are proposed as the centre of diversity for this important industrial plant. Russian plant breeders consider that there are four large centers of variability; Irano-Afghanistan region, Palestine-SW Asia, India-China, and the Arabian Peninsula (Heller, 1996; Moshkin, 1986). While this argument

is acceptable to distinguish centers of diversity, as numerous plants long used or domesticated by man, there exist in East Africa a far greater range of wild types ancestral to all others.

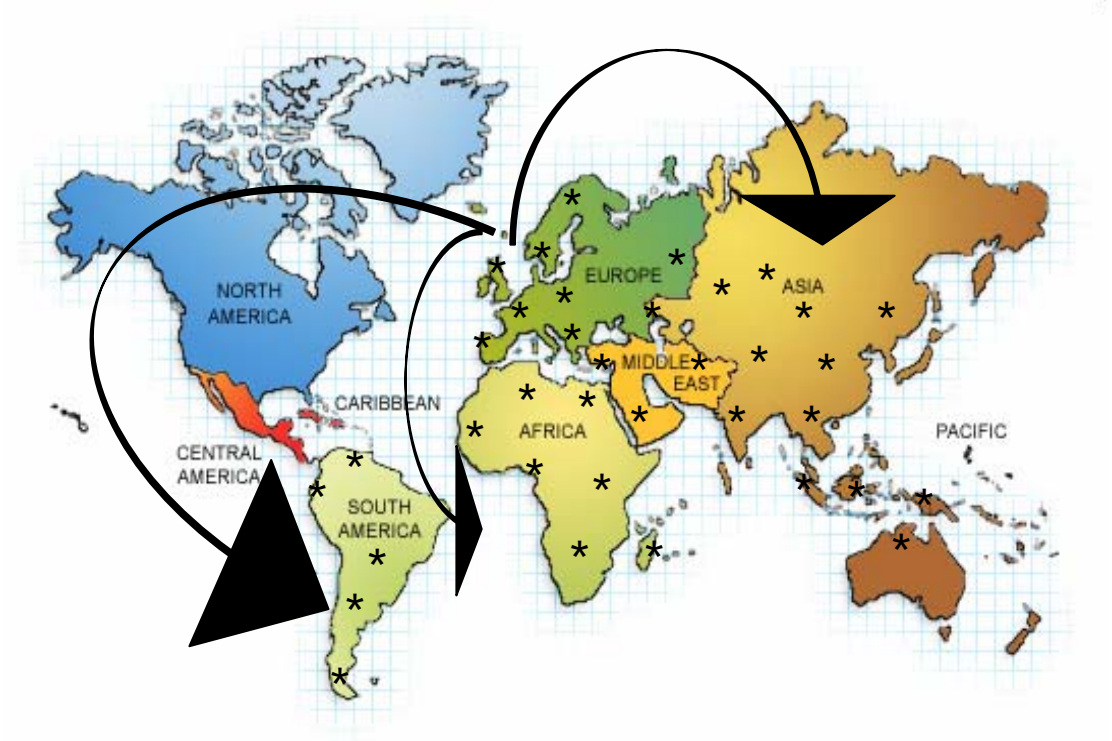


Figure 2.5 Geographical distributions of *R. communis*.

The castor plants reach 30-40 feet tall but all types of castor from giant perennials to short internode dwarfs have the same chromosome number $2n = 2x = 20$. A natural polyploidy is probably absent from monotypic genus. Castor normally monoecious, with pistillate flowers on the upper and staminate on the lower portion of raceme and normally cross-pollinated but self-pollination of monoecious plants can occurs (Weiss, 2000). Castor's produce of female flowers achieved 95% per inflorescence but average is 30-50% (Wahyu and Sumarsih, 2007). Indeed, the number of female flowers reached 99% and less than 5% in very extreme conditions (Balittas, 2000).

Figure 2.6 An inflorescence of *Ricinus* flower; upper part is a female and lower part is mostly male (a), mature male flowers (b) and fruit of *Ricinus* (c).

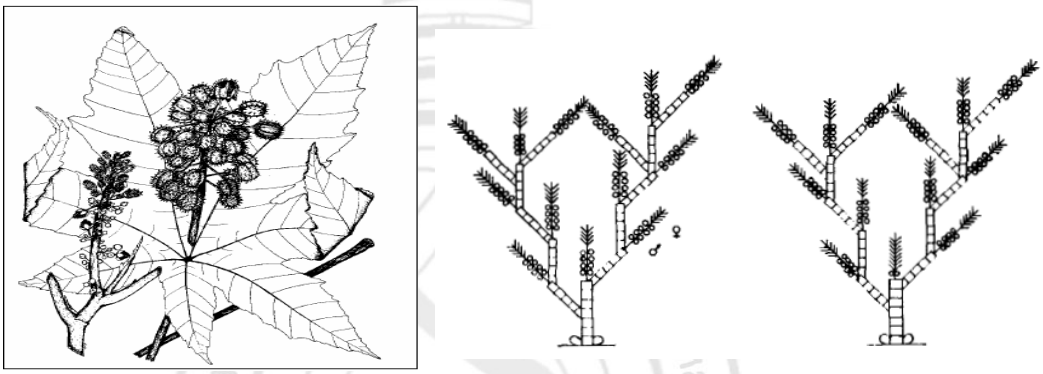


Figure 2.7 Diagrams of some developmental patterns of sex expression in *Ricinus communis* (Shifriss, 1955).

The stem color of this plant is varying, light green to green or red to maroon (Balittas, 2000) and it herbaceous when young but becomes woody with age. The shape of the stem is round, glabrous and covered with a waxy bloom which gives red or green stems a bluish appearance (Weiss, 2000). The castor plants height reaches 1-6 m with a diameter of 3-5 cm and keeps growing and will survive as long as growing factors are available, particularly water (Wahyu and Sri, 2007).

Pollen normally sheds from 2-3 hours before the sunrise until late afternoon, with a temperature of 26-29^{o} C and relative humidity of 60% is the optimum, usually cross-pollinated, wind being the major agent in the tropics (Wahyu and Sri, 2007). Day length also affects the ratio

of male to female flowers and it has a long flowering period, the wild giant types may flower year-round; the lowest flowering raceme on the plant is usually the first to mature, the others following sequentially up to the stem, with a distinct variation in yield of seed and seed oil content; primary racemes generally producing more and larger seeds (Weiss, 2000). Shifriss (1955) found that the number of flowers per raceme varied considerably among different strains but it commonly over 120. The fruit is a globular capsule, usually green and spiny, becoming hard and brittle when ripe and normally containing three seeds (Balittas, 2000).

After the first raceme appears, branches originate at the nodes below it. The number of branches depends on plant spacing and in some cases the cultivar. Under field conditions, two or three branches occur at almost the same time, but generally in the following order: the first branch at the node immediately beneath the primary raceme, the second at the second node, and the third at the third node below the primary raceme. The first racemes formed on the branches are commonly called the "second set" of racemes. Subsequent branches arise from the nodes just beneath the racemes of the second set. This sequence of development continues as long as the plant remains alive and grows actively. Thus, the development of racemes along any one axis is sequential, making it possible for a plant to have racemes in all stages of development from bud stage to complete maturity (Brigham, 1993).

Typically, the racemes usually bear pistillate flowers on the upper 30 to 50% and staminate flowers on the lower 70 to 50% of the raceme. The number of staminate and pistillate flowers can vary greatly, depending upon raceme size. The flowers are without petals. After the pollen is shed, the staminate flowers dry up and usually drop. The pollen, which is discharged forcibly from the anthers, is carried to stigmas mainly by the wind (Brigham 1967). After fertilization, the pistillate flowers develop into spiny capsules, though spineless types are known. At maturity, the hull (pericarp) of the capsule may split along the outside seam (dorsal suture) of each of the three capsule segments (carpels). If splitting is violent, as in wild types, the seed will be ejected and scattered on the ground around the plant. This type of splitting (dehiscence) is not present in cultivars grown for mechanized production.

Seeds of present cultivars are held within the capsule for several weeks after frost with no appreciable loss. Seeds of current cultivars weigh from 3.0 to 3.5 g. The seed color ranges from

light to dark brown, with various mottling patterns. The seed coat makes up about 25% of the weight of the seed. Oil content averages 50% on a dry weight basis.

Oil castor formation began 20 days after flowering on a dwarf hybrid in Canada and two-thirds synthesis in the next 20 days. Cerry (1992) found that the seed contents makes up 65-85% of the weight of the bean and the oil content can range from 35-52%, depending on the variety of seeds and environment. Commercially castor oil consists of triglycerides that contain 90% of ricinoleic acid and it has effective hydroxyl functionality (Cunha, *et al*., 2004). Ricinoleic acid was absent in very young seed, but appeared at 12 days, and at 36 days it represented 90% of fatty acids; the fatty acid composition of oil then remained constant (Weiss, 200).

2.3 Pollen morphology

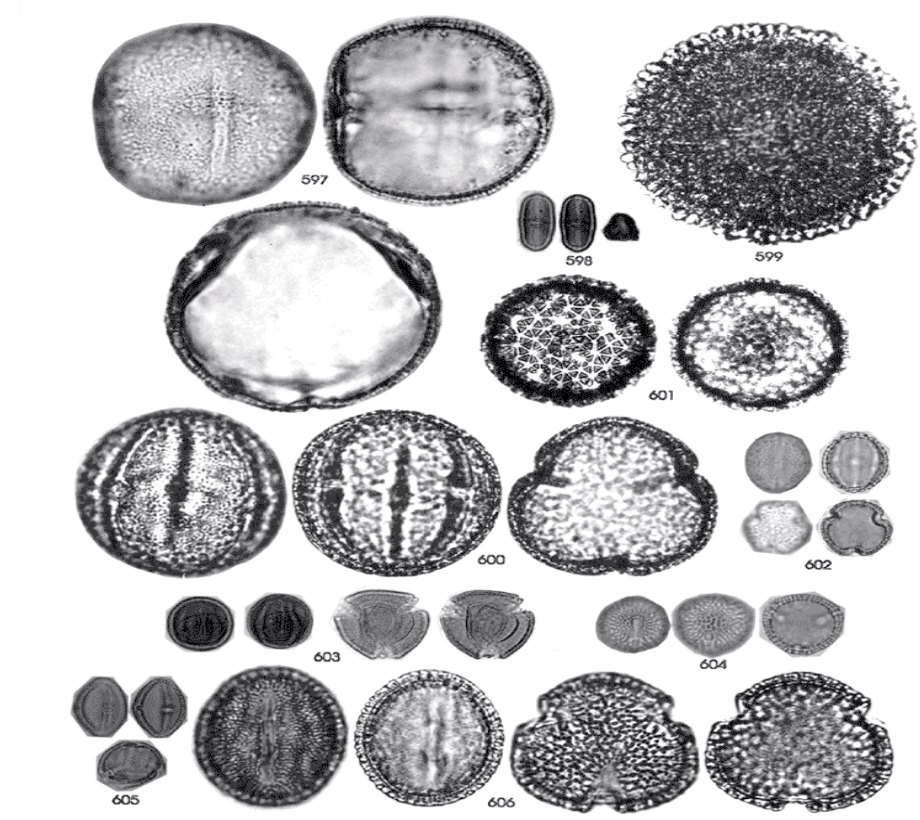
Pollen grains have a multicelullar structure arising by two meiotic divisions in the microspore mother cell, resulting in the formation of a male gametophyte (Batygina, 2002). There are two types of pollen grains in angiosperm; binucletae and trinucleate. Binucleate grains contain a generative cell and a vegetative or tube nucleus, the generative cell dividing during pollen tube growth to form the two male gametes. The trinucleate grain has a vegetative nucleus and two male gametes (sperm cells). Pollen grains of Eauphorbiaceae family are included in binucleate type.

Although angiosperm pollen has a high diversity, it is characterized by certain common features; it contains carbohydrate reserves for gametophyte development and its maintenance up to germination, it has two walls: exine (outer layer) and intine (inner layer), and it contains the gametes or their precursors (Pacini, 2000). The exine is constructed primarily of lipoprotein, sporopollenin, a highly resistant polymer of carotenoids and carotenoid esters, whereas the intine is made of cellulose. Pollen grains vary widely in exine structure, aperture number and shape.

The exine surface exhibits furrows, pores, rimae and colpi, extending down to the intines which are collectively called ‘aperture’ and represent sites of the pollen tube germination. Pollen lacking a clearly defined aperture (inaperturate) is also fairly common; multi-aperturate and inaperturate pollen are sometimes found in closely related species, for example, in the basal angiosperm families Trimeniaceae and Chloranthaceae and in some Alismatales.

The pollen grain has a tough outer wall, like a spore, which typically is sculptured. It provides a porous surface containing the many substances that are deposited on it by the parent sporophyte while it is inside the anther or that are secreted into it by the male gametophyte during pollen development (Dickinson, *et al*., 2000).

The intine envelope of the pollen protoplast is comparable to the primary wall of other plant cells. It is primarily composed of cellulose, hemicellulose and peptic polymers. In some taxa such as grasses, a middle layer between the exine and intine rich in peptic polysaccharides, termed Z-layer, is distinguishable. The intine invariably contains protein in the form of radially elongated tubules generally concentrated in the germ pore region (Shivanna, 2003).



Euphorbiaceae: Hura crepitans (597), Hyeronima laxiflora [H. alchorneoides] (598), Jatropha curcas (599), Mabea occidentalis (600), Manihot esculenta [2x] (601), Margaritaria nobilis (602), Omphalea diandra (603), Phyllanthus acuminatus (604), P. amarus (605), Poinsettia heterophylla [Euphorbia heterophylla] (606)

Figure 2.8 Pollen morphology from some member of Euphorbiaceae family.

The size of pollen is highly variable and ranges from 5 to 200 µm. The smallest grains are recorded in members of Boraginanceae and larger pollen grains are produced in members of

Cucurbitaceae, Malvaceae and Nyctaginaceae. Walker (1971) in Shivanna (2003) reported that the pollen of *Cymbopetalum odoratissimum* (Annonaceae) measuring up to 350 μm is perhaps the largest pollen. In a majority of species pollen size varies between 15 and 50 μm. The size of pollen grains varies to some extent, depending on the method of preparation and its hydration level. However, with other morphological characters, size of the pollen may help in demarcating the taxa.

The shape of pollen grains is also variable. Dessicated grains are frequently subprismatic because of the contraction of the exine, while hydrated grains tend to be orbicular, ellipsoid or oblong but may also lobate or polygonal. Also the shape varies depending on whether it is observed in polar view or equatorial view. Pollen grains of al large number of species in pollar view are circular or elliptic. However, triangular, quadrangular and rectangular pollen are also present.

2.3.1 Pollen and pollination

The role of pollen grains as the male partner in the sexual reproduction of seed plants was established by the end of the 19th century. Pollen-pistils play an important role in sexual reproduction. The pollen grain is screened during pollen-pistil interaction; the pistil facilitates germination of compatible pollen and the growth of the resultant pollen tube until it reaches the embryo sac while incompatible pollen is inhibited before germination or during the growth of the pollen tubes before its entry into the embryo sac (Shivanna, 2003).

Fertilization in flowering plants is a complex process. Whereas in the cryptogams and to a large extent in the gymnosperms the male and female gametes come in direct contact with each other, in the angiosperms the pollen grain (male gametophyte that carries the male gametes or their progenitor cell, the generative cell), does not have direct access to the embryo sac (female gametophyte) that contains the egg. The pollen grains landon the stigma, following effective pollination through autogamy or biotic/abiotic agents. The pollen grain germinates on the stigma and the resulting pollen tube carries the male gametes through the tissues of the stigma and style, enters the ovule and eventually the embryo sac, and discharges the two male gametes to effect double fertilization (Shivanna and Mohan Ram, 2005).

When pollen comes in contact with the stigma, it adheres, hydrates, and germinates. The result is a pollen tube cell that carries the sperm cells, endocytosed into the larger tube cell, thus conveying them to the embryo sac and the egg in the ovule. A flower can produce pollen that penetrates its own pistil and fertilizes its own ovules in most angiosperms, a predominantly self-compatible (SC) group.

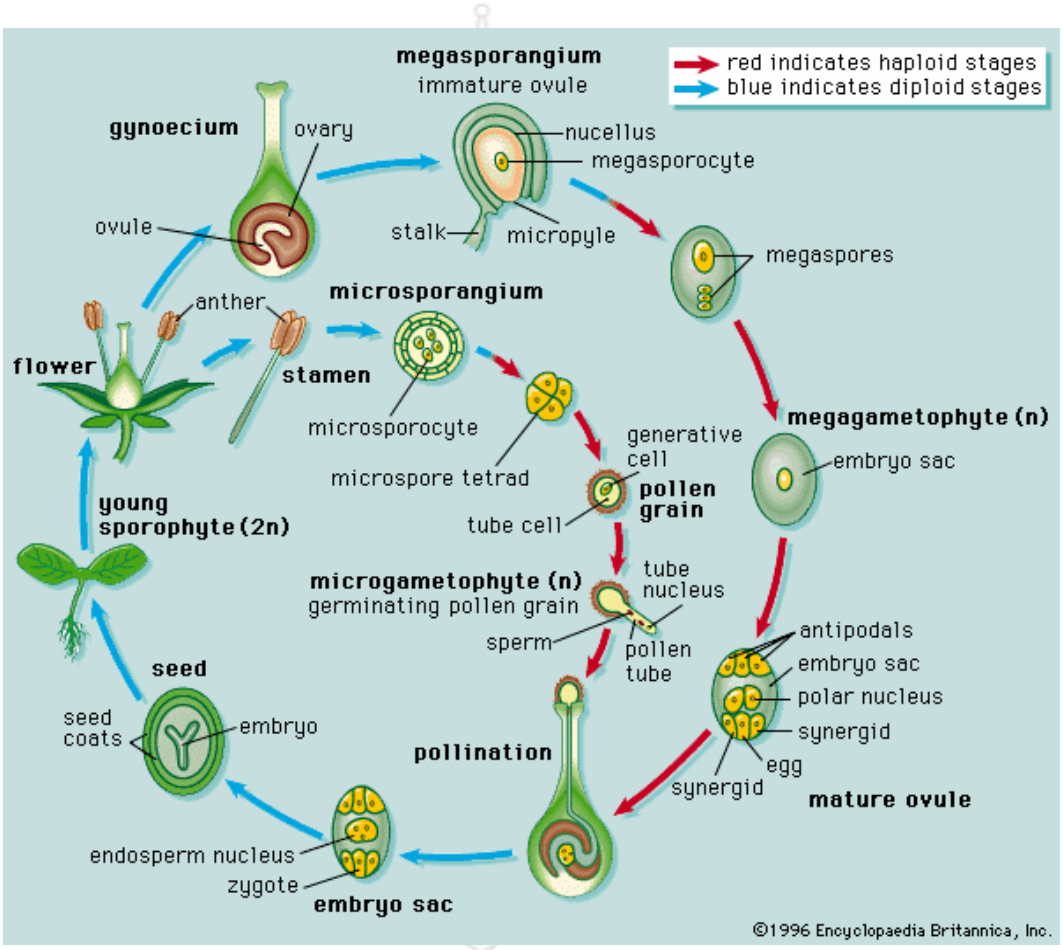


Figure 2.9 The cell cycle and fertilization in flowering plant.

Pollen grains, especially of entomophilous plant such as *Jatropha*, are coated with an oily, sticky, and often coloured material commonly term ’polenkitt’ or ‘tryphine’. The pollen coat substances contain a range of chemicals; lipid, carbohydrate, protein, glycoproteins carotenoids and flavanoids. The coat may have some role in adhesion of pollen to vectors such as insect and

birds because wind-pollinated species have thin pollen coats and may also have evolved as a food source for biotic vectors. Another role for the pollen coat substances is that of facilitating penetration into style. Enzymes residing in the coat play a role in modification of the stigma extracellular matrix (ECM) to allow for penetration into the pistil (Wheeler, *et al*., 2001). The coat is full of proteins that could act as ligands for receptor in the stigma, preparing the way for adhesion, hydration, and germination of compatible pollen, but so far the only known recognition event at the stigma surface is that of self-incompatibility, where active rejection ensues when self pollen lands on the stigma (Dixit and Nasrallah, 2001).

When the pollen grain hydrates, the tube cell cytoplasm becomes activated and some genes, such as MAP kinases of unknown function, may act specifically at this stage (Heberle-Bors, *et al*., 2001). Many of genes necessary for germination have been transcribed, and the pollen tube cell is primed for the next events leading to fertilization, which can take hours or days to occur (Taylor and Hepler, 1997). Once germination occurs, maintenance of polar growth in the pollen tube is also controlled by Rop in a complex signaling network that includes regulation of tip-focused Ca^{+} gradient and F-actin polymerization.

