

รหัสโครงการวิจัย 52101010003

รายงานวิจัยฉบับสมบูรณ์

การศึกษาการสังเคราะห์ลูกผสม amphidiploids ระหว่าง
สบู่ดำ *Jatropha curcus* L. และ ละหุ่ง *Ricinus communis* L.
และการศึกษาความเข้ากันได้ระหว่างสองจีโนม

Investigations on the artificial synthesis of amphidiploids of *Jatropha*
curcus L. with *Ricinus communis* L.;
Intergeneric Genomic Relation

โดย

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งานวิจัยนี้ได้รับทุนอุดหนุนการวิจัยจากมหาวิทยาลัยแม่ฟ้าหลวง
ประจำปี พ.ศ.2551-2552

กิตติกรรมประกาศ

งานวิจัยเรื่องนี้มีจุดเริ่มต้นและสามารถดำเนินการจนสำเร็จจนลุล่วงไปได้ด้วยดีได้ ด้วยความกรุณาของ ศาสตราจารย์ ดร.สุจินต์ จินายนต์ ที่ให้คำแนะนำ ปรัชญา ชี้นะ และให้ความช่วยเหลือในหลายสิ่งหลายอย่างในระหว่างที่ท่านดำรงตำแหน่งที่ปรึกษาอธิการบดีมหาวิทยาลัยแม่ฟ้าหลวง ผู้วิจัยขอกราบขอบพระคุณเป็นอย่างสูงมา ณ ที่นี้

ขอขอบคุณ Mr. Sopian HANDI นักศึกษาแลกเปลี่ยนจากประเทศอินโดนีเซีย ที่ได้ตั้งใจค้นคว้าทำการทดลองทำวิทยานิพนธ์ระดับปริญญาโทที่มหาวิทยาลัยแม่ฟ้าหลวง นางสาวมัลลิกา เนื่องอุดม นักศึกษาปริญญาตรีที่ได้ตั้งใจทำการทดลองรายวิชาปัญหาพิเศษ ซึ่งเป็นส่วนหนึ่งที่สำคัญของงานวิจัยชิ้นนี้ และทำให้งานชิ้นนี้สำเร็จได้ด้วยดี

ขอขอบคุณ เจ้าหน้าที่ทุกท่านในส่วนบริการการวิจัย โดยเฉพาะอย่างยิ่ง นางสาว มาลินี ใจพรหม และนางสาว วรางคณา อินทร์สา ที่ได้ให้คำแนะนำ ช่วยเหลือในด้านการดำเนินการเอกสารต่างๆ อย่างดียิ่ง ตั้งแต่เริ่มต้นจนงานสำเร็จ

ขอกราบขอบพระคุณอาจารย์ทุกท่าน ที่เคยสอนทั้งมหาวิทยาลัยในประเทศไทย และมหาวิทยาลัยในต่างประเทศ ถึงแม้ว่าผู้เขียนจะจบการศึกษามานานแล้ว แต่ท่านอาจารย์ก็ยังคอยให้คำแนะนำ ปรัชญา และช่วยเหลือตลอดมา

สุดท้ายนี้ ผู้วิจัยขอกราบขอบพระคุณบิดาและมารดาที่คอยเป็นกำลังใจให้อย่างสม่ำเสมอ

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ช่วงเวลาดำเนินการปี พ.ศ. 2551-2552