After pollen germination, penetration into the style is the next step, and here the processes vary considerably across species, stigma, and style types. Entrance is always into a specialized ECM of the pistil that appears to guide pollen tubes to the ovule (Lord, 2000). In open styles, typically the stigma is also open and covered with a secreting epidermis that is continuous with that of the stylar canal so no penetration is necessary, just guidance along a secretory dermal layer into the style and then to the ovary.

Evidence for pollen tube attraction has been an elusive target recently, and efforts to demonstrate it using living male and female gametophytes have been inconclusive. Pollen tubes of an *Arabidopsis* were attracted over a distance of a little more than 100 μm to cross the septum and fertilize the ovule (Hulskamp, *et al*., 1995). No attraction was observed in incompletely formed ovules or those lacking embryo sacs. These suggest that embryo sac cells may themselves govern pollen tube guidance. Higashiyama, *et al*., (2000) using only light microscopy, described the pollen tube entering the embryo sac at the micropylar end “thrusting its way between the two synergid cells.”

It is known that pollen tube growth rates vary *in vivo* and *in vitro* and that the ability of a pollen donor to sire seeds varies with the maternal plant in self–compatible species. Thus nonrandom mating occurs in plants. It is obvious that interaction between pollen tubes of varying genetic background in one pistil, as well as pollen/pistil-specific interaction, create a complex environment so that cellular mechanisms that produce nonrandom mating will be difficult to decipher. The major lesson for cell biologist from this population is clear, however, the pollen tube's function in delivering viable sperm cells to the embryo sac involves a close partnership with the transmitting tract of the pistil that must include a myriad of recognition and signaling events (Lord and Russell, 2002).

2.3.2 Interspesific and intergeneric hybrids pollen

In the case of interspesific hybridization between *J. curcas* x *J. integerrima*, were parents highly pollen fertile and monomorphic (75-80 µm), the F_1 plants showed reduced pollen fertility (6.6 to 52.0 %). Pollen grains of the hybrids were dimorphic (30-45 and 70-75 µm) and only the larger sized grains were fertile. Good fruit set was observed in the hybrids but they lacked normal seed development irrespective of the pollen source (Sujatha and Prabakaran, 2003).

The pollen grains of *J. tanjorensis* Ellis & Saroja from a natural interspesific hybrid *J. curcas* and *J. gossypifolia* in Tamil Nadu, India were highly sterile, cytological studies were initiated. The meisosis was abnormal with the formation of I and a few III at methapase and an unequal separation at anaphase I leading to irregular sporads instead of normal tetrads at the end of meisosis II. This resulted in very high pollen sterility with pollen grains exhibiting polymorphism. The possible reason for this abnormal meiosis may be due to presence of dissimilar genomes which are resulting in the formation of III and I (Prabakaran and Sujatha, 1999).

Intergeneric hybrids between *P. ebracteolatus* ($2n = 22$) and *Coffea arabica* ($2n = 44$) were successfully produced by crossing tetraploid level. Observation of pollen reveals morphological variations between *P. ebracteolatus* and *C. Arabica*. In particular, exine morphology is very different in terms of reticulation and perforation but the hybrid showed an intermediate form and pattern. As reported by Lobreau-Callen and Leroy (1980, grains of *P. ebracteolatus* ($2x$) were observed to be either tetracolporate or pentacolporate. However,

hexacoporate pollens were also observed from tetraploid *P. ebracteolatus* (4*x*). In *C. Arabica*, pollens were either tricolporate or tetracolporate. Pollens of the hybrid varied from tricolporate to pentacolporate (Couturon, *et al*., 1998).

2.4 *In vivo* and *In vitro* pollination

The past several years have seen a resurgence of the *in vivo* approach using the remarkable plant *Torenia fournieri* (Scrophulariaceae), in which the embryo sac protrudes from the tip of the ovule exposing the egg apparatus. Higashiyama, *et al*., (1997) recorded the kinetics of fertilization by the direct observation of the two synergids, egg cell and central cell. One item of contention given the apparent participation of sperm-associated myosin with a pre-existing actin corona is how long the unfused sperm cell remains in synergid. Acording to Higashiyama *et al*., (1997), both sperm cells are present only transiently inside the degenerated synergid, but Wallwork and Sedgley (2000) report duration in excess of an hour.

In nature, intergeneric or interspesific hybridization occurs less frequently and the combination of distant hybrids and genetic modification of the artificial zygotes have been a frequently cited a goal of *in vitro* fertilization. Pollination and fertilization under *in vitro* condition offer an opportunity for producing hybrid embryos among plants that cannot cross by conventional methods of plant breeding. This is due to barriers hindering the growth of the pollen tube on the stigma or style. Interspesific hybridization has allowed for the creation of new plants and the transfer of desirable features from one species into another, e.g., via introgression from wild into related cultivated species (Kobayashi, 2003).

In vitro pollination and fertilization provide very effective techniques in fundamental and applied areas of fertilization and seed development. Pollination of cultured pistils is simple and can be used for a number of experiments on seed and fruit development. The *in vitro* system with its controlled conditions provides a much more convenient aspect in particular for ovule physiology before, during and after fertilization than *in vivo* systems.

The technique of *in vitro* pollination involves aseptic pollination of cultured pistils or ovules to achieve pollen germination, pollen tube growth and its entry into the embryo sac, double fertilization and the subsequent development of the embryo. Pollination of cultured pistils

and their growth *in vitro* mature fruits provide a very convenient technique for experimental studies on pollen-pistil interaction, fertilization and development of fruits and seeds. This technique has been successfully achieved in the species of *Nocitiana* (Rao, 1965), *Petunia* (Shivanna, 1965), and *Antirrhinum* (Usha, 1965). The pistils were surface-sterilized, cultured on simple nutrient medium and pollinated with fresh pollen collected aseptically. Pollen grains in compatibility-pollinate d pistils germinated, the pollen tube grew through the style, entered the ovary and effected fertilization. Such cultures developed into normal mature fruits in about 3 weeks, the same period required for fruit development *in vivo*. Pollination of cultured pistils with incompatible pollen did not result in fruit- and seed-set; incompatible pollen tubes were inhibited in the style (Shivanna, 1965). Thus, pollination of cultured pistils was not effective in overcoming sexual incompatibility because the zone of inhibition, namely the stigma and style, was intact. In such cases the style or part of it can be excised and pollen grains either placed on the cut surface of the ovary or transferred through a hole in the wall of the ovary (Leduc*, et al*., 1992; Razdan, 2003). This technique called *intra ovarian* pollination, has been successfully applied in such species as *Papaver somniferum, P. rhoeas, Argemone mexicana* and *A. ochroleuca*. Another approach to overcome the barriers to pollen tube growth is direct pollination of cultured ovules called *in vitro ovular* pollination, which has been developed to produce hybrids among the species Papaveraceae and Solanaceae, or excised ovules together with a placenta called *in vitro placenta* pollination, which has been successfully applied to overcome self-incompatibility in *Petunia axillaris*.

2.5 Reproductive incompatibility

Reproductive isolating mechanisms prevent interbreeding between two species whose ranges overlap. The evolution of reproductive isolating mechanisms between a population and other members of that species is an important step in the speciation process.

Generative reproduction in plants allows for variable forms of selection during the phase from the formation of gametes to their fusion in zygotes and the initial stages of seed development. Although this reproductive phase normally covers only a comparatively small portion of an organism’s total life span, its overall selective potential may be considerable. Effects

of selections on mating processes (selective mating) have long been discussed in the population genetics literature. They include aspects such as differential intensity and synchrony of flowering as a source of differential mating participation and mating preferences, segregation distortion as implied by selection during gamete formation, gametophytic selection, and early prezygotic incompatibility or selective embryo abortion (Steiner and Gregorius, 1999). In flowering plants, the term of incompatibility in crossing between species or genera was called interspesific or intergeneric incompatibility. Interspesific incompatibility as a reproductive barrier results in the failure of seed-sets between two reproductively isolated species. Interspesific incompatibility may therefore operate before fertilization (prefertilization) and/or after fertilization (post-fertilization). This system prevents gene flow between species and maintains species identity.

Prezygotic barriers include temporal isolation, habitat isolation, behavioral isolation, mechanical isolation, and gametic isolation. Three other prezygotic barriers are behavioral isolation, mechanical isolation, and gametic isolation. In plants, mechanical isolation often occurs in flowering plants pollinated by insects. The flowers of black sage and white sage are structurally different and are pollinated by different species of insects. In this example, each insect species pollinates flowers of only one of the sage species. Therefore, interbreeding does not occur. If the gametes of two species meet, fertilization may not occur because of gametic isolation, in which the egg and sperm of different species are incompatible.

Postzygotic barriers include hybrid inviability, hybrid sterility, and hybrid breakdown. Postzygotic behaviors—hybrid inviability, hybrid sterility, and hybrid breakdown—prevent gene flow in the unlikely event that fertilization occurs between two closely related species. In hybrid inviability, the hybrid offspring of two species do not mature normally and usually die in the embryonic stage of development. In crosses between different species of irises, for example, the embryos die before seeds form. Sometimes hybrid offspring mature normally but are not able to reproduce successfully. Hybrid sterility often occurs because the gametes are abnormal in some way. If a mating between two F_1 hybrids produces a second hybrid generation, this F_2 generation may be unable to reproduce because of hybrid breakdown. The second-generation hybrids are defective in some way that prevents successful reproduction. Hybrid breakdown has been demonstrated in sunflower hybrids.

Although interspesific incompatibility has been reported in a large number of crosses, most of them are based on seed-set data; only limited studies have been done to understand the details of incompatibility or its mechanism.

2.6 Oil content and oil profile

Oil can be extracted from many raw materials, but not all contain edible oil (Akinoso, *et al*., 2006). Some contain poison and unpleasant flavors. Oil from plants is classified as vegetable oil. The oil content of vegetable oil-bearing materials varies between 3 and 70% of the total weight of the seed, nut, kernel or fruit (Bachman, 2004). The largest sources of vegetable oils are annual plants; include soybean, corn, cottonseed, groundnut, sunflower, rapeseed, melon and sesame seed (Frank, 1998). Oil is obtained from oilseed by either solvent extraction or mechanical expression or the combination of the two processes.

Table 2.2 Structure of the major fatty acids in edible oil (Belitz, *et al*., 2004)

Abbreviated	Common name	Proportion (100%)
14:0	Mystiric acid	2
16:0	Palmitic acid	11
18:0	Stearic acid	4
18:1(9)	Oleic acid	34
18:2 (9,12)	Linoleic acid	34
18:3 (9,12,15)	Linoleniic acid	5

Noted: A percentage estimate based on world production of edible oils

Unsaturated fatty acid tends to have lower melting points than saturated fatty acids do with the same length of chain. This is because natural unsaturated fatty acid molecules, with their *cis* configuration (and their linked structure), cannot pack together as tightly as the straight-chain saturated fatty acid molecules. This means that it takes less heat energy to disrupt the ordered

structure of such unsaturated fatty acids in the solid state and convert them into the less ordered liquid state. The more unsaturated fatty acids there are in fat, the more likely it is to be a liquid (and so technically oil) at room temperature. Coconut, palm and palm kernel oil (or tropical oil) contains 81%, 87% and 57% saturated fatty acids, respectively; yet they are liquid in room temperature. This is because many of their fatty acids are short-chain, which tend to lower the melting point of fat (Guthrie, *et al*., 1995).

2.6.1 Fatty acid composition of *J. curcas*

Table 2.3 Fatty-acid composition of the seed of *J. curcas* in four provenances of Mexico and two plantations in Nicaragua.

Fatty Acid	*J. curcas* of Mexico[a]				*J. curcas* of Nicaragua[b]	
	Castillo de Teayo, Veracruz	Puebiblo, Veracruz	Coatzacoalcoz, Veracruz	Yautepec, Morelos	Caboverde	Nicaragua
Myristic acid	0.18	0.15	1.18	0.3	0.1	0.1
Palmitic acid	11.4	12.3	13.0	10.5	15.1	13.6
Palmitoleic acid	0.44	0.55	0.52	0.32	0.9	0.8
Stearic acid	2.27	2.80	2.53	2.45	7.1	7.4
Oleic acid	45.0	47.1	48.8	41.5	44.7	34.3
Linoleic acid	40.3	36.7	34.6	44.4	31.4	43.2
Linolenic acid	0.11	0.18	0.12	0.21	0.2	0.2

Note: [a] Martinez-Herrera, *et al*., (2006)

[b] Foidl, *et al*., (1996)

The fatty acids found common in all the oil samples were oleic, linoleic, palmitic and stearic acids. Martinez-Herrera, *et al*., (2006) reported the major fatty acid in the sample from Veracruz was oleic acid, whereas in the sample from Morelos it was linoleic acid. This variation

is possibly due to soil and climate conditions. It is very important to mention that the phorbol esters were found in high levels only in the sample from Coatzacoalcos (3.85 mg/g). In the oils from Castillo de Teayo, Pueblilo, and Yautepec seed samples, phorbolesters were not detected. The results obtained are similar to those reported for the *J. curcas* seed provenances from different countries, such as Foidl, *et al*., (1996) who reported that the major fatty acids in the Nicaragua variety was linoleic acid, whereas in the Caboverde variety oleic acid is predominant.

2.6.2 Fatty acid composition of *R. communis*

The most important seed constituent is the oil, usually 40-60% in commercial cultivars high in triglycerides. The main fatty acid component is ricinoleic acid (12-hydroxy oleic acid) whose three hydroxyl groups confer on the oil unique property of solubility in alcohol. Ricinine is a white crystalline alkaloid usually extracted from a seed, but it is also contained in leaves. It is synthesized mainly in the growing tissues of young and developing organs of older plants. Castor residue or meal contains toxins and allergens which remain behind in the residual meal. These toxins are soluble in water, but not in castor oil. Despite these unhealthy toxins, when detoxified, they make an important by-product that is potentially beneficial in horticultural and agricultural applications (McKeon, *et al*., 2007).

However, due to the presence of the toxic protein ricin and hyperallergenic 2S albumins, the production of castor oil is problematic. One logical approach to solving the biohazard problem is to produce ricinoleate in plants lacking these noxious components. Several researchers have attempted to produce ricinoleate oil by transgenic expression of the oleoyl-12-hydroxylase, the enzyme that is responsible for ricinoleate biosynthesis (McKeon, *et al*., 2007).

Table 2.4 Analysis of castor oil from selected countries (Weiss, 2000)

Source	C_{16}	C_{18}	Oleic	Linoleic	Linolenic	Eicosenoic	Ricinoleic	Dihydroxystearic
Angola	1.0	0.7	2.9	4.3	0.6	0.5	89.4	0.6
Brazil	1.0	0.8	3.3	4.2	0.5	0.4	88.9	0.9
India	1.1	1.0	3.1	4.7	0.5	0.5	88.4	0.7
USA	0.9	0.8	2.0	4.3	0.6	0.3	90.3	0.8

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials

This experiment had 3 parts; pollen study, *in vivo* and *in vitro* pollination, and oil extraction. The seeds of the *R. communis* commercial-type from the Thai Castor Oil Company from Nonthaburi Province, Thailand, were kindly provided by former Prof. Sujin Jinahyon (President of Naresuwan University, Thailand), which are being maintained in the germplasm of Mae Fah Luang University, were used in this study.

3.1.1 Pollen materials

Table 3.1 Plant materials used for pollen size and morphology observation

Accessions	Number of plant sample	Flower sample per plant	Country of origin
J. curcas toxic	10	5 female flowers	Thailand
J. curcas nontoxic	10	5 female flowers	Mexico
R. communis commercialf-type	10	5 male flowers	Thai Castor Oil Company, Thailand
R. communis wild-type	10	5 male flowers	MFU germplasm, Thailand

3.1.2 Intergeneric hybridization materials

The *in vivo* pollination between *J. curcas* toxic x *R. communis* commercial-type was done in the garden of Saboo Dam Thai Company (Bangkok) which kindly helped and provided by Aj. Orachun Kewkangwan. Thirty two plants of *J. curcas* toxic (Thai origin) and 5 plants of *R. communis* commercial-type (Thai origin) were used in this experiment. Two female flowers of

J. curcas toxic per inflorescence per plant and a hundred male flowers of *R. communis* commercial-type as pollen source were taken as a sample.

The *in vivo* pollination between *J. curcas* nontoxic x *R. communis* wild-type was done in MFU germplasm, Chiang Rai. Thirty plants of *J. curcas* nontoxic (Mexico origin) and twenty plants of *R. communis* were used. Two female flowers of *J. curcas* nontoxic per inflorescence per plant and a hundred of male flowers of *R. communis* nt were taken as a sample.

Table 3.2 Plant materials used for *in vivo* pollination

Accessions	Number of plant sample	Total sample flower	Country of origin
J. curcas toxic	32	63 female flowers	Thailand
J. curcas nontoxic	30	60 female flowers	Mexico
R. communis commercial-type	5	100 male flowers	Thai Castor Oil Company, Thailand
R. communis wild-type	20	100 male flowers	MFU germplasm, Thailand

The *in vitro* pollination between *J. curcas* nontoxic x *R. communis* commercial-type was done in the Tissue Culture laboratory, School of Science, MFLU, Chiang Rai, Thailand. Fifty plants of *J. curcas* nontoxic (Mexico origin), 50 plants of *J. curcas* toxic (Thai origin) and twenty plants of *R. communis* were used in this experiment. Five female flowers of *J. curcas* nontoxic and toxic per plant and 200 male flowers of *R. communis* from 20 plants as pollen source were taken as a sample.

Table 3.3 Plant materials used for *in vitro* pollination

Accessions	Number of plant sample	Total sample flower	Country of origin
J. curcas toxic	50	250 female flowers	Thailand
J. curcas nontoxic	50	250 female flowers	Mexico
R. communis commercial-type	20	200 male flowers	Thai Castor Oil Company, Thailand

3.1.3 Seed materials

The seeds of *R. communis* commercial-type were kindly provided by Thai Castor Oil Company from Nonthaburi Province, Thailand and were planted in MFU germplasm. The seeds material of *J. curcas* toxic, nontoxic and *R. communis* wild-type collected from germplasm of Mae Fah Luang University were used in this study.

Table 3.4 Seed materials used for physical measurement and oil extraction

Accessions	Number of plant sample	Total sample seed	Country of origin
J. curcas toxic	10	300	Thailand
J. curcas nontoxic	10	300	Mexico
R. communis commercial-type	10	300	Thai Castor Oil Company, Thailand
R. communis wild-type	10	300	MFU germplasm, Thailand

Table 3.5 Plant material for each experiment

Accessions	LM observation	SEM analysis	*In vivo* pollination	*In vitro* pollination	Seed diversity	Oil extraction	FA analysis
J. curcas toxic (Thailand)	✓	✓	✓	✓	✓	✓	✓
J. curcas nontoxic (Mexico)	✓	✓	✓	✓	✓	✓	✓
R. communis dwarf-type	✓	✓	✓	✓	✓	✓	✓
R. communis wild cultivar	✓	✓	✓		✓	✓	✓

3.2 Pollen study

3.2.1 Fresh pollen measurement

This method has been used to study the pollen grains morphology, shape and size of *J. curcas* and *R. communis* in hydrated conditions. Observing fresh pollen grains aims to study the real shape of grains before the lipid removed, carbohydrates and some proteins in the exine layer. The diameter of a hundred pollen grains of *Jatropha* and castor were counted to be compared under a Light Microscope with 40x and 10x lens. Pictures of pollen grains were taken and diameters were measured by a Motic camera program. The sizes of fresh pollen grains were measured and record as basic information to determine the male and female parent plant in the hybridization experiment. Statistical analysis of pollen grains data was performed by Univariate ANOVA using SPSS (ver. 16.0 for Windows).

3.2.2 Pollen acetolysis

The acetolysis method was introduced by Erdtman (1954, with minor modification) is still a highly successful technique to destroy pollen exine material coat with the exception of sporopollenin which forms in the exine (Hesse and Waha, 1989). Pollens grains were poured into graduated clean centrifugal tubes and rinsed with 70% Ethyl alcohol (MERCK, Germany), centrifuged for 5 min (14,000 rpm) then acetolyse with Acetic and Sulphuric Acid (9:1). Shake and mixed pollen-solution well, boiled in water bath 94-100^{o}C (10 minutes) and centrifuged (14,000 rpm) for 5 minutes. This was repeated once to ensure the pollen grains were clean. Acetic and Sulphuric Acid (1:1) were used for next solution and centrifuged (14,000 rpm) for 5 minutes and this was repeated three times. By placing these droplets on a cover slide and drying them overnight, the morphology of pollen grains was observed under a Scanning Electron Microscope.

3.3 Intergeneric hybridization

3.2.1 *In vivo* pollination

Sixty three of the *J. curcas* toxic and sixty of the non toxic female flowers were pollinated with castor pollen grains. The pollinations were performed by emasculating the male flowers before the female flowers opened. Fresh castor pollen grains were removed with a paintbrush and then brushed on to the stigma of the female parent. The pollinated flowers were labeled and numbered then bagged in order to prevent undesired pollination. the pollinated female flowers were extracted at 4 hours, 48 hours, 3 days, 5 days, and 7 day after their pollination and were then fixed in acetic acid (Lab-Scan, Ireland): alcohol (MERCK, Germany) (1:3) v/v for 12 hour. The fixed pistils were rinsed thoroughly in tap water and treated with aceto archein (Univar, UK). The pistils were sectioned by a razor blade and kept on slides and cover slip to observe the pollen tube germination and zygote development. The development of zygote growth was observed following the microtome section technique (Ruzin, 1999) using the fixed flowers in formalin (Lab-Scan, Ireland): alcohol (MERCK, Germany): acetic acid (Lab-Scan, Ireland): distillated water (10:50:5:35).

The investigation by microtome section was the further method to investigate the intergeneric compatibility, and the steps of microtome section were: pollinated female flower of

J. curcas which was fixed overnight at 4°C-Room Temperature in a FAA (50% EtOH (MERCK, Germany), 5% glacial acetic acid (Lab-Scan, Ireland), 10% formalin (Lab-Scan, Ireland), 35% water) solution. The tissues were killed and hardened within 18-24 hours when treated at room temperature and then were washed in 50% EtOH (MERCK, Germany), 15 minutes. The fixing tissues were dehydrated and transferred into paraffin using TBA (tert-butyl alcohol) (Fisher scientific, USA) as the intermediate solvent which is two hours for each step as the following order:

a. EtOH 95%:H_2O:TBA= 50:40:10

b. EtOH 95%:H_2O:TBA= 50:30:20

c. EtOH 95%:H_2O:TBA= 50:15:35

d. EtOH 95%:TBA= 50:50

e. EtOH 100%:TBA= 25:75

f. TBA= 100

The dehydrated tissues were infiltrated with paraffin by pour off 1/3 volume and replaced with an equal volume of melted paraffin. Form a paraffin 'cap' on top of the TBA, uncap the vial and place in the paraffin oven (58°C). At four hours intervals, ½ a volumes of paraffin/TBA mixture was poured out into paraffin waste container and bring up to volume with liquid paraffin for 3 times. All the paraffin/TBA mixture was poured off and adds pure liquid paraffin. Fresh and pure paraffin was added for three times at one hour intervals (overnight for the last step).

Tissue samples were embedded in paraffin using hand-made Aluminium foil boats. Paraffin was poured into the boats slowly to prevent the bubbles being revealed. Mayer's adhesive preparation as the section adhesive. Mix 1:1 fresh egg white and glycerol plus one gram sodium salicylate (MERCK, Germany). The glass microscope slides were coated very lightly and let it dry.

1) The paraffin embedded tissue was section at 8 µm on a rotary microtome. The ribbon was cut into lengths of about 75% of the available slide length to allow for expansion. The ribbon floated on the warm water at 58°C.
2) The ribbons were attached onto slide surfaces which were already mounted by Mayer's adhesive. The slides were placed horizontally overnight in a 42°C oven.
3) At the staining step the ribbon was soaked into Coplin jars which each contain:

a. Xylene (Fisher scientific, USA), 10 min.
b. Xylene + Abs. alcohol (1:1), 5 min.
c. Alcohol 95%, 5 min.
d. Alcohol 70%, 5 min.
e. Alcohol 50%, 5 min.
f. Alcohol 30%, 5 min.
g. Water, 5 min.
h. Safranin O (MERCK, Germany) 1% Safranin O in distillated water, 2 h.
i. Water 3 times, 10 s each.
j. Alcohol 30%, 5 min.
k. Alcohol 50%, 5 min.
l. Alcohol 70%, 5 min.
m. Alcohol 95%, 5 min.
n. Fast green (MERCK, Germany) 1% Fast green in Alcohol 95%, 15 s.
o. Absolute alcohol, 2 min.
p. Xylene + Abs. alcohol (1:1), 10 min.
q. Xylene 3 times, 5 min each.

4) Coverslip was mounted with permount and observed under light microscope.

3.2.2 *In vitro* pollination

3.2.2.1 Media culture

The basal medium (see more details on APPENDIX A) consisted of Murashige and Skoog (1962) 30 g L^{-1} sucrose and 0.7% agar. The medium was supplemented with 0.125 mg L^{-1} of BA (cytokinin) and 0.125 mg L^{-1} of NAA (auxin) singly or a combination. Subculture medium callus induced supplemented with 0.5 mg L-1 of BA (SIGMA, USA) and NAA (SIGMA, USA) combination. The pH of each medium was adjusted to 5.7 ± 0.5 and autoclaved (Sanyo, Japan) at 121^{o}C for 15 minutes. All culture was incubated at 24 ± 2^{O}C under a 16-hours photoperiod using cool, white fluorescent light.

3.2.2.2 Flower sterilization and pollination

Emasculated unopened flowers 2-3 days before anthesis were sterilized and pollinated in the laminar flow cabinet. The flowers were soaked in distilled water: sodium hypochlorite (3:1) for 5 minutes and rinse three times in sterile glass-distilled water. Then the whole pistil (sepals and petals are included) and mature unopened male flowers were sterilized by flaming them with 95% Ethyl alcohol for a minute. Pollen grains were obtained by excised the male flower and exposed in to the laminar light on a sterile Petri dish. Pollen grains were dispersed from the anther and put on to stigma parts. Female flowers cultured in media and were kept on the sterile room to prevent contaminants. Subculture every 3 weeks was necessary to maintain the growth.

3.3 Physical characterization of seed and seed oil analysis

3.3.1 Seed measurement

The seed of the *J. curcas* (non toxic and toxic) variety and the *R. communis* (commercial and wild type) were measured by Varnier caliper. This method was used aim to investigate the variation seed size of two species of *J. curcas* and two varieties of *R. comunis*. 100 seeds of each species were measured for their length, breadth, and the thickness. Three hundred seeds were weighted to obtain the average weight gram per 100 seeds. Statistical analysis of seed measurement and seed weight data was performed by Univariate ANOVA using SPSS (ver. 16.0 for Windows).

3.3.2 Analysis of oil content and oil profile

3.3.2.1 Sample preparation

The seeds from each species were cracked from the seed-coat, than crushed with pestle and mortar. Samples were cleaned and were placed in the clean bottles separately avoiding the contamination from another sample. Two grams from each sample were weighed and measured by the sample being placed into the Whatman No. 4 filter paper (Toyo Roshi

Kaisha ltd., Japan). The thin cup were washed and dried in the hot oven for 20 min and cooled in desiccators (Gibthai Co ltd., Bangkok) for 30 min. The weight of the thin cup was recorded as a basis cup weight before the sample was placed into the cup. Oil was extracted from the sample and was repeated three times to obtain the average weight. The statistical analysis of oil weight data was performed by Univariate ANOVA using SPSS (ver. 16.0 for Windows).

3.3.2.2 Determination of oil content

The extraction of oil from the seed sample was carried out by the Soxhlet method using Foss Soxtec apparatus (Sitiporn Asc., Bangkok). The steps included boiling, rinsing, recovery and drying. For operating this, 70 ml of normal Hexane was added into cups and 2 grams of the sample was put into thimbles, which were subsequently placed in the apparatus. The extraction was performed at 135^{O}C. The solvent was boiling for 20 minute, during this time the solvent evaporated up to the condenser where it condensed, dropped onto the sample in the thimble and leached oil out through the paper thimble repeatedly. Then the rinsing step was carried out for 60 minutes to extract the papers and the rest of the oil in sample into the cup. Afterwards, solvent was evaporated out and the cups were dried in a hot air oven at 105^{O}C for 20 minutes. The cups were cooled in desiccators and weighed to determine the oil and moisture content using the following equation.

$$\text{Oil content (\%)} = \frac{\text{weight of oil (g)}}{\text{weight of seed (g)}} \times 100$$

$$\text{Moisture content (\%)} = \frac{\text{Wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100$$

Extraction was triplicate. The extracted oil was kept in amber vials under refrigeration conditions to determine the fatty acid compositions. Statistical analysis of oil content data was performed by Univariate ANOVA using SPSS (ver. 16.0 for Windows).

3.3.2.3 Determination of fatty acid compositions

The fatty acid compositions of extracted oils were determined by converting all fatty acids in triglycerides into the corresponding fatty acid methyl esters followed by gas chromatography-mass spectroscopy (GC-MS). The transformation of fatty acids was performed as follow; ten micro liters of oil were methylated with 0.5 NaOH in methanol (500 µl), vortex (Scientific Industries, USA) for 1 minute and then incubated for 20 minutes at 60^0C after cooling. 0.5 µl of *n*-hexane was added into the tube, then vortex for 1 minute. washing the hexane layer by added 200 µl of distillated water, vortex for 30 seconds. Followed by centrifuge (Tuttlingen, Germany) at 1,500 rpm for 30 seconds to separate them. The hexane (upper) phase was transferred to another tube and was dried over Na_2SO4 anhydrous. This solution was subjected for GC-MS analysis using DB-5 column. The condition for GC was analyzed by statistic using Univariate ANOVA.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Pollen Study

4.1.1 Fresh pollen and light microscopic observation

Observation using light microscope found some variation in *J. curcas* pollen size. The average pollen size of *J. curcas* toxic variety was the biggest among the four accessions. The diameter of pollen grains of toxic *J. curcas* and nontoxic averages 96.01 μm (SD= 0.1620) and 84.26 μm (SD= 0.0075), respectively. The results of the ANOVA showed the significant difference of the pollen grain size between toxic and non toxic (P>0.05). The pollen size of *J. curcas* in Vikashapatnam District, India, about 81-89 μm, was published by Raju and Ezradanam (2002) and some palynologists from India reported that the size of *J. curcas* pollen was about 94.5 μm (Bhattacarya *et al*., 2005), but the information about variation in pollen size was never revealed before. From the previous report, it seems that the size of Jatropha pollen grains varies upon the environment. Our toxic variety pollen grain was bigger than Raju and Ezradanam (2002) have reported; the grain of *J. curcas* pollen is 89 μm, but nontoxic variety was smaller.

Moreover, the presence of small grains (35-40 μm) compared to the average size was ssumed as infertility/sterile pollen grains (see further detail in Fig. 4.1 and 4.2). The sterile pollen has been reported in many species. In *Mimulus guttatus*, viable grains are greater than 35 μm, while most inviable grains are less. The mean diameter of viable grains is 41.9 μm and the diameter of inviable grains is 28.9 μm (Kelly et al, 2002). From this observation, it is suggested that the size of the pollen grains can be used to estimate pollen viability.

Table 4.1 Pollen grains size

Plant accession	N	Average		
		1	2	3
R. communis wild-type accession	100	30.03[a]		
R. communis commercial accession	100	32.84[a]		
J. curcas nontoxic	100		84.26[b]	
J. curcas toxic	100			96.01[c]

Note: different letter indicate significant difference between accessions

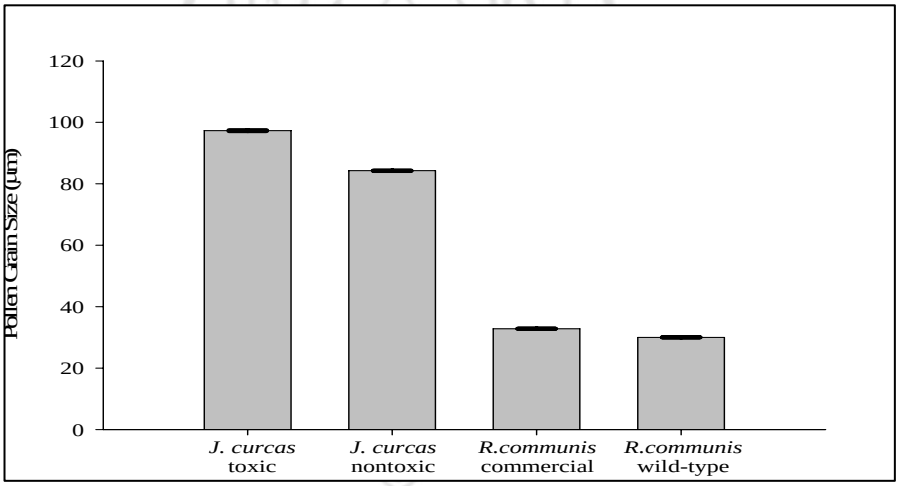


Figure 4.1 Comparative size hydrated pollen grains.

Ricinus communis commercial type pollen grains are 32.84 µm (SD= 0.0373) and wild-type are 30.03 µm (SD= 0.0201). Castor pollen grains were used as the male parent due to the fact that the grains are smaller than *J. curcas* and assumed easier to penetrate the style and micropyle pore.

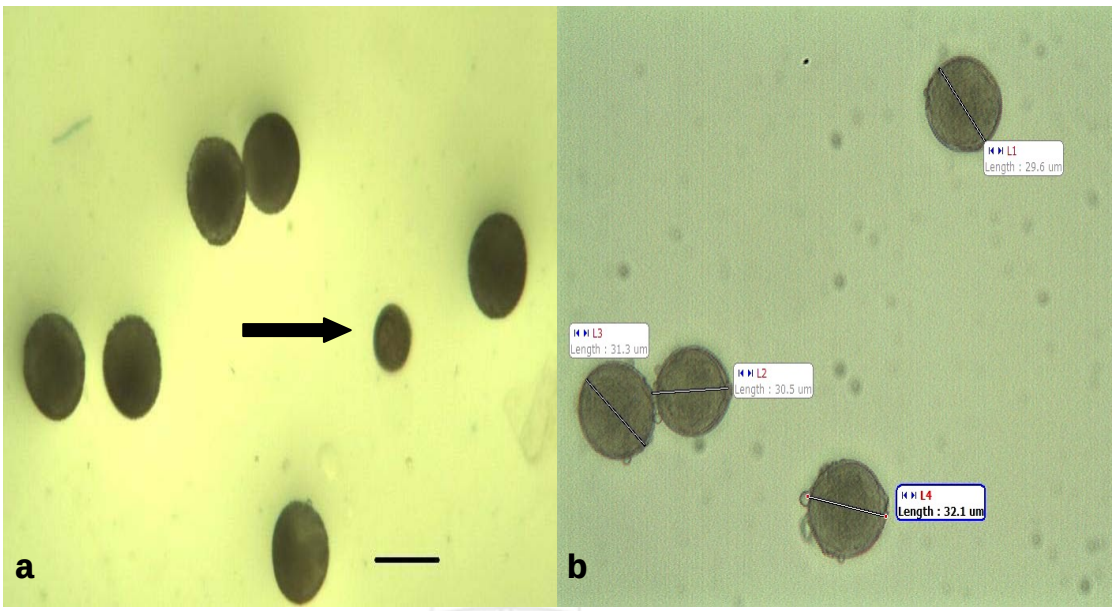


Figure 4.2 Hydrated pollen grains of *J. curcas* and *R. communis*; the arrow indicate sterile pollen in *J. curcas* (a), bar 100 µm and *Ricinus communis* commercial-type hydrated pollen grains (b), bar 50 µm.

Observation under light microscopy (LM) pollen grains of *J. curcas* and *R. communis* as in figure 4.2 exhibit distinctions in shape and size, but exine structural pattern was unclear. Pollen grains of *R. communis* has unique characteristic. These grains would change their shape from ellipsoidal into globular whenever contact with a drop of water. The pollen shape changing was as a response to the water intake through the exine (Shivana, 2003). But inversely, the grains of *J.curcas* did not change their shape a lot like *R. communis*. *Jatropa curcas* hydrated pollen grains still remain globular before or after contact with a drop of water on a microscope slide. This different characteristic may be due to the different ways of pollination. Pollen grains such as *J. curcas* pollen is transferred by insect or zoophilous (Heller, 1996), generally are coated with an oily, sticky and often colored material commonly termed 'pollenkit'. Pollenkitt threads have many functions: keeping the pollen in anther until dispersal, forming pollen clumps and sticking pollen to insect (Pacini, 2000). Some substances covering the pollen grains play an important role in animal pollination, e.g., the lipid, sticky and viscous pollenkit (Hesse, 1986). These substances usually cover the aperturates, which are visible only after acetolysis. Anemophilous plants such as

R. communis produce an enormous amount of pollen (Weiss, 2000). Pollen grains are light and non-sticky, and have a high surface-to-volume ratio (Shivanna, 2003).

4.1.2 Pollen acetolysis and scanning electron microscopy (SEM) observation

Scanning electron microscope (SEM) was carried out with acetolysis, a method already used in identifying ultra-structure in the pollen surface of many varieties. The acetolysis process destroys all pollen material with the exception of sporopollenin that forms the outer wall, the exine (Hesse and Waha, 1989). The results from SEM, the morphology of two genera are clear to observe. The aperturate part both genera ia also distinguishable from the pictures.

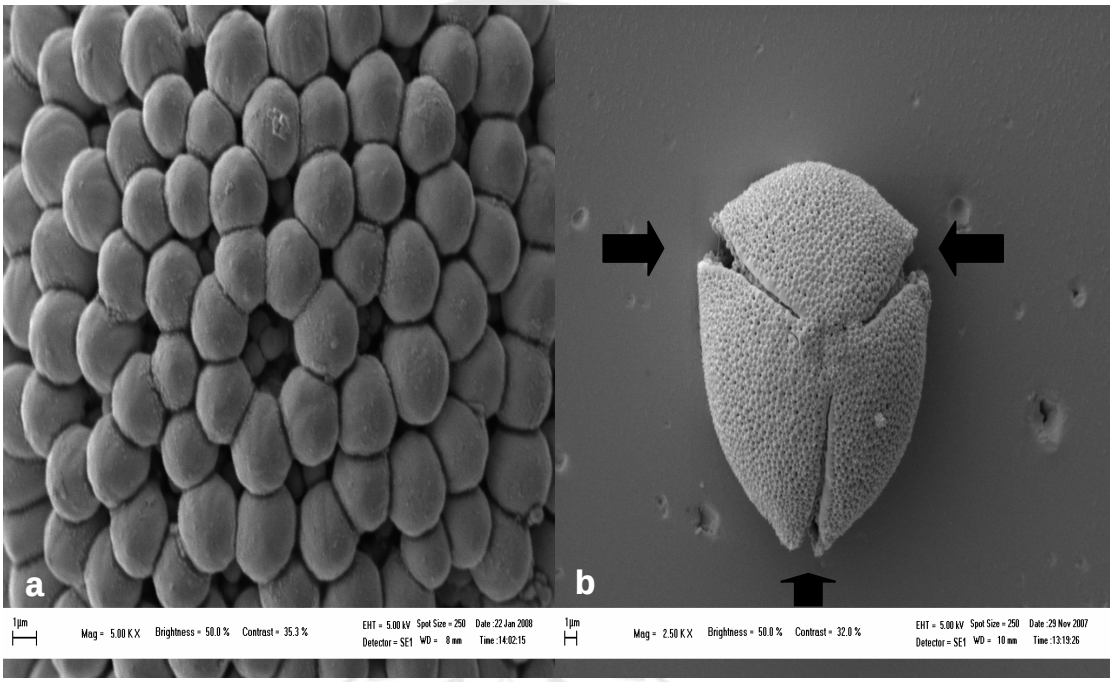


Figure 4.3 Apertures in the pollen exine layer. *Jatropha curcas* inaperture grain (a) and three arrows show of *R. communis* tricolporate or posses three apertures (b).

Apertures are key characters of pollen grains with systematic importance in angiosperms. For pollen terminology and for the systematic palynology the apertural conditions are very important. In *J. curcas*, the mature pollen grains in figure 4.3 are inaperturate. Inaperturate pollen grains are usually tin-walled, and suggest that all pollen grains without exinal differentiation are

able to germinate at any area of the pollen surface (Furness and Rudal, 2004) or the pollen tube can potentially emerge at any point. Nevertheless, the significant initial differentiations in pollen of some genera mean that inaperturate pollen grains (without exine differentiation) sometime act as mono- or even diaparture grains (Thanikaimoni, 1986).