ปัญหาที่ทําวิจัยและความสำคัญ

เนื่องจากการปรับตัวเพิ่มสูงขึ้นอย่างต่อเนื่องของราคาน้ำมันดิบในตลาดโลก การพัฒนาพลังงานทดแทนจากพืชจึงเป็นทางออกทางหนึ่ง ที่เหมาะสมสำหรับประเทศไทย โดยพืชที่สามารถใช้ผลิตน้ำมันได้มีหลายชนิด อาทิ เช่น พืชตระกูลถั่วต่างๆ กา ทานตะวัน ปาล์มน้ำมัน ละหุ่ง และสบู่ดำ ละหุ่งเป็นพืชน้ำมันที่มีความสำคัญทางเศรษฐกิจชนิดหนึ่งของประเทศไทย น้ำมันละหุ่งมีคุณสมบัติที่ดีคือ มีความเหนียวและไม่แข็งง่าย และมีความหนืดดีกว่าน้ำมันชนิดอื่นๆ จึงมีการนำน้ำมันละหุ่งไปใช้เป็นส่วนผสมในอุตสาหกรรมหลายประเภท เช่น สี หมึก พิมพ์ พลาสติก จาระบี และน้ำมันหล่อลื่น เนื่องจากน้ำมันละหุ่งที่สกัดได้ยังไม่เพียงพอกับความต้องการของอุตสาหกรรมภายในประเทศ ประเทศไทยมีการนำเข้าน้ำมันละหุ่ง และเมล็ดละหุ่งจากต่างประเทศเพื่อใช้ในอุตสาหกรรม และอุตสาหกรรมต่อเนื่องปีละไม่น้อยกว่า 400 ล้านบาท ปัจจุบันการผลิตละหุ่งของประเทศไทยมีแนวโน้มลดลงทุกปีทั้งพื้นที่ปลูกและผลผลิต เนื่องจากมีปัญหาการผลิตที่สำคัญคือ พันธุ์ที่เกษตรกรปลูกละหุ่งมีความเป็นพิษสูง และแมลงศัตรูพืชระบาดรุนแรง ทำให้เกษตรกรไม่นิยมปลูกละหุ่งเป็นแปลงใหญ่ โดยมากมักจะปลูกตามริมรั้วหรือหัวไร่ปลายนา และไม่มีการคัดสรรปรับปรุงพันธุ์

สบู่ดำเป็นพืชน้ำมันที่กำลังได้รับความสนใจจากทั่วโลก เนื่องจากสามารถขึ้นได้ในที่แห้งแล้งและมีความอุดมสมบูรณ์ของดินต่ำ สบู่ดำมีความได้เปรียบกับปาล์มน้ำมันตรงที่สามารถขึ้นได้ทั่วไป ไม่มีความจำเพาะเจาะจงต่อสภาพแวดล้อม แต่อย่างไรก็ตามปริมาณน้ำมันที่ได้ต่อไร่จากสบู่ดำยังค่อนข้างต่ำ มีรายงานสบู่ดำ 4 กิโลกรัมสามารถผลิตน้ำมันได้ 1 ลิตร การวิจัยและการพัฒนาเพื่อการปรับปรุงพันธุ์เพื่อการผลิตจึงมีความจำเป็น เนื่องจากสบู่ดำสายพันธุ์ต่างๆ ที่รวบรวมได้ในประเทศไทย รวมถึงหลายประเทศในทวีปเอเชีย เช่น อินเดีย มีรายงานความหลากหลายในระดับประชากรค่อนข้างต่ำ ทำให้การสร้างสายพันธุ์ใหม่ โดยการใช้การผสมแบบดั้งเดิม (conventional breeding) เป็นไปได้ยาก