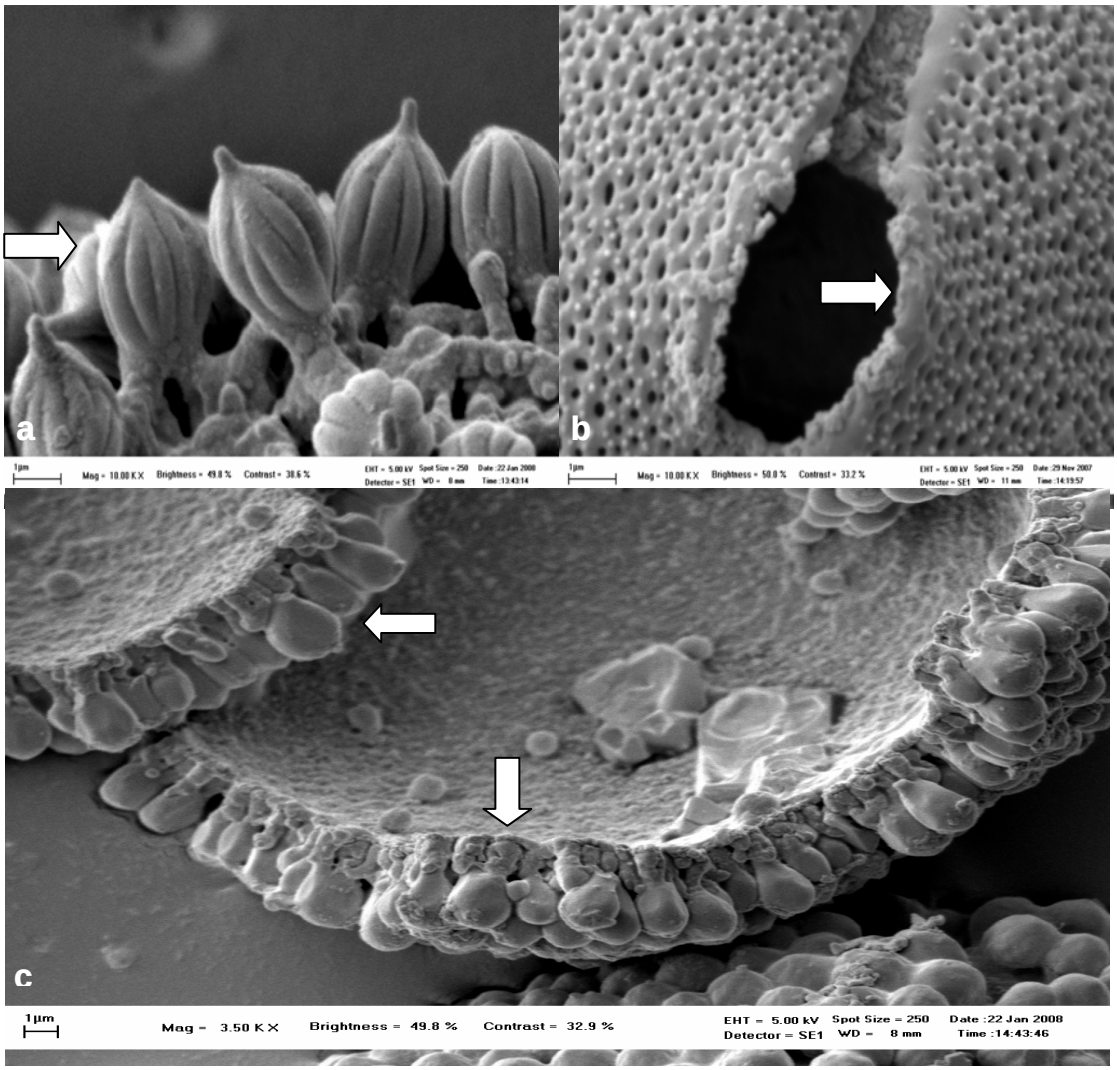


Figure 4.4 Small spinules in exine layers of *J. curcas* (a) and small dots in exine of *R. communis* (b). Fig. c top arrows show the exine part and bottom arrow show intine part of *J. curcas* pollen.

Interestingly, castor pollen grains are tricolporate or clearly have three apertures (Fig. 4.3). The function of aperture such as sites for pollen tube exit, water uptake, transfers of

recognition substances and accommodation of volume change (Furness, 2007; Shivana, 2003). Possession of three or more pollen aperturate like castor (Takahashi, *et al*., 2000) might offer a selective advantage because of the increased fertilization rate, thus facilitating pollen and stigmatic surface contact (Furness and Rudal, 2004).

From the SEM pictures shown clearly, the exine sculptures of *Jatropha* pollen grains are semitectate (Fig. 4.3); the tectum (the layer of sexine, which forms a roof over the collumelae) covers the pollen. The diameter of pollen grains were 42-53 µm, spinules height in exine layer around 3.2-4.5 µm and intine thickness was1.4-1.9 µm (Fig. 4.3 c).

Ricinus pollen grains have a diameter of around 19-22 µm with colpus length 3.2-4-9 µm and breadth 2.4-3.2 µm. Colpus along aperture clearly observable under SEM and the exine sculpture are semitectate (Fig. 4.4).

Shaheen (2002) investigated morphological variation of pollen of *R. communis* from 12 areas in Egypt (1. Egate, 2. Gabel Elba, 3. Wadi Defeit, 4. Wadi Allaqi, 5. University campus, 6. El-Shalal, 7. El-Kubbania, 8. Assuit, 9. Barrages, 10. El-Mounfia 11. New Demitta and 12. North Sinal) and by SEM he grouped it into two; pollen grains from area 1-5 have trilet aperture type, globose pollen shape and perforated exin sculpture, and from area 7-12 the characteristics were dicolporate aperture type, oblate pollen shape and granulated exin sculpture. Meanwhile, *R. communis* pollen grains (commercial and wild-type A, B, C, D) from MFU field (Appendix A) are similar; tricolporate.

4.2 Intergeneric hybridization

4.2.1 *In vivo* pollination

Pollinated flowers were observed and counted to investigate the compatibility/incompatibility between genera. *In vivo* pollinations were applied by *J. curcas* toxic (63 flowers) and nontoxic (60 flowers) variety as a female parent (see more details in Table 4.2). The pollinated female flowers remained healthy for about a week, but later turned yellow, then brown and dried up. The *in vivo* test indicated 100% of flowers both of *J. curcas* varieties x *R. communis* commercial-type were aborted in average 9 (11.4%) and 10 (23.6%) days after pollination (Fig. 4.5 and table 4.2).

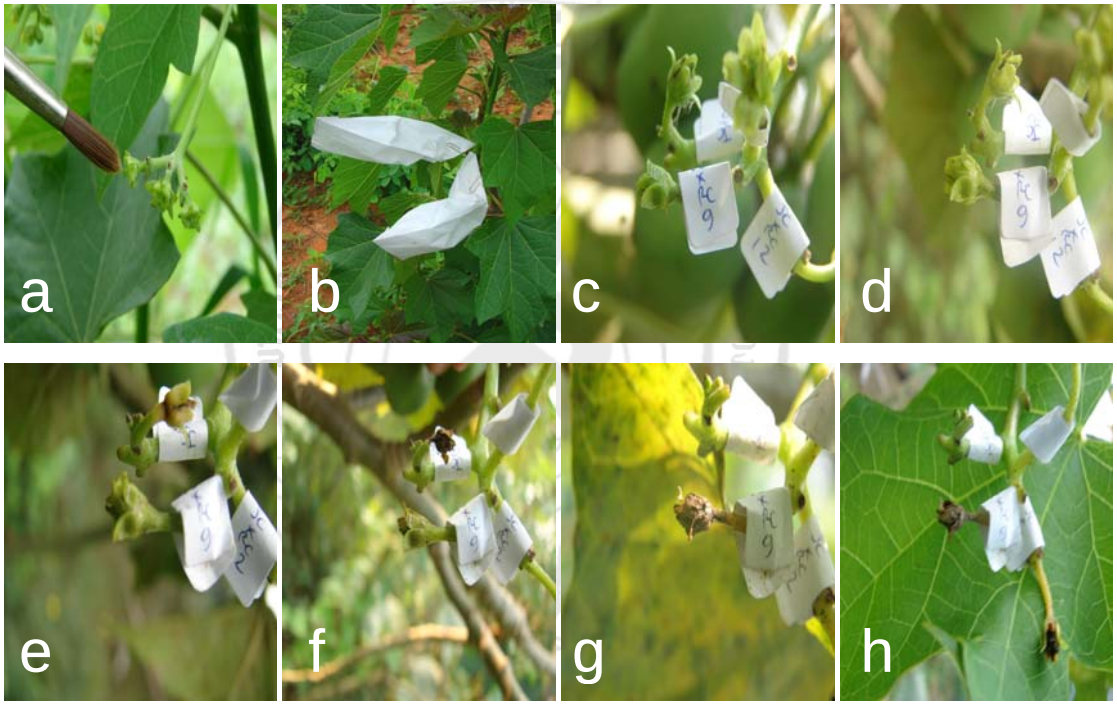


Figure 4.5 *Jatropha curcas* female flowers after *in vivo* pollination. Flower still remained healthy for 3 days (c), 5 days (d) and 7 days (e). Flower turned yellow in 9 days (f), brown in 11 days (g) and was dry in 13 days after pollination (h).

Table 4.2 *In vivo* pollination observation data in the field

Day After pollination	*J. curcas* toxic x *R. communis*. pollinated abortion flower	Total abortion per day (%)	*J. curcas* nontoxic x *R. communis* pollinated abortion flower	Total abortion per day (%)	Total abortion (%)
5th	-		-		
6th	11	17	6	10	13.8
7th	11	17	10	16	17
8th	6	9	3	5	7.3
9th	7	11	7	11.7	11.4
10th	17	26.9	12	20	23.6
11th	4	6	8	13.3	9.8
12th	3	4	5	8.3	6.5
13th	6	9	2	3	6.5
14th	-	-	5	8.3	4.1
Total flowers	63		60		100
Total seed set	0		0		

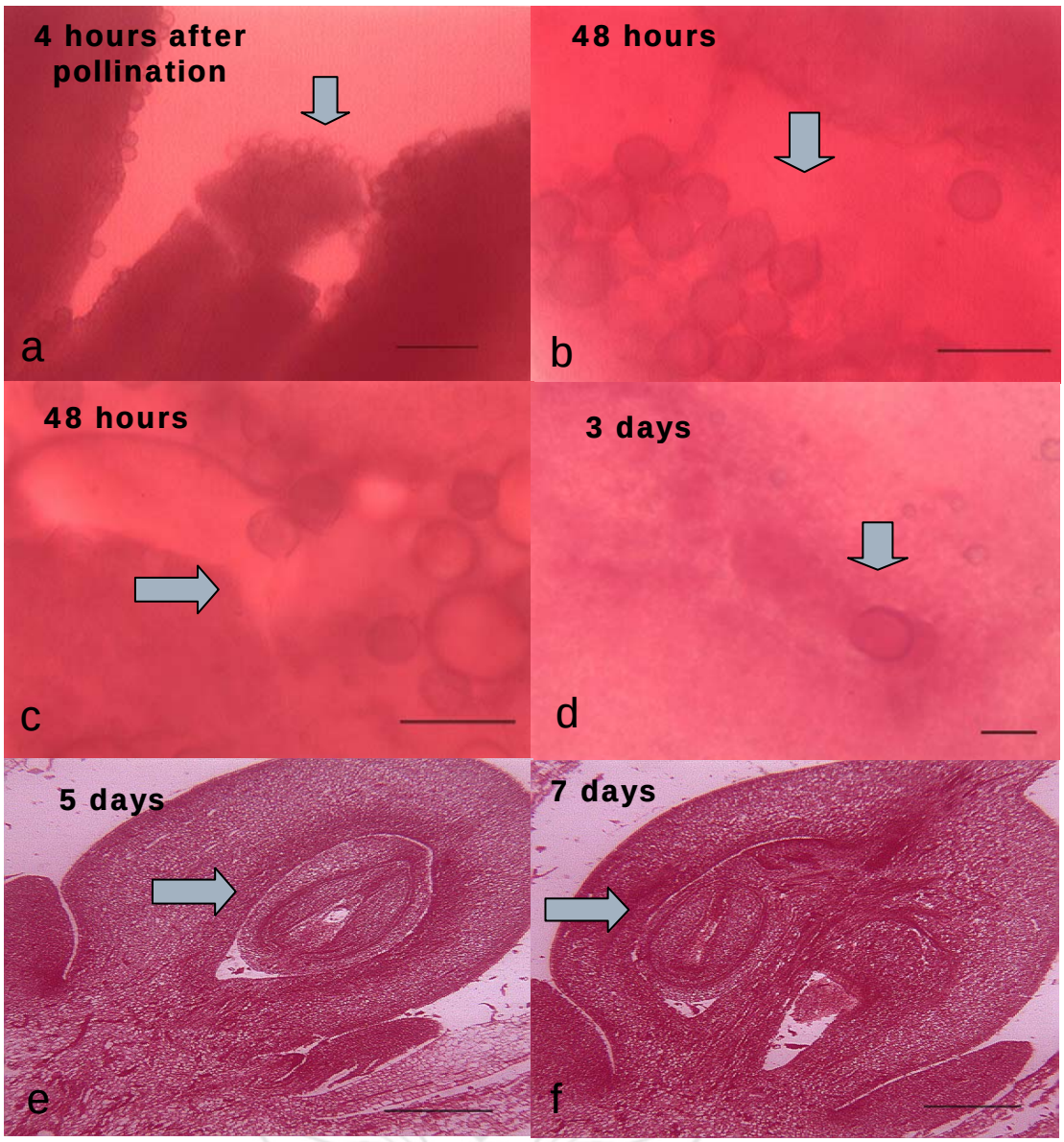


Figure 4.6 Pollen tube growth and hybrid zygote development. *Ricinus* pollen grains on *Jatropha* stigma 4 hr after pollination, bar 150 µm (a). Pollen tube germinating in the placenta 48 hours after pollination, bar 60 µm (b&c). Hybrid zygote assumed developed in embryo sac 3rd day after pollination, bar 10 µm (d). Seed development in 5th day after pollination, bar 5 µm (e) and cotyledon, endosperm, radicle and seed coat development 7th day after pollination (f).

The pollen-pistil interactions were observed in terms of pollen germination and pollen tube growth in the ovary of *J. curcas.* The results of the microtome section showed up 4 hours after pollination pollen was attached onto stigma with no pollen tube germination appearance (Fig. 4.6 a). These early events of adhesion and hydration of pollen on the stigma have been observed intensively in this study.

The *Ricinus* pollen tube was germinated after 48 hours of pollination (Fig. 4.6 b & c). The tubes were germinating through the apertures into style part of the *J. curcas* flower. Richard (1977) reported that in most plants, growth of the pollen tube lasts between 12-48 hours from pollen germination to fertilization. Pollen tubes were seen entering *J. curcas* ovules in the pistil section after they had been pollinated for 48 hours. Unfortunately, we were unable to observe how the male gamete penetrates female gametophytes through the micropyle pore which would be direct proof that the developed globular embryos were a hybrid origin (Fig.4.6 a-f). However, pollen tube growth through the style seems to be necessary for guiding the tubes to the ovule micropyle. Although the stages in double fertilization were not seen by us, it may be assumed to take place normally, for the development of the embryo and endosperm followed the normal course and the subsequent ovule development 3 days after pollination indicated at least partial success of fertilization in the *J. curcas* ovary. This phenomenon showed that *J. curcas* was capable of supporting and guiding the *Ricinus* pollen tube growth. This indicates that the male (pollen) of *R. communis* and the female organ (ovary) of *J. curcas* are compatible with a lack of pre-fertilization barriers.

Even though the zygote presenced (figure 4.6 d, e and f) in 3 days and developed in 7 days after pollination, but it seemed failed to set the seed more than 9-10 days. The endosperm development retarded more than 10 days is often the cause of spontaneous abortion of hybrid embryos after interspesific pollination (Sharma et al, 1996). Interspecific hybridization studies in *Alstromeria* show that embryos do not mature, due to a failure of the endosperm to develop properly (Buitendijk, 1995). Jaskani *et al*., (2005) noted the equal fact: disfunction of endosperm, or it is absence, causes an abortion of the embryo. The problem in interploid crosses of *Citrus* embryo abortion because the absence or of an interrupted supply of food materials from the endosperm, and embryo do not complete their normal development. Such flowers abortion caused probably due to the impairment of ovule function (Kokubin, *et al*., 2001) or hybrids breakdown

are thought to be because the gene pools within population have been selected overtime for their harmonious interaction (Hancock, 2004).

The abortion of *J. curcas* flowers in this test might be caused by failure of the tissue to feed the endosperm seed zygote or imbalance between hybrid zygote and *Jatropha* maternal cell. Twenty two chromosomes pair of *J. curcas* and 20 pairs of *R. communis* chromosomes might become an obstacle in this hybridization. If 10 chromosomes pairs become homologue in metaphase, than one chromosome remain have no pair, as Hodnett, *et al*., (2005) stated that interaction that includes inhibition of successful fertilization in interspesific crosses may be considered as a result of inharmonious genetic interaction due to the genetic divergence among the species (Hodnett, *et al*., 2005). This indicated the post zygotic barriers in intergeneric hybridization inhibited by different genome of each plant. As the *in vivo* pollinations results were not effective in the realization of hybrid seed, the barrier in this cross appeared to operate at post-fertilization stages. Very often interspesific or intergeneric crosses fail due to the arrested development of embryos resulting from either the abortion at an early stage or from the generation of endosperm such as in Cucurbita interspesific crosses (Kwack and Fujiedi, 1987). Seed abortion in intergeneric crosses between *J. curcas* and *R. communis* may be due to the abnormalities in the fertilization and/or compatibility between the embryo and the endosperm. Moreover, the published data about the hybrid between these two genera in nature has never been reported so far. Despite Kobayashi (2003) statement that interspesific hybridization is possible in the greenhouse but it cannot occur naturally.

More evidence from intergeneric hybrids in coffee trees have been reported by Counturon, *et al*., (1998) with success dealing with different amounts of chromosomes *Psilanthus ebracteolatus* (2n = 22) x *Coffea Arabica* (2n = 44). Although 41 hybrid plants were obtained, only nine plants survived after 5 months growth. The natural intergeneric hybrid was also found by Tara (1979) in *Aster* Alliance as a new backcross hybrid between *A. agerotoides* (2n = 36) x *Kalimeris incise* (2n = 72). Despite Kobayashi (2003) stated where interspesific hybridization is possible in the greenhouse but not occur naturally.

4.2.2 *In vitro* pollination

Stigmatic or pistil pollination *in vitro* is potentially useful in species crosses. *In vitro* pollination procedures provide controlled systems for studying fertilization and embryological development with varying levels of nutrient, growth regulators, temperature and light. Theoretically, the removal of reproductive organs from the maternal parent could aid in avoiding post-fertilization barriers to hybridization contributed by maternal genotype (Richards and Rupert, 1980).

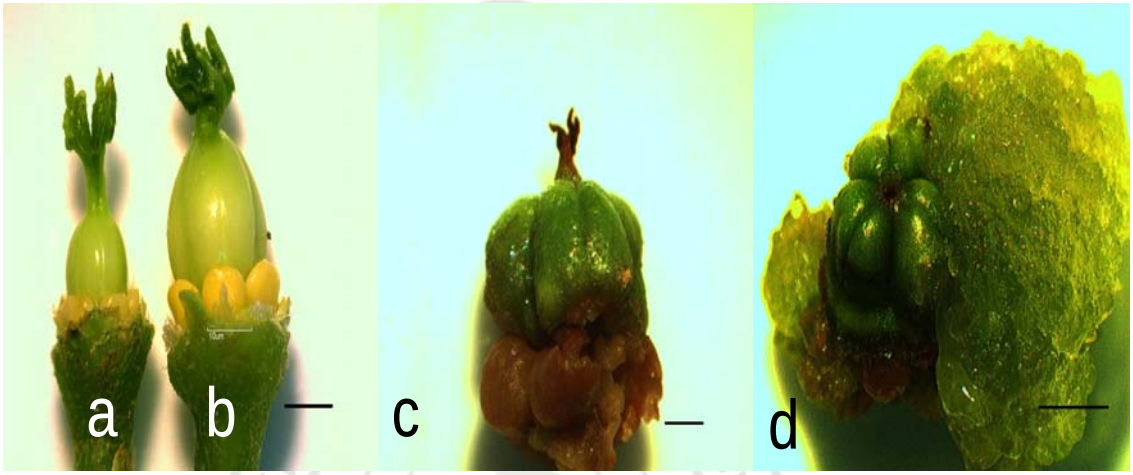


Figure 4.7 *Jatropha curcas* nontoxic (a) flower and toxic (b) varieties, bar 10 µm. *In vitro* pollinated toxic flower (c) in 57 days in media culture 0.125 mg/L BA, bar 5 µm. *Jatopha curcas* flower induced to callus (0.5 mg/L BA+NAA) after 3 week after *in vitro* pollination (d) bar 10 µm.