การนำสายพันธุ์จากแหล่งต่างๆ กันเข้ามาผสมเพื่อการปรับปรุงพันธุ์จึงเป็นทางเลือกที่น่าสนใจ อาทิเช่น สายพันธุ์ที่ไม่เป็นพิษจากประเทศเม็กซิโก ที่มีแหล่งกระจายพันธุ์อยู่บนเทือกเขาที่มีความสูงถึง 4000 เมตร โดยสายพันธุ์นี้มีรายงานว่าเมื่อทำให้เมล็ดสุกสามารถใช้บริโภคได้ ในขณะที่สายพันธุ์จากประเทศไทยมีความเป็นพิษ ถึงแม้ว่าสบู่ดำจะมีความทนทานต่อโรคและแมลง การที่จะนำสบู่ดำเข้าสู่การเป็นอุตสาหกรรมพืชน้ำมัน ยังต้องการการปรับปรุงพันธุ์ในอีกหลายหลายด้าน ทั้งในแง่ของการเพิ่มผลผลิตและคุณภาพของน้ำมัน เนื่องจากสบู่ดำมีอัตราดอกตัวเมียต่อตัวผู้ค่อนข้างต่ำ (1 : 29) และการออกดอกติดผลเป็นไปในลักษณะทยอยออก จึงน่าจะมีการปรับปรุงลักษณะการออกดอกเพื่อเพิ่มจำนวนผลผลิต และเพื่อง่ายต่อการเก็บเกี่ยว ควรปรับปรุงต้นให้มีลักษณะเตี้ยลง เป็นที่ทราบกันว่าลูกผสมที่ได้จากพ่อแม่ที่มีความแตกต่างกันทางสายพันธุ์มักจะมีลักษณะที่เด่นกว่าทั้งพ่อและแม่ (heterosis) การผสมกันระหว่างสบู่ดำกับละหุ่งจึงอาจจะได้ลูกผสม (hybrid) ที่มีลักษณะที่น่าสนใจ อาทิเช่น เพิ่มปริมาณดอกตัวเมียในสบู่ดำส่งผลให้ผลผลิตเพิ่มขึ้น เนื่องจากละหุ่งมีปริมาณดอกตัวเมียมากกว่าตัวผู้ เพิ่มความทนต่อโรคและแมลงในละหุ่ง ลดความเป็นพิษในละหุ่ง เพิ่มคุณภาพน้ำมันในสบู่ดำ นอกจากนี้ในละหุ่งสายพันธุ์เพื่อการค้ายังได้มีการคัดสายพันธุ์ต้นเตี้ยได้สำเร็จ ดังนั้นถ้าเราสามารถผสมและคัดลักษณะที่ต้องการจากพืชสองชนิดนี้ได้จะทำให้ได้พืชน้ำมันที่เหมาะสมกับการปลูกเพื่ออุตสาหกรรม แต่สบู่ดำกับละหุ่งมีความต่างกันในระดับสปีชีในบางครั้งจึงอาจมีปัญหาการเข้ากันได้ระหว่างต่างชนิด (compatibility) การผสมข้ามชนิดจึงต้องศึกษาถึงพื้นฐานความเข้ากันได้ ในระหว่างสองสปีชี และถ้าหากพืชทั้งสองชนิดมีกลไกการป้องกันการผสมกัน การสร้างพืชลูกผสมอาจจะต้องอาศัยเทคนิคการหลอมรวมเซลล์ (protoplast fusion)

วัตถุประสงค์

1. เพื่อศึกษาหาความเข้ากันได้ (compatibility) ระหว่าง สบู่ดำ กับละหุ่ง
2. เพื่อศึกษาหาเทคนิคที่เหมาะสมในการสร้างลูกผสมระหว่างสบู่ดำกับละหุ่ง โดยใช้เทคนิคการหลอมรวมเซลล์ (ในกรณีที่พืชทั้งสองชนิดมีความเข้ากันได้)

ผลการวิจัย

1. การศึกษาความเข้ากันได้ (compatibility) ระหว่างสนับดำและละหู่

การศึกษาลักษณะเกสรตัวผู้ เกสรตัวผู้ของสนับดำมีขนาดเส้นผ่านศูนย์กลางประมาณ 100

ไมโครเมตร มีลักษณะเป็นทรงกลม ไม่มีรูงอกที่จำเพาะเจาะจง ผิวเป็นตุ่มหนามเล็กๆ เกสรตัวผู้ของละหู่มีลักษณะคล้ายลูกกรกบี้ เมื่อโดนน้ำมีลักษณะเป็นทรงกลม มีรูงอกสามรู ผิวเรียบ

การผสมพันธุ์ในธรรมชาติ (*In vivo* pollination) เกสรตัวผู้ของสนับดำมีขนาดใหญ่กว่าเกสรตัวผู้ของละหู่สามเท่า และมีขนาดใหญ่กว่าเกสรตัวเมียของละหู่ การผสมข้ามจึงใช้ดอกสนับดำตัวเมียเป็นแม่ และใช้เกสรตัวผู้ละหู่เป็นพ่อเท่านั้น จากการทดลองผสมในแปลงปลูก พบว่าดอกสนับดำที่ได้รับการผสมจะเปลี่ยนเป็นสีเหลืองและดำ ตายภายใน 14 วัน เฉลี่ยการตายสูงสุดในวันที่ 10

จากการสังเกตการงอกของ pollen tube ภายใต้กล้องจุลทรรศน์ พบว่า pollen ของละหู่สามารถงอกไปผสมกับไข่ของสนับดำได้และตัวอ่อนสามารถเจริญได้จนถึงวันที่ 7 ของการผสม