Female flowers of two varieties of *Jatropha* species are distinguished; the toxic variety is larger than the nontoxic variety. The average of the pistil's lengths of the toxic variety 43 mm and breadth 22 mm was significantly bigger than nontoxic variety pistil flower (32 mm and breadth 14 mm). The difference in female flower size between *J. curcas* toxic (Thai origin) and nontoxic (Mexico origin) have not been repoted yet. Two varieties of *J. curcas* in MFU germpalsm have been maintained in two separate places, but with a relatively similar ecotype. This result may suggest that the Mexico variety is smaller than Thai variety and possibly effected by genotype and/or the effect of the acclimation to the new environment.

After pollination, the fowers of *J. curcas* started to develop within the first week of culture. The oval form or ellipsoidal form and change into a trilocular form. Increase in fruit size occurred after 4 weeks of culture. The majority of the pistils at least enlarge their ovary size. Subculture on every 3 week was done to maintain the fruit growth. During 57 days, the fruit enlarged as the response to the nutrient in the media.

Using stigmatic and ovule *in vitro* pollination, *Jatropha* female flowers were survived more than 57 days after pollination but failed to continued development after 60 days. The growth of the pollinated flowers may be as the effect of nutrition uptake and plant growth regulator. This result suggested *in vitro* pollination techniques can prolong the pollinated flower 4 weeks longer than the *in vivo* pollination technique. Due to the failure of the fruit to grow and develop longer more than 60 days, three weeks of fruit need to induced to callus by using a MS medium supplemented 0.5 mg/L of BA+ 0.5 mg/L of NAA.

The young hybrid zygotes were induced to callus through subculture the pistils in to MS medium supplemented 0.5 mg/L of BA+ 0.5 mg/L of NAA. After two weeks of culture, the young seeds were induced to callus (Fig. 4.7). In the callus induction step, the methodfor recognizing the hybrid cell from maternal callus needs to establish. Because in the zygote culture has three characterizations of cells; *Jatropha* cell, *Ricinus* cell and their hybrid cell.

Previous studies from Malika (2008) reported that callus of *J. curcas* and *R. communis* showed the different characteristics in different mediums supplied with different plant growth regulators concentrations. The *Jatropha* callus grew properly in MS medium with 0.5 mg/L BA+NAA and after eight weeks the calluses remained green and continue to develop. The most suitable medium for castor callus growth was medium with 0.25 mg/L or half from *Jatropha* calluses. Moreover, *Ricinus* callus became either brown or white after eight weeks of culture. From this result suggested that the *in vitro* hybrid cell could be distinguishable from the callus characteristic. *Jatropha* cells are green (photosynthetic cell) but *Ricinus* cells are white (non-photosynthetic cell) and the hybrid was assumedly supposed to be a combination of the two cells.

However, attempts to overcome these barriers by *in vitro* methods were early successful based on developing and prolong surviving periods of the young hybrid zygote. The sterilization method and plant growth regulator concentrations are still required to be established in further study. Sujatha *et al*., (2008) reported that the auxin and cytokinin combination induced

adventitious shoot along with the callus of *Jatropha* but the combination of TDZ+BA+IBA was found to suppress callus growth and promote direct adventitious regeneration. Sujatha summarized the requirement of auxin and cytokine ratio for propagation of *Jatropha* in MS media, but the protocols mention only frot he somatic cell of *Jatropha*. Richard and Rupert (1980) noted fertilization and partial embryo development occurred in interspesific crossed between *T. ambiguum* x *T. hybridum* indicating that embryological development under *in vitro* conditions closely parallels *in situ* development although growth regulator requirement may vary among species.

Auxin promote, mainly combination with cytokinin, the growth of calli, cell suspension, and division and elongation (Machakova, *et al*., 2008) whereas the cytokinin effects is most noticeable in tissue cultures, together with auxin, to stimulate cell division and control morphogenesis (van Staden, *et al*., 2008).

In the future from the callus, chromosomal analyses is required to proof where a hybrid origin as Mujeeb-Kazi, *et al*., (2007) observed cytogenetics from the planlet of some *Triticum aestivum* and *T. turgidum* x *Aegilops variabilis* intergeneric hybrids, root tips were collected and somatically analyzed to validate hybridity.

Interestingly, embryo rescue experiments were done by the undergraduate students in tissue culture laboratory of Mae Fah Luang University. They show the interesting preliminary result using MS medium supplemented with auxin and cytokinin 0.5 mg/L, which was successful in growing the young endosperm from various sizes (0.7 cm – 1.5 cm) pollinated *Jatropha* pistils. This preliminary result from culturing the embryo of *J. curcas* brought promising methods to attempt hybridization between two genera in the future. The embryo rescue might be applied before the flower was aborted. However, the study about the various methods of sterilization and embryo culture in media still need to be completed.

4.3 Physical characteristics and oil analysis of seeds

4.3.1 Physical characteristics of seeds

Table 4.3 shows the maximum of seed length (18.64 mm) obtained in *J. curcas* nontoxic followed by *J. curcas* toxic (17.49 mm). In contrast, the breadth seed of *J. curcas* toxic (11.21 mm) was wider than *J. curcas* nontoxic (10.99). These phenotypic characteristic were similar from Popluechai, *et al*., (2008) reported length and breadth *Jatropha* seeds from Thailand were 19.2 and 11.5 mm, respectively. The seed thickness between two *Jatropha* species was not different.

Whereas, maximum seed length of *R. communis* wild-type (16.52 mm) and its breadth (10.67 mm) was observed to be significantly different from the length of *R. communis* commercial (14.30 mm) and seed breadth was 8.95 mm. Seed thickness between two *Ricinus* cultivar was not significant either. Weiss (2000) stated the seed of *R. communis* varied greatly in size, from 5 to 250 mm long in giant types, in breadth from 5 to 16 mm and the size of seed varies not only between cultivars, but from different racemes on the same plant.

Kausik, *et al*., (2007) stated various ecotypes/provenances/seed source of *J. curcas* may exhibit variation in seed morphological traits and from Mae Fah Luang germplasm accessions showed almost similar variation traits between two varieties, it might becauce both *Jatropha* species has grown in the same environment.

Table 4.3 Seed size and seed oil content in *J. curcas* and *R. communis*

Accession	Seed length (mm)	Seed breadth (mm)	Seed thickness (mm)	100-seed weight (g)	Moisture content (%)	Oil content* (%)
J. curcas toxic	17.49[a]	11.21[a]	8.41[a]	73.17[b]	5.9	53.03[c]
J. curcas nontoxic	18.64[a]	10.99[a]	8.72[a]	65.43[a]	5.2	39.47[b]
R. communis commercial	14.30[b]	8.95[b]	6.01[b]	50.83[c]	3.4	27.03[a]
R. communis wild-type A	16.52[b]	10.67[b]	6.40[b]	55.96[c]	5.6	23.25[a]

Note: * oil content on dry weight basis

* Different letter in the same column indicate significant difference between accessions

4.3.2 Seed weight and oil content

For 100 seeds weight, the top ranking accession was *J. curcas* toxic seed with 73.17 g (SD= 1.90) followed by nontoxic with 65.43 g (SD= 5.29), *R. communi*s wild type A with 55.96 g (SD=1.47) and commercial type with 50.83g (SD= 0.73) as showed on Table 4.3. The seed weight of *J. curcas* toxic variety was higher than that reported by Khausik et al (2007) from 24 accessions in Haryana (India), which was around 49.20-69.20 g.

Jatropha curcas toxic variety showed the highest oil content (53.03 %) while nontoxic variety yielded 39.47% (Fig. 4.8). From this result, the oil content of Thai *J. curcas* toxic was comparable to Caboverde variety (52.9%) but higher than the seeds of Haryana state (29.26-38.60%) as Winayanuwattikun, *et al*., (2008) reported that *J. curcas* seeds from the north-eastern part of Thailand contained an average oil content of 43.8%.

Oil contents in *R. communis* (Table 4.3 and Fig. 4.9) were lower than in *J. curcas*. The lowest oil content was founded in *R. communis* wild-type A (23.25%). These results were very low according to other reports which indicated that the castor bean contained 40% of oil (Melo, *et al*., 2008) or equal to 65-85% of the bean weight (Cherry, 1992).

The variation in oil contents that yield from both *J. curcas* and *R. communis* seeds might because by season, region or plantation area, and hybrid (genetically) as in corn oil case (Jellum and Marrion, 1966). It was also indicated that climatic differences were responsible for castor seed oil content and that harvesting too early with high proportion of immature or still green capsules can drastically reduce oil yield/ha (Weiss, 2000).

Moisture content is also useful information in the drying process. All accessions but *R. commercial* type (3.4%) have an average moisture content value of about 5.2-5.9% (Table 4.3). The low moisture content value from commercial type might be caused by the long storage period, or the temperature in the storage room was not suitable.

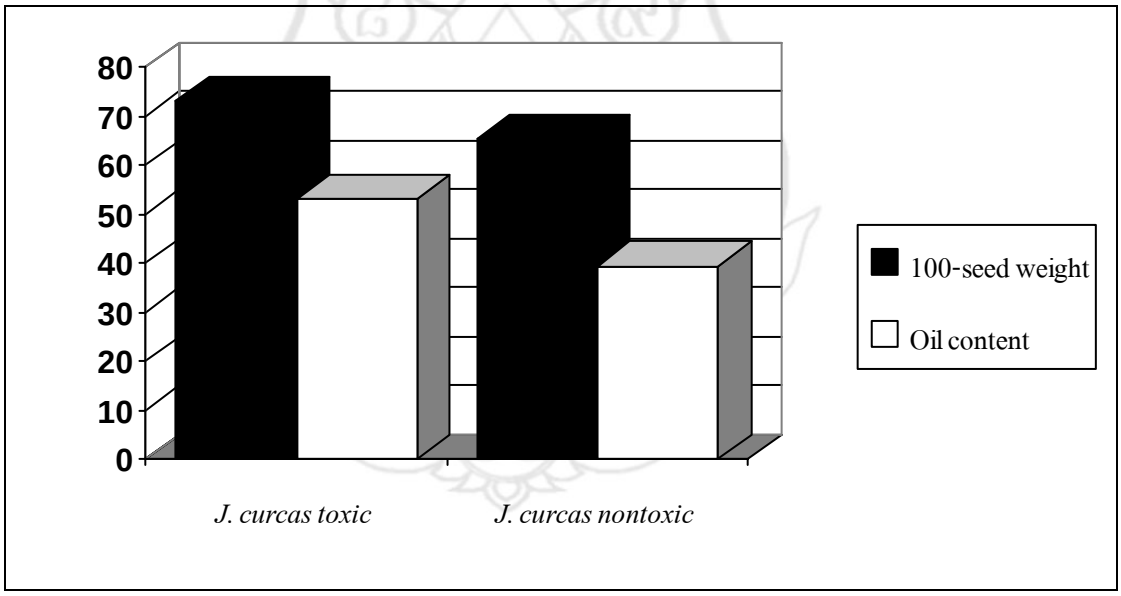


Figure 4.8 Average of 100-seed weight (g) and oil content (%) of *J. curcas*.

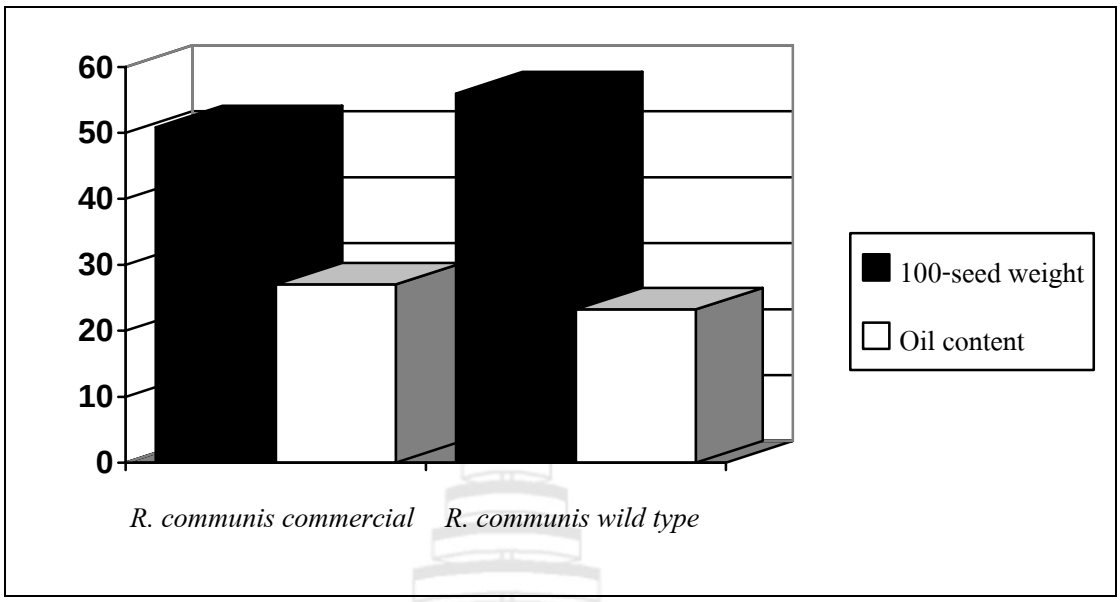


Figure 4.9 Average of 100-seed weight (g) and oil content (%) of *R. communis*.

Some researcher reported a correlation between seed size and oil content especially; when the range of seed size is large, kernel percentage is more closely correlated with oil content than with other factors (Weiss, 2000; Khausik *et al.*, 2007). However, seeds study in the MFU germplasm showed a negative correlation between seed weight and oil content (Table 4.3). It is understandable that the commercial type has higher oil content than the wild-type A according to the improvement and breeding program. The breeding programs for wild-type A might increase the breed a big seed with contain higher oil content to be achieved in the future.

Genetic variation in seed morphology and oil content of *J. curcas* and *R. communis* can be a great potential in the tree improvement program, particularly a selection of genotypes having more oil content and yield. The variation would be useful as a source of future genetic selection provided the desired idea types for agroforestry system are clearly defined (Khausik, *et al*., 2007).

4.3.3 Fatty acid analysis

Jatrophas are rich sources of hydrocarbons (27.0 to 48.5% seed oil content) and have created interest all over the world for the use of its seed oil as a commercial source of fuel (Sujhata, *et al*., 2008). Numerous sources are available on the fatty acid composition of physic nut

oil originating from different countries. The fatty acids found common and important in all *Jatropha* samples were oleic, linoleic, palmitic and stearic (Martinez-Herrera, *et al*., 2006; Heller, 1996) and free fatty acid (FFA) in *Jatropha* contains about 14% (Tiwari et al, 2007).

The GC analysis results showed in Table 4.4, that there was no significant difference in fatty acids between *J. curcas* toxic and nontoxic varieties whereas oleic acid and linoleic acid were the majority (Table 4.4) followed by palmitic, stearic and palmitoleic acid, respectively. The linoleic acid content in *J. curcas* toxic variety from this study (37%) was higher than the Caboverde variety (31.4%) but lower than the Nicaragua variety (43.2%) (Foidl, *et al*., 1996).

The *J. curcas* nontoxic variety originating in Mexico that was planted in MFU germplasm contained 35% linoeic acid which was in the range 34.6-44.4% as reported by Martinez-Herrera *et al*., (2006) stated from the observation in four provenances in Mexico. Linoleic acid is an important polyunsaturated fatty acid in human food because of its prevention of distinct heart vascular disease (Balhouwer, 1983). If is the most abundant fatty acid in *Parkia bigblobbossa* oil, and *J. curcas* however it is predominantly made up of oleic acid (Akintayo, 2004).

Table 4.4 Fatty acid profile of 4 accessions

Fatty acid	*J. curcas* toxic	*J. curcas* nontoxic	*R. communis* commercial	*R. communis* wild-type
Palmitoleic acid	0.552	0.586	-	-
Palmitic acid	13.926	14.096	2.615	0.965
Linoleic acid	35.090	37.666	8.033	3.861
Oleic acid	40.317	39.999	7.841	5.157
Stearic acid	7.207	7.076	1.757	0.825
Ricinoleic acid	-	-	79.864	89.183

There was no significant difference in the oleic acid content between the two varieties of *J. curcas.* The content of *J. curcas* toxic variety was 40.3% and the non toxic was 39.9%, these

results were lower than the literature reported (41.3%) (Akintayo, 2004), also lower than the Caboverde variety (44.7%) and higher than the Nicaragua variety (34.3%) (Foidl, *et al*., 1996).

The oleic acid content from four provenances in Mexico varied between 40.5-48.8% (Martinez-Herrera, *et al*., 2006), which were higher than that in the MFU germpalsm. Popluechai, *et al*., (2008) compared the content of the main fatty acids such as oleic and linoleic acid in Jatropha seeds from India, Nigeria and Thailand. They found that oleic acid in Thai Jatropha seeds were lower than India and Nigeria, but higher than these two countries for linoleic acid.

Jatropha curcas oil would require refining to make it edible (Akintayo, 2004) because the seeds of *J. curcas* are highly toxic and the toxicity is ascribed to phorbol ester (Makkar, *et al*., 1998). The toxicity of the seeds is mainly due to the following components: a toxin protein (curcin) and diterpene esters. Curcin is similar to ricin and it is hinders protein synthesis *in vitro* and diterpene have been isolated from seeds and roots. These substances promoted skin tumors in a mouse experiment (Heller, 1996). Harinder, *et al*., (2008) investigated phorbol ester concentration from toxic and nontoxic varieties and they were found for nontoxic genotype, phorbol ester were virtually zero but the toxic variety varied from 1.17-1.76 mm/g of oil. Mexico variety contained 0.11 mg/g of phorbol ester (Makkar, *et al*., 1998) which was lower than the toxic *J. curcas* from Nigeria (2.17) and Nicaragua (2.30). Interestingly, the highest concentration of phorbol ester 3.85 mg/g was detected in the sample from Coatzacoalcos (Martinez-Herrera, *et al*., 2006) which is one of four provenances different agro-climatic regions in Mexico. It is not clear whether the high levels of phorbol ester from Coatzacoalcos are caused by genetic or environmental factors.

Among the vegetable oils, castor oil is distinguished by its high content of hydroxilylate fatty acid, ricinoleic acid (D-12-hydroxy-*cis*-9-octadecanoic acid). Castor bean contains 60% oil of unique composition (McKeon et al, 2003). It is the only source of an 18-carbon hydroxilated fatty acid with one double bond (Caupin, 1997; Bafor, *et al*., 1991). Many literatures reported that this acid contains 87-90% of castor oil (McKeon, *et al*., 2007; Akpan, *et al*., 2006) and 10% of non functional acid (Cunha, *et al*., 2004). From GC analysis the content of ricinoleic acid in *R. communis* commercial cultivar was 79.8%, and was apparently higher in wild-type (89.1%). Ricinoleic acid has many industrial uses but it is undesirable in vegetable oil for human consumption (McKeon, *et al*., 2007) and also poisonous to animals because it contains the potent

toxin ricin and has highly allergenic proteins (Weiss, 2000). The recent isolation of a natural mutant of castor bean with high oleic and low ricinoleic acid concentration diversifies potential uses for castor oil (Rojas-barros, *et al*., 2004). Using classical mutagenesis techniques, random mutations are introduced into the castor genome and screened to determine the mutations that have the ricin genes knocked out. Published data on oil composition from different world regions varies substantially, and could be due to climate, cultivar, cultural methods, or processing (Weiss, 2000).

Commercially, the industry demands oil with a maximum concentration of the desired fatty acid because the use of such oils contributes to a reduction in the amount of waste and represent considerable savings in processing costs (Rojas-Barros, *et al*., 2004). The disadvantages of castor oil for use as biofuel are a significantly higher viscosity at temperatures under 50^{O}C, higher compressibility than other vegetables oils and hygroscopity. Despite the high viscosity of raw castor oil, the kinematic viscosities of transesterified castor oil are comparable to other vegetable oils making it suitable as a bodiesel blend (Conceicao, *et al*., 2007).