การผสมพันธุ์ในหลอดทดลอง (*In vitro* pollination) จากการผสมในหลอดทดลองพบว่าฮอร์โมนออกซิน และอาหารสูตร MS สามารถยืดระยะเวลาการเจริญของผลอ่อนได้ถึง 60 วัน การชักนำให้เป็น Callus สามารถทำได้โดยใช้อาหารสูตร MS + 0.5 mg/L NAA, 0.5 mg/L BA

2. การศึกษาด้านคุณสมบัติและการตอบสนองของเซลล์ การหลอมรวมเซลล์

จากการทดลองเลี้ยงเซลล์ของสนับดำและละหู่ เซลล์ของพืชทั้งสองชนิดพบว่าพืชทั้งสองชนิดมีลักษณะเซลล์ที่ตอบสนองต่อฮอร์โมนพืชต่างกันมาก นอกจากนี้ เซลล์ของสนับดำเมื่อผ่านการชักนำเป็น callus แล้วเลี้ยงไปนานกว่าสองเดือน เซลล์จะยังคงความสามารถในการสังเคราะห์แสง (photosynthetic cell) ในทางกลับกันเซลล์ของละหู่เมื่อถูกชักนำให้เป็น callus และเลี้ยงไปนานกว่าสองเดือน เซลล์จะหมดความสามารถในการสังเคราะห์แสง (non-photosynthetic cell)

จากการสกัดเซลล์โปรโตพลาสจากคัลลัสและจากใบอ่อนพบว่าเซลล์โปรโตพลาสจากใบอ่อนมีคุณภาพที่ดีกว่าจากเซลล์คัลลัส

จากการทดลองหาลอรวมเซลล์ในเบื้องต้นพบว่า ทำได้ยากได้เซลล์จำนวนน้อย การรวมเซลล์ในเบื้องต้นสามารถทำได้โดยใช้เอนไซม์ PEG 10% แต่ว่าเซลล์ลูกผสมไม่สามารถเจริญเติบโตต่อได้ คาดว่าเนื่องจากความไม่เข้ากันอย่างสิ้นเชิงของเซลล์ทั้งสองชนิด

วิเคราะห์และสรุปผลการวิจัย

สับดูดำและละหุ่งมีความแตกต่างกันอย่างมาก ทั้งลักษณะของดอกตัวผู้และดอกตัวเมีย ตลอดจนขนาดของเกสรตัวผู้ ซึ่งลักษณะเหล่านี้เป็นปัจจัยขวางการผสมพันธุ์กันตามธรรมชาติในเบื้องต้น (pre-zygotic barrier)

นอกจากนี้เมื่อได้รับการผสมเทียมในสภาพธรรมชาติแล้ว ผลอ่อนสามารถเจริญเติบโตได้ประมาณ 10 วัน แสดงถึงว่ามีกลไก (post-zygotic barrier) เกิดขึ้น การใช้การผสมในหลอดแก้วภายใต้อาหารและฮอร์โมนที่เหมาะสมสามารถยืดอายุของผลอ่อนได้ไปถึง 60 วัน

การหาลอรวมเซลล์สามารถทำได้แต่เซลล์ลูกผสมไม่สามารถเจริญเติบโตต่อได้ อาจเนื่องมาจากจำนวนชุดโครโมโซมที่ต่างกัน หรือปัจจัยที่ขวางกันอื่นๆ เช่น การไม่เข้ากันและการตกตะกอนของสารในนิวเคลียส

ข้อเสนอแนะที่คาดว่าจะควรจะมีการวิจัยเพิ่มเติม และวิธีการที่ควรพัฒนาต่อยอดสู่ภาคปฏิบัติจริง การสร้างลูกผสมสับดูดำ ควรทดลองผสมพันธุ์สับดูดำกับพืชชนิดอื่นที่มีความใกล้เคียงกันทางสายพันธุ์