Castor and *Jatropha* can supply materials for biodiesel and can turn marginal lands into valuable economic lands (Sujatha, *et al*., 2008). The published data on oil composition from different world regions varies substantially, and could be do to climate, cultivar, cultural methods, or processing (Weiss, 2000). The review of Gressel (2008) on various feedstock indicate that these two non-edible oilseed plants could be converted to efficient biofuel crops by rendering them less toxic biotechnology. Thus, the potential of introducing novel traits into higher plant crop species allowed researchers to consider the concept of the "designer oilseed," in which traits were not present in a current oilseed crop by genetic engineering. The unique characteristics of castor oil fatty acid compositions was a tool to distinct it from *Jatropha* oil. Furthermore the investigation of a hybrid plant can be determined through the changing of fatty acid compositions since Napier (2007) stated the possibilities to use genetic engineering to modify the fatty acid profile of plants.

CHAPTER 5

CONCLUSIONS

This is the first report on intergeneric hybridization between *J. curcas* x *R. communis* through *in vivo* and *in vitro* techniques. Based on the results obtained in this study there are some certain points to summarize as follows:

1. The pollen grain diameters of *J. curcas* were three times bigger than *R. communis*. Therefore, *J. curcas* is suitable to be a female parent for hybridization.

2. *In vivo* pollination test shows the abortion in all pollinated flowers in two weeks indicating their intergeneric incompatibility.

3. *In vitro* pollination test shows that the flowers can survive four weeks longer than *in vivo* test.

4. Incompatibility between *J. curcas* and *R. communis* shows lack of pre zygotic barriers. The incompatibility caused by post zygotic barriers.

5. Oilseed weight (g) of *J. curcas* toxic was top ranking followed by nontoxic variety, *R. communis* commercial type and *R. communis* wild-type.

6. The major fatty acids in *J. curcas* both varieties were oleic and linoleic acid. Ricinoleic acid was a main fatty acid in *R. communis* seeds both types.

Further work relevant to investigation intergeneric cross to complete the project based on this thesis results include:

1. Embryo rescue techniques need to be established before the zygote abortion, due to the size of the zygote which was too small to rescue. The proper time to rescue is 7-10 days after pollination.

2. Doubling the chromosomes to obtain amphidiploid plant.

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APPENDIX A

Jatropha curcas and *Ricinus communis*

A.1 *Jatropha curcas* diversity in Mae Fah Luang germplasm.

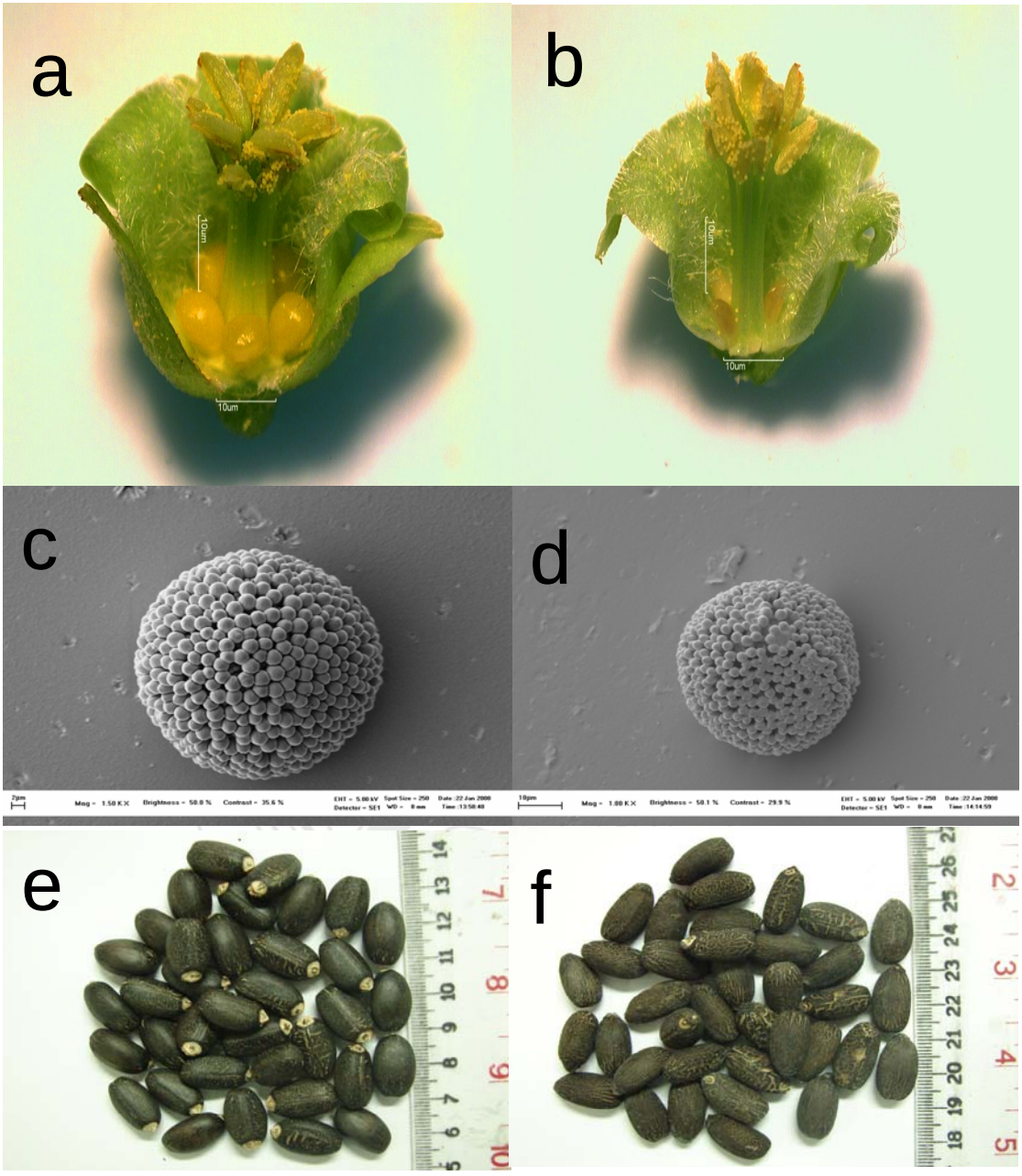

Figure A1. *Jatropha curcas* toxic variety; a. male flower c. pollen (inaperture) d. seed (smooth skin). *J. curcas* nontoxic variety; b. male flower d. pollen (inaperture) f. seed (rough skin)

A2. *Ricinus communis* wild-type in MFU germplasm

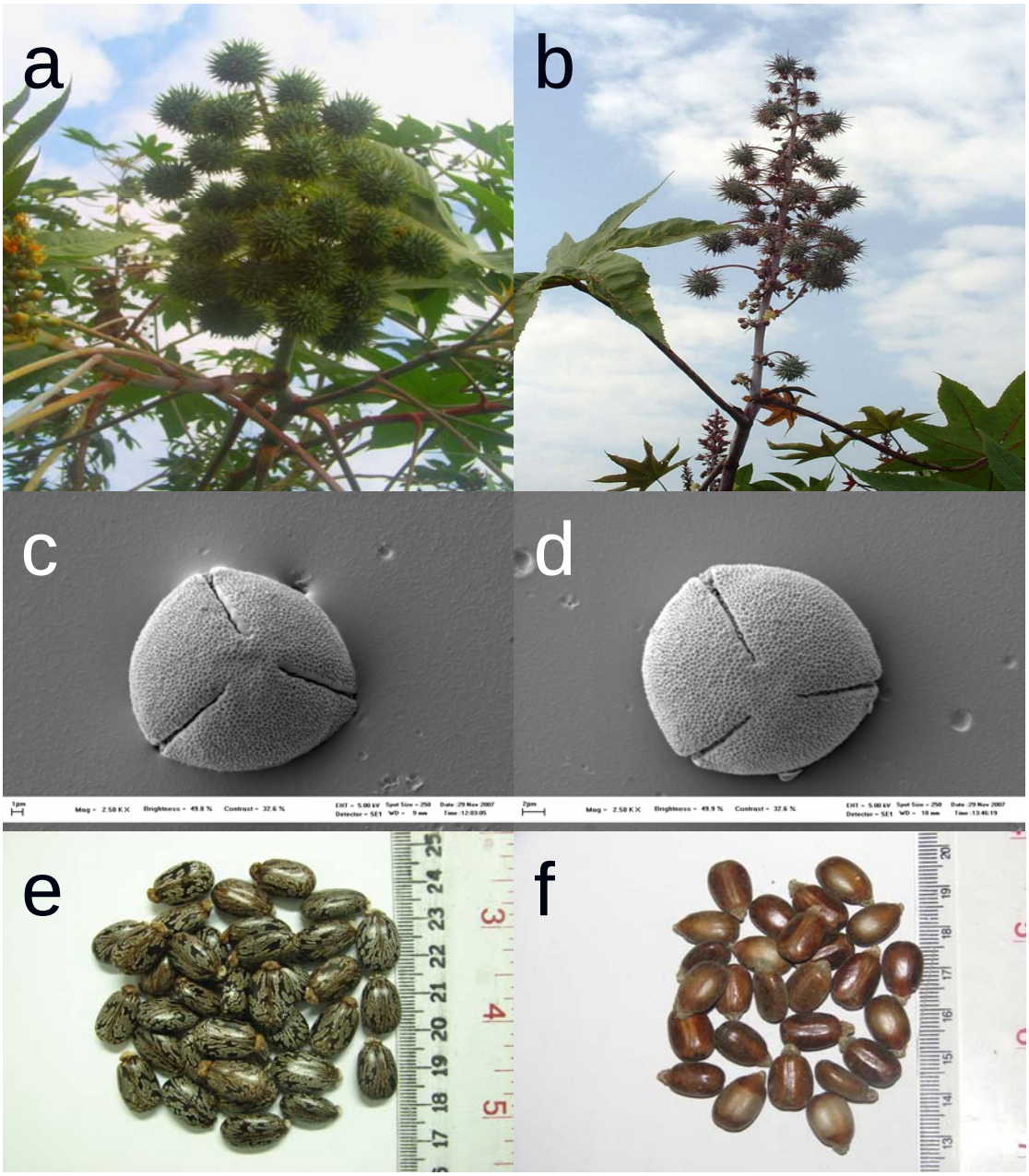

Figure A2. *Ricinus communis* wild-type A; a. umbrella spike and compact c. tricoloporate pollen e. seed: big size and black. *Ricinus communis* wild-type B; a. conical spike and semi compact c. tricoloporate pollen e. seed: medium size, brown and spotless.

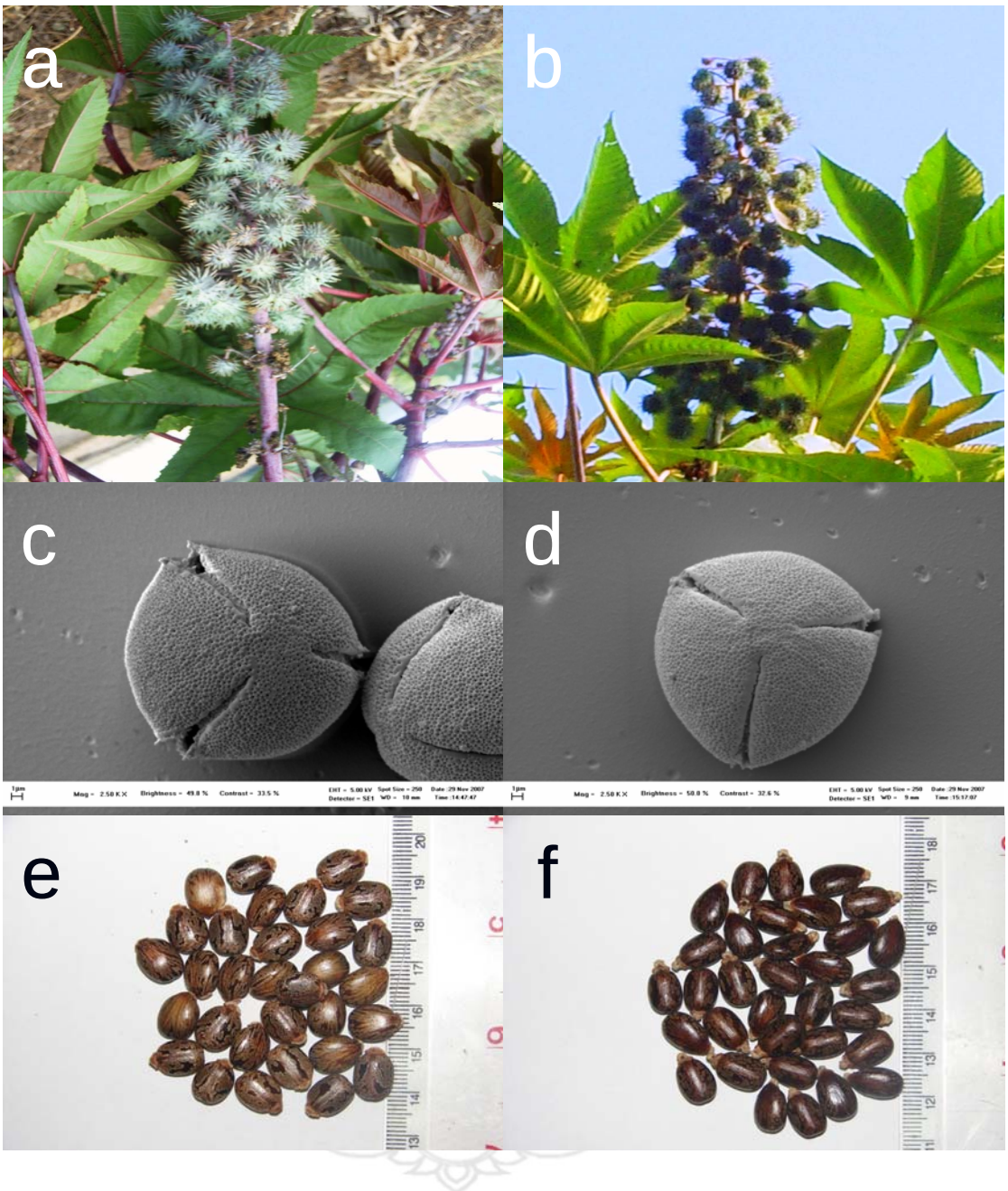

Figure A3. *Ricinus communis* wild-type C; a. cylindrical spike and very compact c. tricoloporate pollen e. seed: small size and brown. *Ricinus communis* wild-type D; a. cylindrical spike and semi compact c. tricoloporate pollen e. seed: medium size and brown.

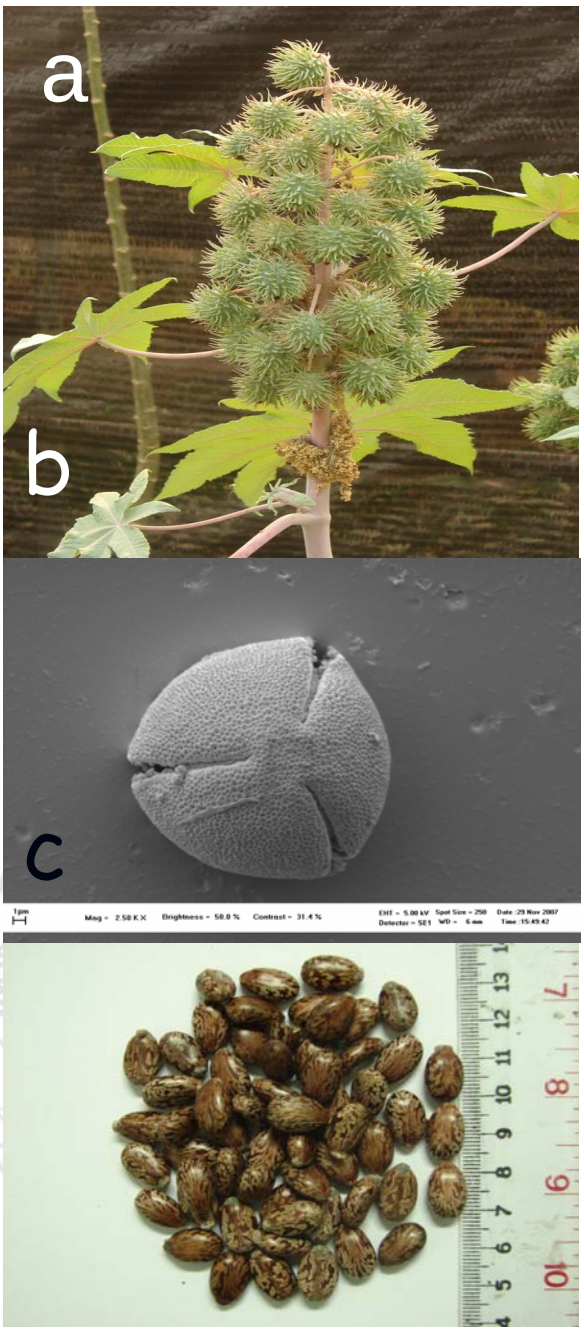

Figure A3. *Ricinus communis* commercial-type; a. umbrella spike and compact c. tricoloporate pollen e. seed: medium size and brown.

APPENDIX B

MEDIA COMPOSITION

Table B.1 MS (Murashige & Skoog, 1962) medium stock solution

Chemical compounds	Supplier	Concentration in stock solution (mg/l)	Concentration in medium (ml/l)
MS Major Salts			20
Potassium nitrate (KNO_3)	UNIVAR, Australia	50	
Ammonium nitrate (NH_4NO_3)	UNIVAR, Australia	82.5	
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	UNIVAR, Australia	22.0	
MS Minor Salts			10
Boric acid (H_3BO_3)	UNIVAR, New Zealand	0.62	
Potassium phosphate (KH_2PO_4)	UNIVAR, New Zealand	17.0	
Potassium iodide (KI)	UNIVAR, New Zealand	0.083	
Sodium molybdate ($Na_3MoO_4 \cdot 2H_2O$)	UNIVAR, New Zealand	0.025	
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	UNIVAR, New Zealand	0.0025	
Magnesium sulfate ($MgSO_4.7H_2O$)	UNIVAR, New Zealand	37.0	
Manganese sulfate ($MnSO_4 \cdot 4H_2O$)	UNIVAR, New Zealand	2.23	
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	UNIVAR, New Zealand	0.86	
Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	UNIVAR, New Zealand	0.0025	
EDTA sodium ferric salt			5
EDTA	FLUKA, Switzerland	7.45	
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	FLUKA, Switzerland	5.57	
MS vitamins			10
Glycine (recrystallized)	Fisher scientific, USA	0.2	
Nicotimic acid	Fisher scientific, USA	0.05	
Thiamine · HCl	Fisher scientific, USA	0.01	
Pyridoxine · HCl	Merck, Germany	0.05	
Myo-Inositol	Merck, Germany	10	
Auxin	SIGMA, USA	1.0	
Cytocynin	SIGMA, USA	1.0	
Gibberellic acid	SIGMA, USA	1.0	
Sucrose	UNIVAR, UK	30	
Agar		7.0	

APPENDIX C

FATTY ACID PROFILE ANALYSIS

C 1. Chromatogram and fatty acid profiles.

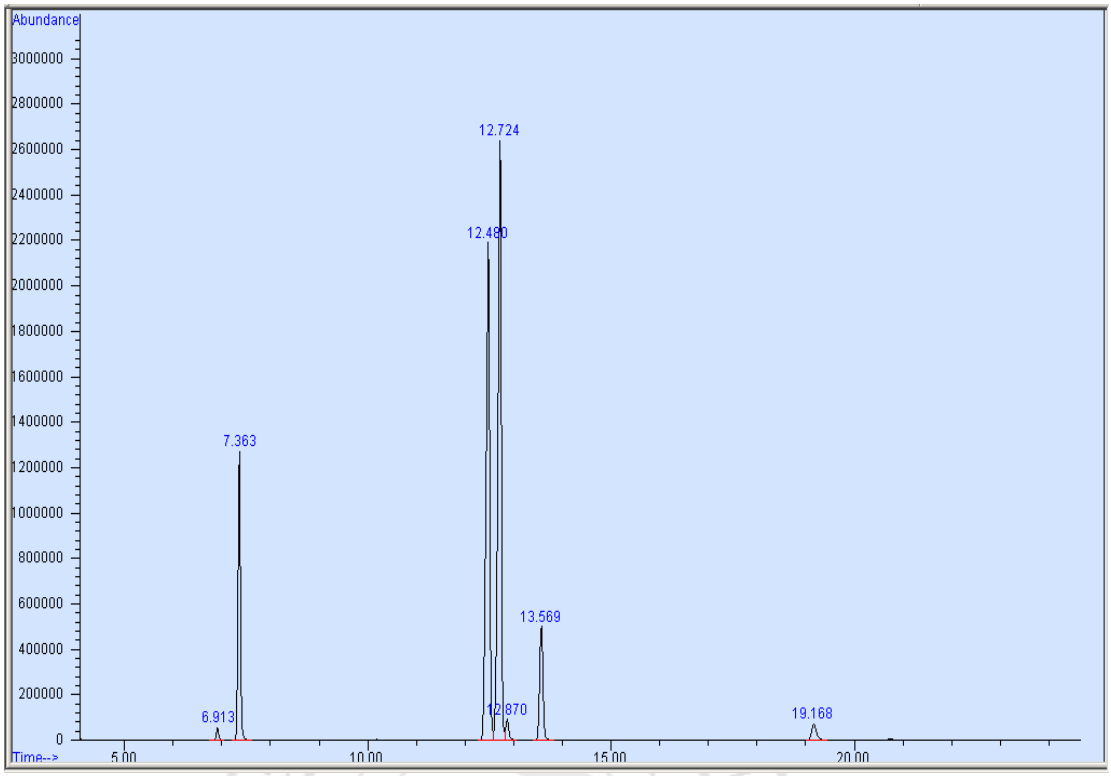

Figure C1.1 Chromatogram of fatty acid from *J. curcas* toxic variety (replication 1)

Table C1.1 Fatty acid profile of *J. curcas* toxic variety (replication 1).