บทคัดย่อ

สบู่ดำ *Jatropha curcas* ($2n=2x=22$) และละหุ่ง *R. communis* ($2n=2x=20$) เป็นพืชต่างสกุล ที่มีเมล็ดให้น้ำมันมากและมีศักยภาพสูงในการใช้ผลิตไบโอดีเซล ในการวิจัยนี้ต้องการพัฒนาสายพันธุ์โดยสร้างลูกผสมระหว่างพืชทั้งสองชนิดที่มีลักษณะต้นเดี่ยว ให้ผลผลิตเร็ว ออกดอกพร้อมกัน ให้น้ำมันที่มีคุณภาพดีและมีปริมาณน้ำมันมาก โดยทดลองผสมระหว่างละหุ่งต้นเดี่ยวสายพันธุ์การค้า และสายพันธุ์ป่า กับสบู่ดำสายพันธุ์พื้นเมืองของไทย และสบู่ดำสายพันธุ์ไม่มีพิษ จากการศึกษาลักษณะของละอองเรณูพบว่าละอองเรณูของสบู่ดำ (เส้นผ่านศูนย์กลาง $96\ \mu\text{m}$) มีขนาดใหญ่กว่าละอองเรณูของละหุ่ง (เส้นผ่านศูนย์กลาง $32\ \mu\text{m}$) ถึง 3 เท่า ดังนั้นจึงใช้สบู่ดำเป็นต้นแม่และใช้ละอองเรณูจากละหุ่งมาผสม จากการทดลองผสมในแปลงพบว่า หลังจากการผสมเกสรเกิดการงอกของละอองเรณูภายใน 48 ชั่วโมง มีการพัฒนาของเซลล์ไซโกตอย่างต่อเนื่องประมาณ 2 อาทิตย์ แต่หลังจากนั้นก็จะเริ่มเหี่ยวและแห้งไป จากการทดลองผสมจำนวน 100 ดอก โดยเฉลี่ยแล้วจะแห้งประมาณวันที่ 10 หลังจากการผสมเกสร ดังนั้นปัจจัยขวางกั้นการผสมระหว่างทั้งสองสายพันธุ์นี้น่าจะเกิดขึ้นหลังการพัฒนาของตัวอ่อน (post-zygotic barrier) จากการทดลองเลี้ยงเซลล์ตัวอ่อนในอาหารที่มีองค์ประกอบของฮอร์โมน $0.125\ \text{mg.L}^{-1}$ benzyladenine (BA) และ $0.125\ \text{mg.L}^{-1}$ 1-naphthaleneacetic acid (NAA) พบว่าตัวอ่อนสามารถพัฒนาต่อได้อีกจนถึง 57 วัน ดังนั้นถ้ามีการพัฒนาสูตรอาหารที่เหมาะสมก็อาจจะสามารถเลี้ยงเซลล์จนได้พืชลูกผสมได้ การทดลองสกัดโปรโตพลาสจากเซลล์ใบอ่อนพบว่าได้เซลล์โปรโตพลาสที่มีคุณภาพดีกว่าสกัดจากเซลล์ลัส จากการทดลองเอนไซม์ที่ความเข้มข้นต่างๆ PEG เข้มข้น 10% ให้ผลดีในการชักนำให้เกิดการรวมเซลล์สองเซลล์ (binary fusion)

ABSTRACT

Jatropha curcas ($2n=2x=22$) and *Ricinus 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. Pollen grain of *J. curcas* (96 μm) was 3 times bigger than *R. communis* (32 μm). *Jatropha curcas* was introduced as female parent plant as determined by pollen grains and ovule size. *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 pollen tube were observed in 48 hours after pollination. The zygote development was observed during the first week but was failed to set the seed after 2 weeks. This indicates this intergeneric hybridization was lack of pre-zygotic barrier. The post-zygotic barrier plays an important role in the hybrid zygote. *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 feasible method in order to obtain new intergeneric plants. A greater yield of protoplast isolating from a young leaf is than the isolation of protoplasts from the young callus cell. The enzyme solution 10% PEG shows the best result of binary fusion.