Peak no.	Fatty acid	Retention time (min)	% of total
1	Palmitoleic acid (C16:1 n-9)	6.910	0.55
2	Palmitic acid (C16:0)	7.362	13.923
3	Linoleic acid (C18:2, n-9)	12.483	35.043
4	Oleic acid (C18:1, n-9)	12.723	40.265
5	11-Octadecenoic acid (C18:1, n-11)	12.872	1.312
6	Stearic acid (C18:0)	13.569	7.381

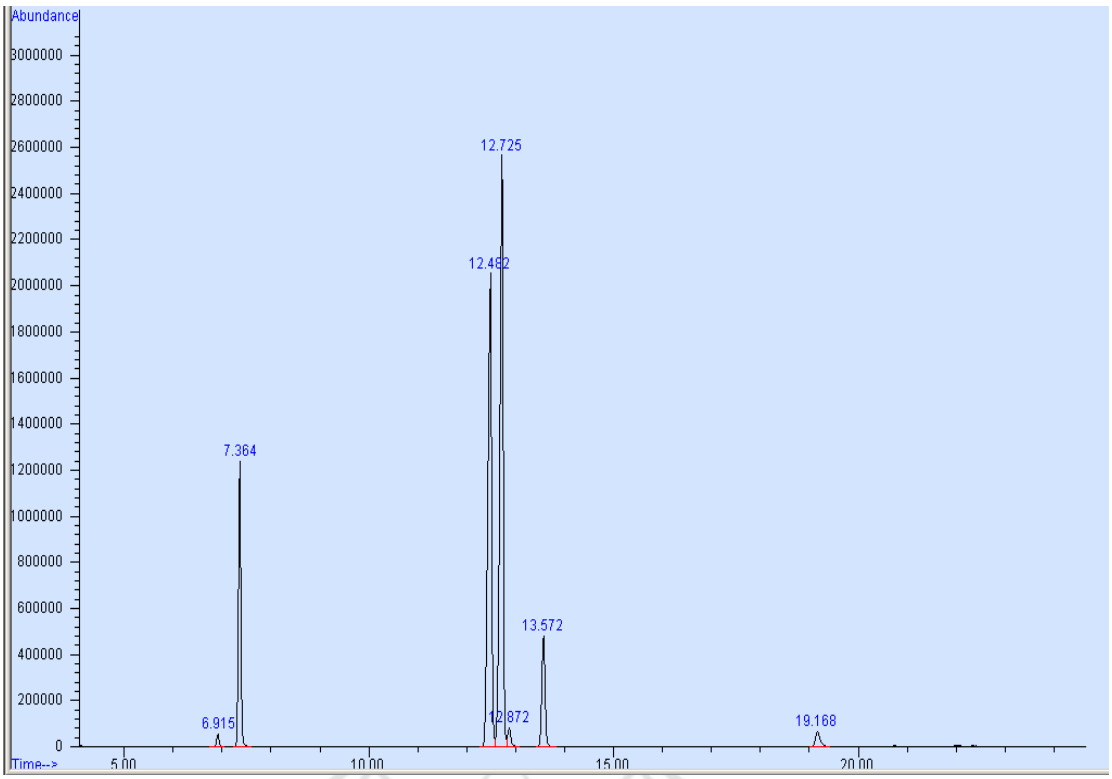

Figure C1.2. Chromatogram of fatty acid from *J. curcas* toxic variety (replication 2)

Table C1.2 Fatty acid profile of *J. curcas* toxic variety (replication 2).

Peak no.	Fatty acid	Retention time (min)	% of total
1	Palmitoleic acid (C16:1 n-9)	6.916	0.569
2	Palmitic acid (C16:0)	7.362	14.012
3	Linoleic acid (C18:2, n-9)	12.483	35.237
4	Oleic acid (C18:1, n-9)	12.723	40.388
5	11-Octadecenoic acid (C18:1, n-11)	12.872	1.289
6	Stearic acid (C18:0)	13.575	7.019

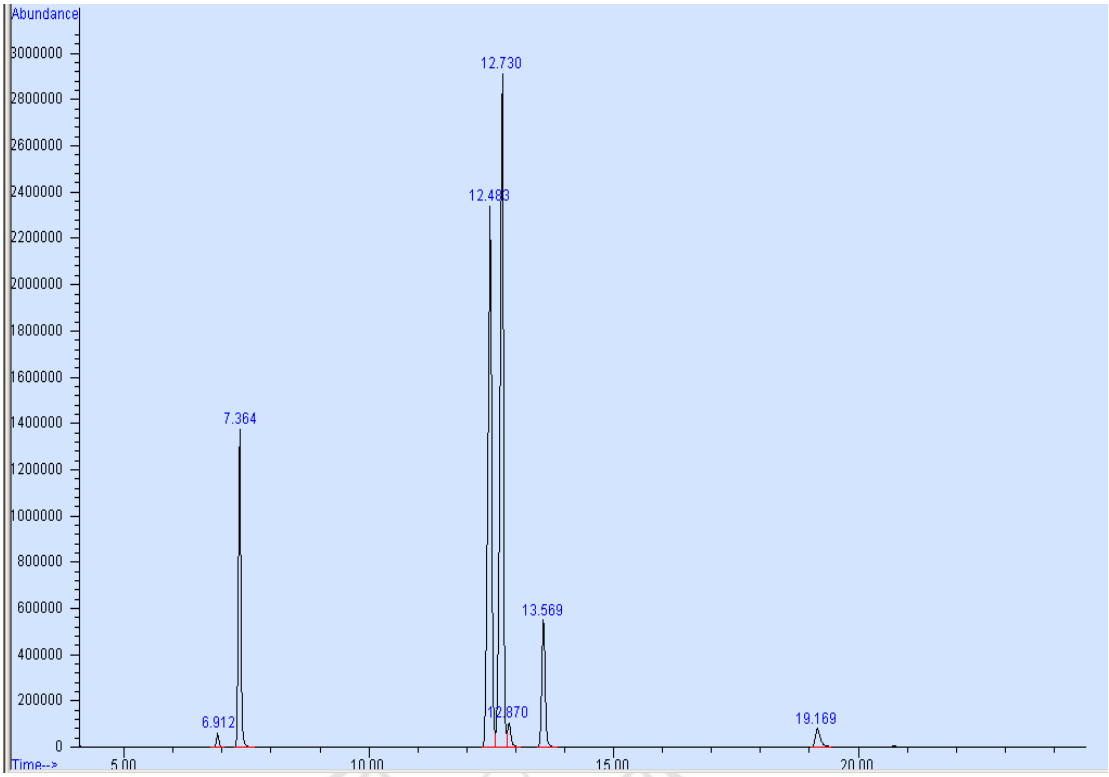

Figure C1.3. Chromatogram of fatty acid from *J. curcas* toxic variety (replication 3)

Table C1.3 Fatty acid profile of *J. curcas* toxic variety (replication 3).

Peak no.	Fatty acid	Retention time (min)	% of total
1	Palmitoleic acid (C16:1 n-9)	6.911	0.579
2	Palmitic acid (C16:0)	7.362	13.842
3	Linoleic acid (C18:2, n-9)	12.483	34.992
4	Oleic acid (C18:1, n-9)	12.729	40.298
5	11-Octadecenoic acid (C18:1, n-11)	12.872	1.458
6	Stearic acid (C18:0)	13.569	7.222

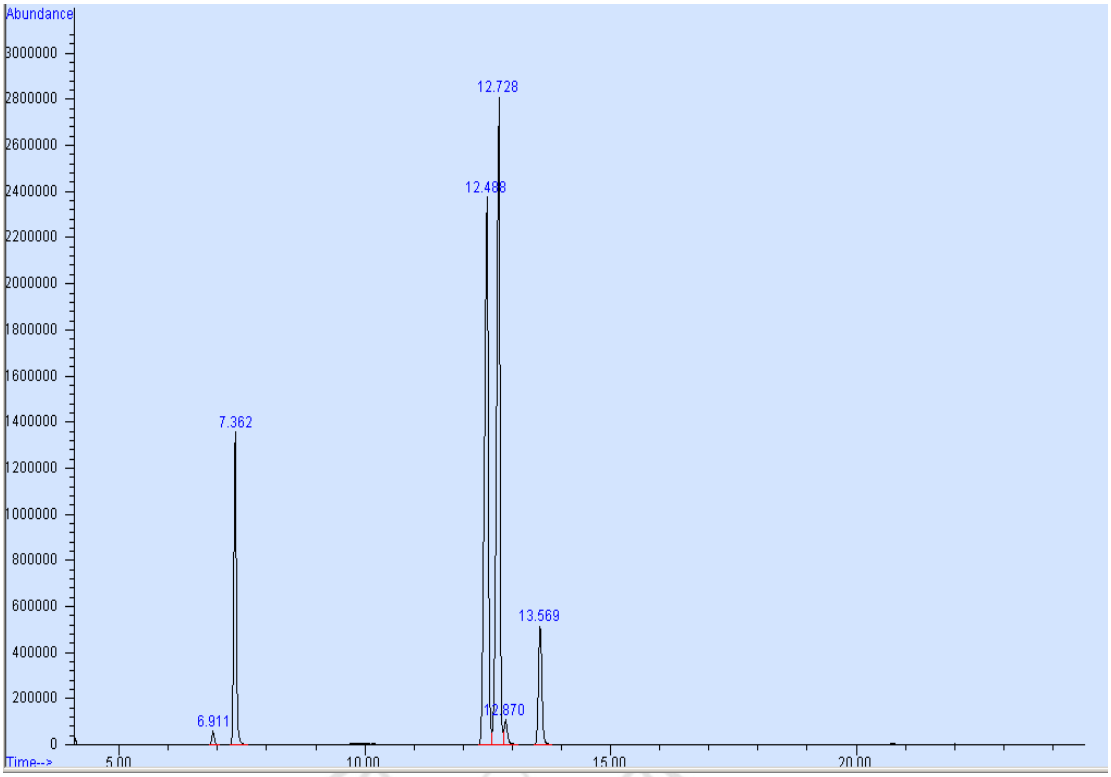

Figure C1.4 Chromatogram of fatty acid from *J. curcas* nontoxic variety (replication 1)

Table C1.4 Fatty acid profile of *J. curcas* nontoxic variety (replication 1).

Peak no	Fatty acid	Retention time (min)	% of total
1	Palmitoleic acid (C16:1 n-9)	6.911	0.585
2	Palmitic acid (C16:0)	7.362	14.115
3	Linoleic acid (C18:2, n-9)	12.489	37.244
4	Oleic acid (C18:1, n-9)	12.729	39.533
5	11-Octadecenoic acid (C18:1, n-11)	12.872	1.514
6	Stearic acid (C18:0)	13.569	7.008

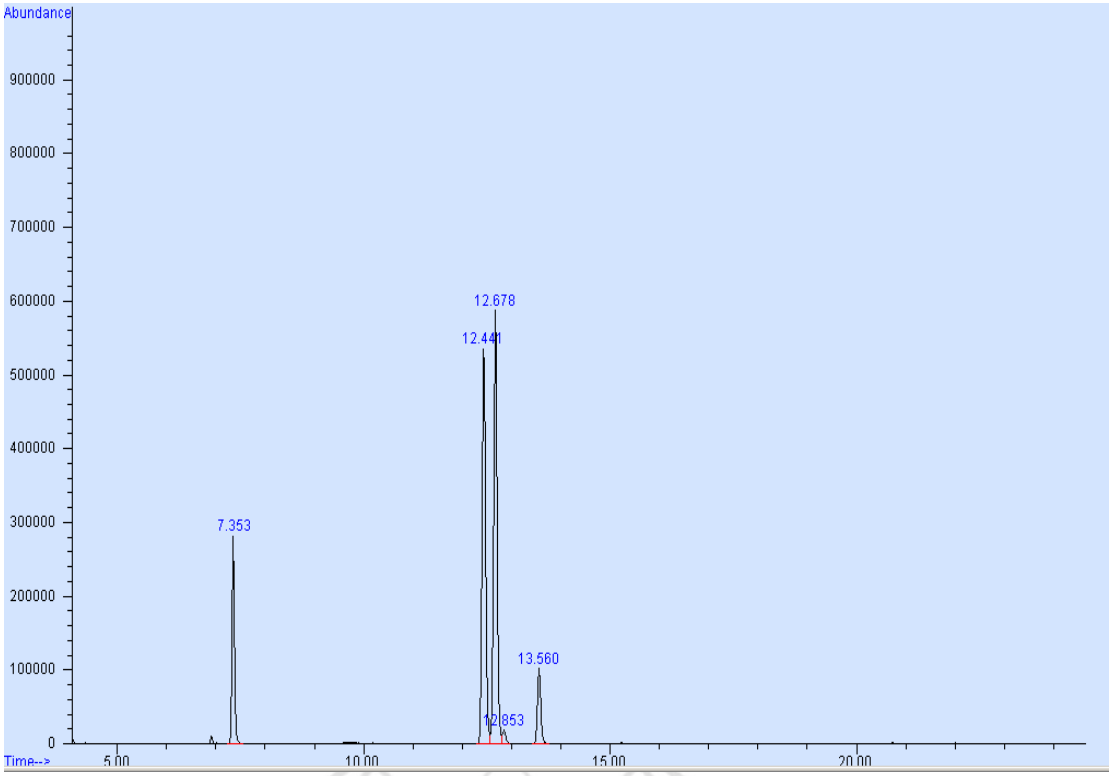

Figure C1.5 Chromatogram of fatty acid from *J. curcas* nontoxic variety (replication 2).

Table C1.5 Fatty acid profile of *J. curcas* nontoxic variety (replication 2).

Peak no	Fatty acid	Retention time (min)	% of total
1	Palmitic acid (C16:0)	7.351	14.656
2	Linoleic acid (C18:2, n-9)	12.443	38.083
3	Oleic acid (C18:1, n-9)	12.677	40.317
4	Stearic acid (C18:0)	13.558	6.944

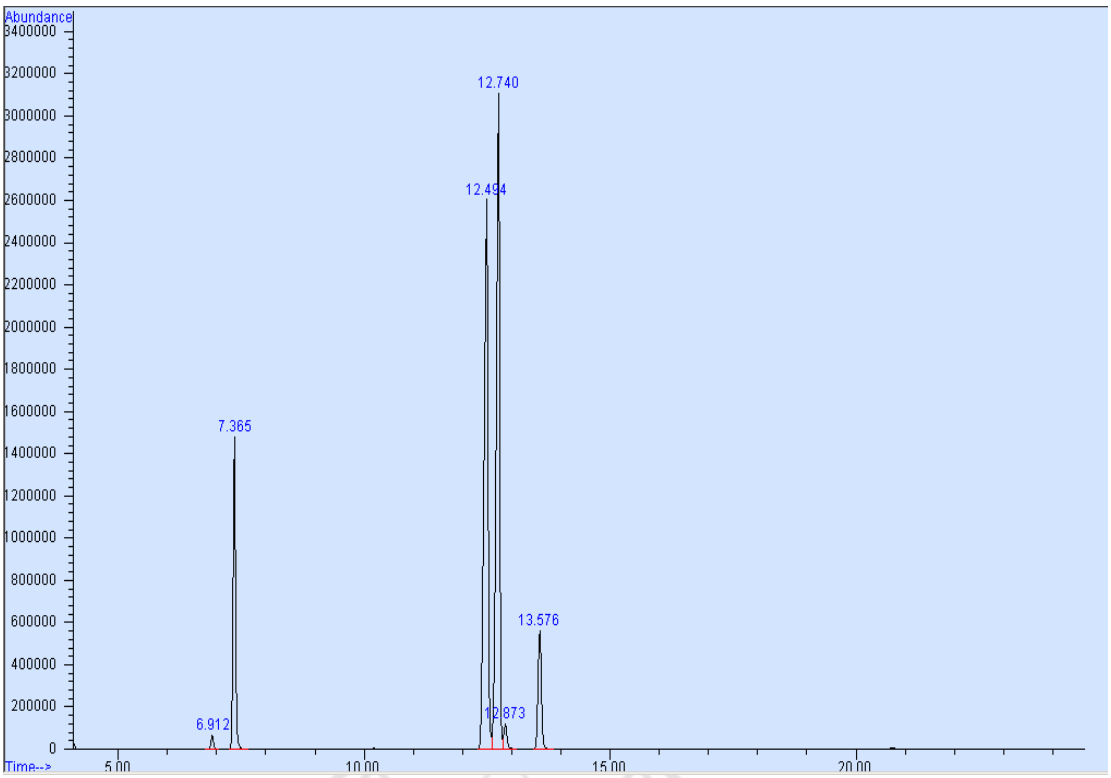

Figure C1.6 Chromatogram of fatty acid from *J. curcas* nontoxic variety (replication 3).

Table C1.6 Fatty acid profile of *J. curcas* nontoxic variety (replication 3).

Peak no.	Target Compounds	Retention time (min)	% of total
1	Palmitoleic acid (C16:1 n-9)	6.911	0.588
2	Palmitic acid (C16:0)	7.368	13.519
3	Linoleic acid (C18:2, n-9)	12.494	37.671
4	Oleic acid (C18:1, n-9)	12.740	39.979
5	11-Octadecenoic acid (C18:1, n-11)	12.872	1.473
6	Stearic acid (C18:0)	13.575	6.771

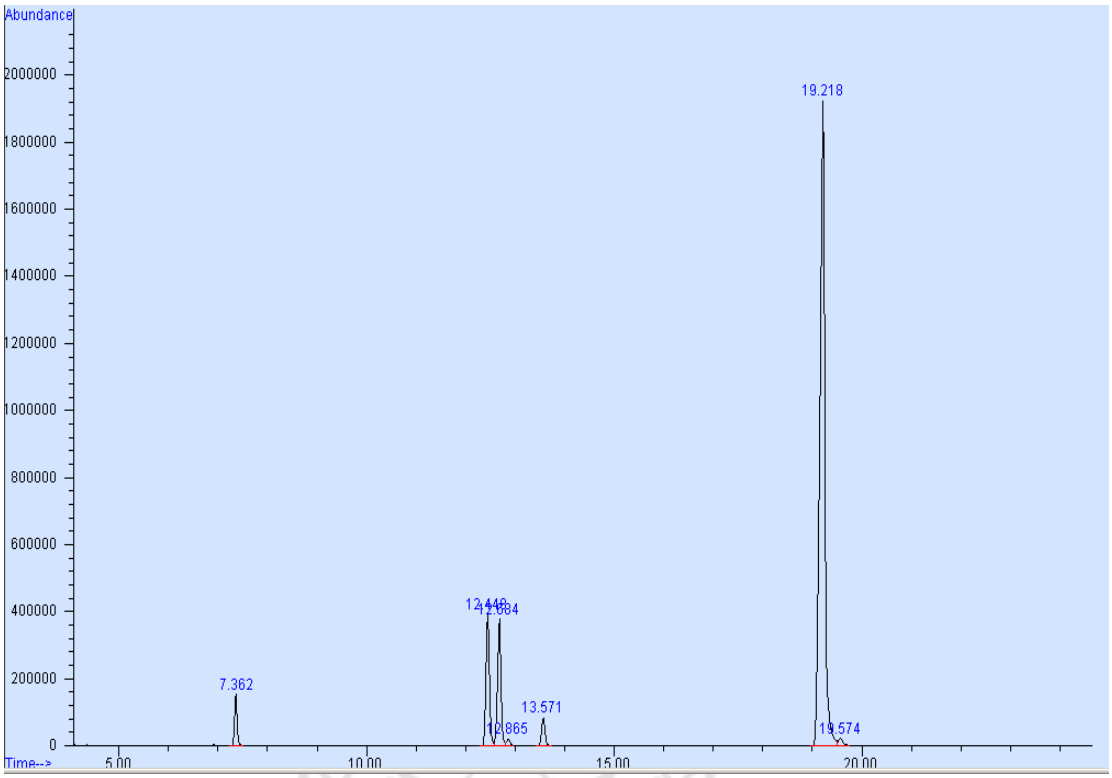

Figure C1.7 Chromatogram of fatty acid from *R.communis* commercial-type (replication 1).

Table C1.7. Fatty acid profile of *R.communis* commercial-type (replication 1).

Peak no.	Fatty acid	Retention time (min)	% of total
1	Palmitic acid (C16:0)	7.362	3.006
2	Linoleic acid (C18:2, n-9)	12.449	10.262
3	Oleic acid (C18:1, n-9)	12.683	9.734
4	Stearic acid (C18:0)	13.659	2.116
5	Ricinoleic acid	19.216	74.882

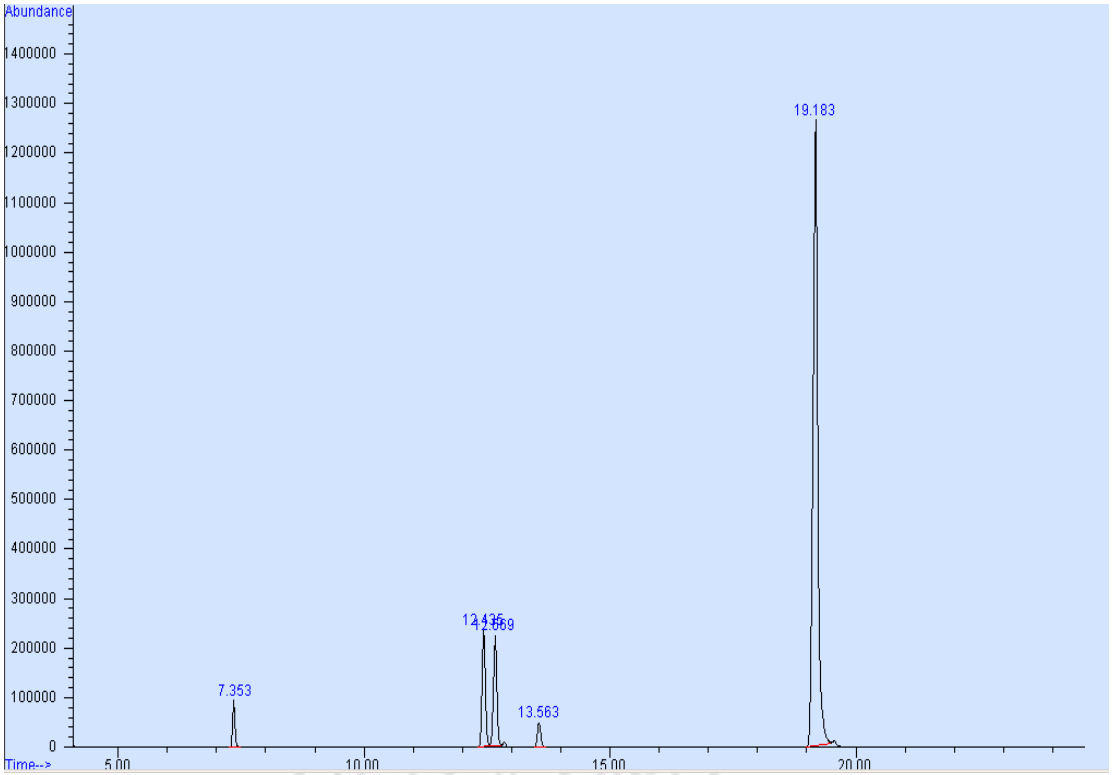

Figure C1.8 Chromatogram of fatty acid from *R.communis* commercial-type (replication 2).

Table C1.8 Fatty acid profile of *R.communis* commercial-type (replication 2).

Peak no	Target Compounds	Retention time (min)	% of total
1	Palmitic acid (C16:0)	7.351	3.845
2	Linoleic acid (C18:2, n-9)	12.437	9.479
3	Oleic acid (C18:1, n-9)	12.672	9.351
4	Stearic acid (C18:0)	13.563	2.027
5	Ricinoleic acid	19.181	76.298

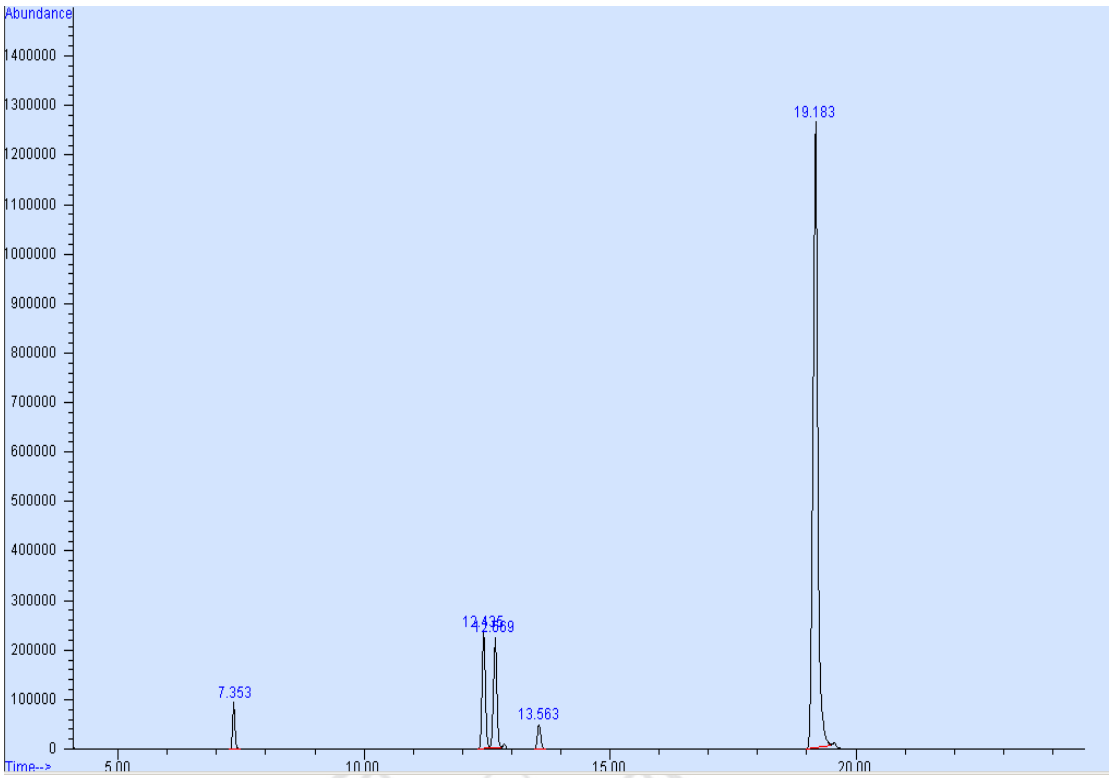

Figure C1.9 Chromatogram of fatty acid from *R.communis* commercial-type (replication 3).

Table C1.9 Fatty acid profile of *R.communis* commercial-type (replication 3).

Peak no.	Fatty acid	Redemption time (min)	% of total
1	Palmitic acid (C16:0)	7.356	0.974
2	Linoleic acid (C18:2, n-9)	12.449	4.357
3	Oleic acid (C18:1, n-9)	12.677	3.747
4	Stearic acid (C18:0)	13.563	1.219
5	Ricinoleic acid	19.393	89.248

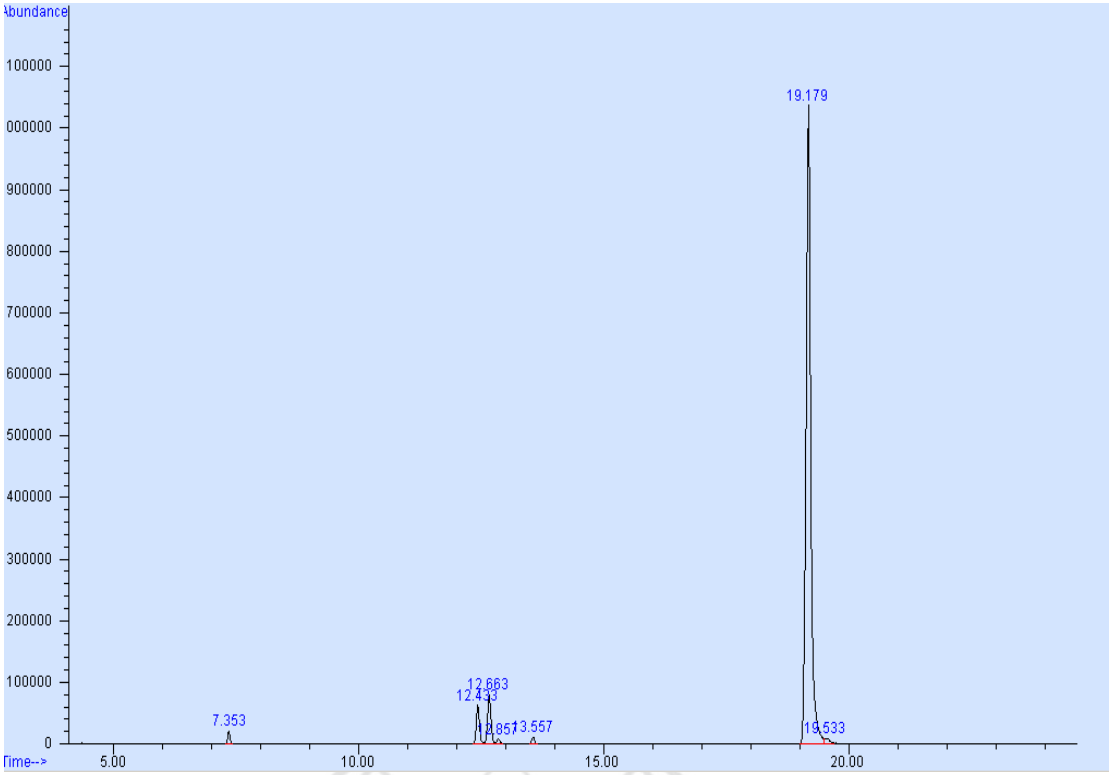

Figure C1.10 Chromatogram of fatty acid from *R.communis* wild-type A (replication 1).

Table C1.10 Fatty acid profile of *R.communis* wild-type A (replication 1).

Peak no	Fatty acid	Retention time (min)	% of total
1	Linoleic acid (C18:2, n-9)	12.432	3.589
2	Oleic acid (C18:1, n-9)	12.666	4.819
3	Ricinoleic acid	19.181	91.592

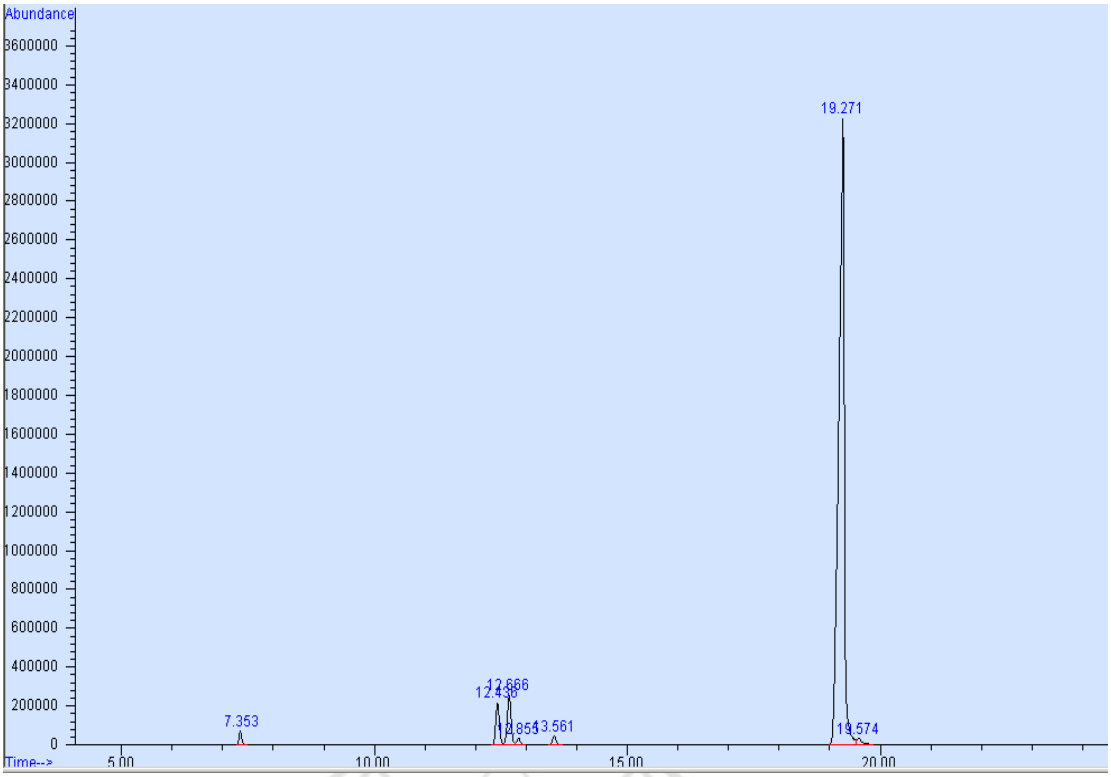

Figure C1.11 Chromatogram of fatty acid from *R.communis* wild-type (replication 2).

Table C1.11 Fatty acid profile of *R.communis* wild-type A (replication 2).

Peak no	Fatty acid	Redemption time (min)	% of total
1	Palmitic acid (C16:0)	7.351	0.915
2	Linoleic acid (C18:2, n-9)	12.437	3.728
3	Oleic acid (C18:1, n-9)	12.666	4.794
4	Oleic acid (C18:1, n-9)	12.854	0.571
5	Stearic acid (C18:0)	13.563	0.744
6	Ricinoleic acid	19.273	89.247

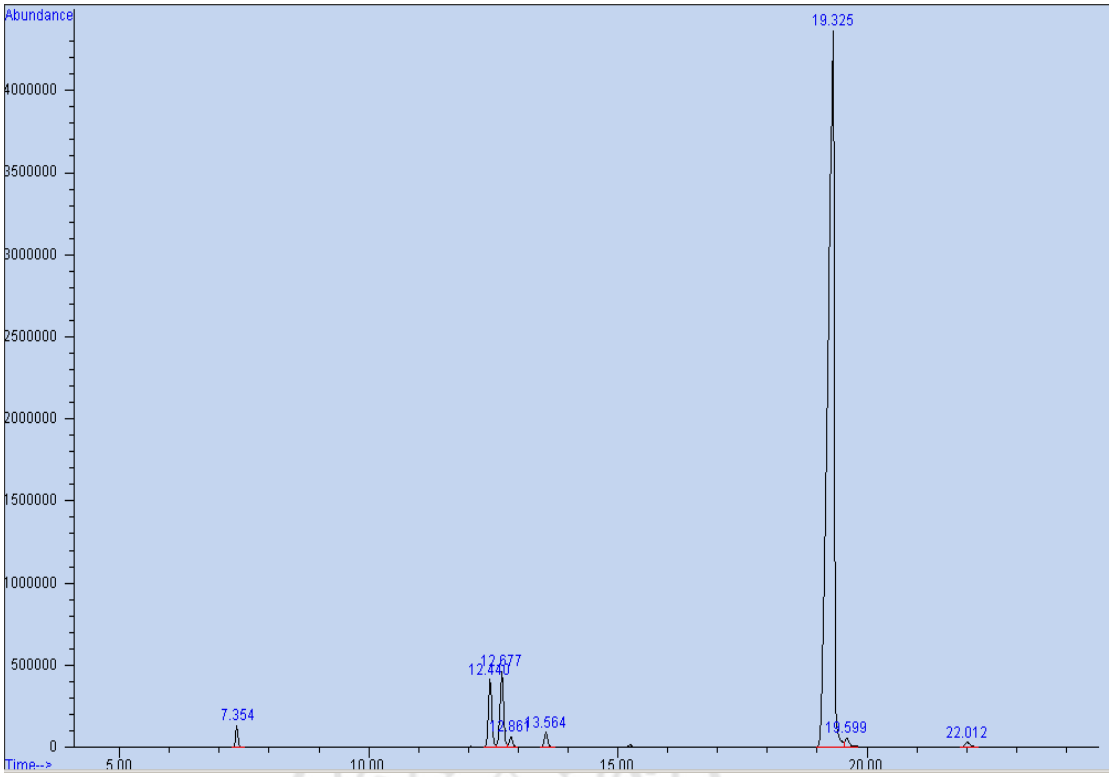

Figure C1.12 Chromatogram of fatty acid from *R.communis* wild-type (replication 3).

Table C1.12 Fatty acid profile of *R.communis* wild-type A (replication 3).

Peak no	Fatty acid	Redemption time (min)	% of total
1	Palmitic acid (C16:0)	7.356	1.015
2	Linoleic acid (C18:2, n-9)	12.437	4.267
3	Oleic acid (C18:1, n-9)	12.677	5.192
4	Oleic acid (C18:1, n-9)	12.860	0.667
5	Stearic acid (C18:0)	13.563	0.906
6	Ricinoleic acid	19.324	87.495

C2. Average amount of fatty acid composition per plant

Table C2.1 Fatty acid composition of *J. curcas* (toxic variety)

Compound	Fatty acid content (%)			Average	SD
	Rep 1	Rep 2	Rep 3		
Palmitoleic acid (C16:1 n-9)	0.51	0.569	0.579	0.552	0.037
Palmitic acid (C16:0)	13.923	14.012	13.842	13.925	0.085
Linoleic acid (C18:2, n-9)	35.043	35.237	34.992	35.090	0.129
Oleic acid (C18:1, n-9)	40.265	40.388	40.298	40.317	0.063
Stearic acid (C18:0)	7.381	7.019	7.222	7.027	0.181

Table C2.2 Fatty acid composition of *J. curcas* (nontoxic variety)

Compound	Fatty acid content (%)			Average	SD
	Rep 1	Rep 2	Rep 3		
Palmitoleic acid (C16:1 n-9)	0.585	-	0.588	0.391	0.002
Palmitic acid (C16:0)	14.115	14.656	13.519	14.096	0.568
Linoleic acid (C18:2, n-9)	37.244	38.083	37.671	37.665	0.419
Oleic acid (C18:1, n-9)	39.533	40.317	39.979	39.943	0.393
Stearic acid (C18:0)	7.514	6.944	6.771	7.076	0.388

Table C2.2 Fatty acid composition of *R. communis* (commercial-type)

Compound	Fatty acid content (%)			Average	SD
	Rep 1	Rep 2	Rep 3		
Palmitic acid (C16:0)	3.006	3.845	0.974	2.608	1.476
Linoleic acid (C18:2, n-9)	10.262	9.479	4.357	8.033	3.207
Oleic acid (C18:1, n-9)	9.734	9.351	4.167	7.754	3.109
Stearic acid (C18:0)	2.116	2.027	1.129	1.757	0.545
Ricinoleic acid	74.882	76.298	88.413	79.864	7.437

Table C2.2 Fatty acid composition of *R. communis* (wild-type A)

Compound	Fatty acid content (%)			Average	SD
	Rep 1	Rep 2	Rep 3		
Palmitic acid (C16:0)	-	0.915	1.015	0.643	0.070
Linoleic acid (C18:2, n-9)	3.589	3.728	4.267	3.861	0.358
Oleic acid (C18:1, n-9)	4.819	4.794	5.859	5.157	0.607
Stearic acid (C18:0)	-	0.744	0.906	0.55	0.114
Ricinoleic acid	91.529	89.242	86.778	89.183	2.376

CURRICULUM VITAE

NAME	Mr. Sopian Hadi
DATE OF BIRTH	17 April 1979
ADDRESS	Perumka. PTP XIII Nusantara, Pasir. East Kalimantan Indonesia
TELEPHONE	+66975094168
E-mail	hadisopian1779@yahoo.com
EDUCATION BACKGROUND	Agriculture Faculty, Mulawarman University, Samarinda, East Kalimantan. Indonesia
RESEARCH EXPERIENCE	The effects of soil management and varies concentration of glyfosat herbicide to the growth of the weed *Micania micrantha* and the yield of *Capsicum annum* L.
PRESENTATION	October 28-30, 2008. As a speaker in International Conference on Mathematics and Natural Science. Institut Teknology Bandung. Jawa Barat, Indonesia.