Keywords: *Jatropha curcas*, *Ricinus communis*, pollen grain, intergeneric hybridization, post fertilization barriers, pre fertilization barriers, protoplast fusion

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	
ABSTRACT	
LIST OF TABLES	
LIST OF FIGURES	
LIST OF ABBREVIATIONS	
CHAPTER	
1 INTRODUCTION	1
1.1 Introduction	1
1.2 Objectives	5
1.3 Scope of work	5
2 LITERATURE REVIEW	7
2.1 <i>Jatropha curcas</i> L.	7
2.2 <i>Ricinus communis</i> L.	13
2.3 Pollen morphology	19
Pollen and pollination	21
2.4 <i>In vivo</i> and <i>In vitro</i> pollination	24
2.5 Reproductive incompatibility	28
2.6 Protoplast fusion	30



TABLE OF CONTENTS (continued)

CHAPTER	Page
3 MATERIALS AND METHODS	31
3.1 Pollen study	32
Fresh pollen measurement	32
Pollen acetolysis and SEM investigation	32
3.2 Hybridization	33
<i>In vivo</i> pollination	33
<i>In vitro</i> pollination	35
3.3 Protoplast fusion	37
4 RESULTS AND DISCUSSIONS	40
4.1 Pollen study	40
Fresh pollen grains and LM observation	40
Pollen acetolysis and SEM observation	43
4.2 Intergeneric hybridization	47
<i>In vivo</i> pollination	47
<i>In vitro</i> pollination	53
4.3 Physical characterization of seed and seed oil analysis	56
Seed physical characteristic	56
Seed weight and oil content	57
Protoplast fusion	62

TABLE OF CONTENTS (continued)

	Page
5 CONCLUSIONS	67
REFERENCES	68
APPENDIX A <i>Jatropha curcas</i> and <i>Ricinus communis</i>	81
APPENDIX B Media composition	86
APPENDIX C Fatty acid profile analysis	88



LIST OF TABLES

Table		Page
1	Nutritional analysis of <i>J. curcas</i> oil seed-cake and manure (%)	11
2	The chemical analysis of <i>J. curcas</i> oil	12
3	The chemical analysis of <i>R. communis</i> oil	18
4	Analysis of castor oil from selected countries	18
5	Plant material for each experiment	31
6	Pollen grains size	41
7	Hand-pollinate field <i>Jatropha curcas</i>	48
8	Seed size and seed oil content in <i>J. curcas</i> and <i>R. communis</i>	58
9	Isolation of protoplast from young leaves and callus tissues of <i>J. curcas</i> and <i>R. communis</i>	63

LIST OF FIGURES

Figure		Page
1	Estimation of energy demand next 20 years	1
2	Geographical distribution of <i>J. curcas</i>	8
3	An inflorescence, pollinated female flower and fruit of <i>J. curcas</i>	9
4	Different component of the physic nut (<i>J. curcas</i> L.)	10
5	Geographical distribution of <i>R. communis</i>	14
6	An inflorescence of <i>R. communis</i> flower	15
7	Diagrams of some developmental patterns of sex expression in <i>R. communis</i>	15
8	Pollen morphology from some member of Euphorbiaceae family	20
9	The cell cycle and fertilization in flowering plant	25
10	Comparative size hydrated pollen grains	41
11	Hydrated pollen grains of <i>J. curcas</i> and <i>R. communis</i>	42
12	Apertures in the pollen exine layer	44
13	Small spinules in exine layers of <i>J. curcas</i> and small dots in exine of <i>R. communis</i>	45
14	<i>Jatropha curcas</i> female flowers after pollination	47
15	Pollen tube growth and hybrid zygote development	49
16	<i>J. curcas</i> nontoxic flower and toxic varieties	53

17	Average of 100-seed weight (g) and oil content (%) of <i>J. curcas</i>	59
18	Average of 100-seed weight (g) and oil content (%) of <i>R. communis</i>	59
19	Protoplast cell of <i>Jatropha curcas</i> (a) Protoplast cell of <i>Ricinus communis</i> (b)	64
20	Multiple fusion of <i>Jatropha curcas</i> and <i>Ricinus communis</i> (a) Binary fusion (b)	66



ABBREVIATIONS

ANOVA	=	Analysis of Variance
BA	=	Benzyladenine
FAA	=	formalin-aceticacid-alcohol
FFA	=	Free Fatty Acid
GC	=	gas-liquid chromatography
IAA	=	Indole-3-aceticacid
LM	=	light microscope
MAP	=	Mitogen-activated protein
MS	=	Murashige and Skoog
NAA	=	1-naphthalineaceticacid
rpm	=	revolution per minute
SD	=	Standard Deviation
SEM	=	Scanning Electron Microscope
TBA	=	tert-butyl alcohol
TDZ	=	Thidiazuron
v/v	=	volume by volume

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.

Energy demand by fuel type

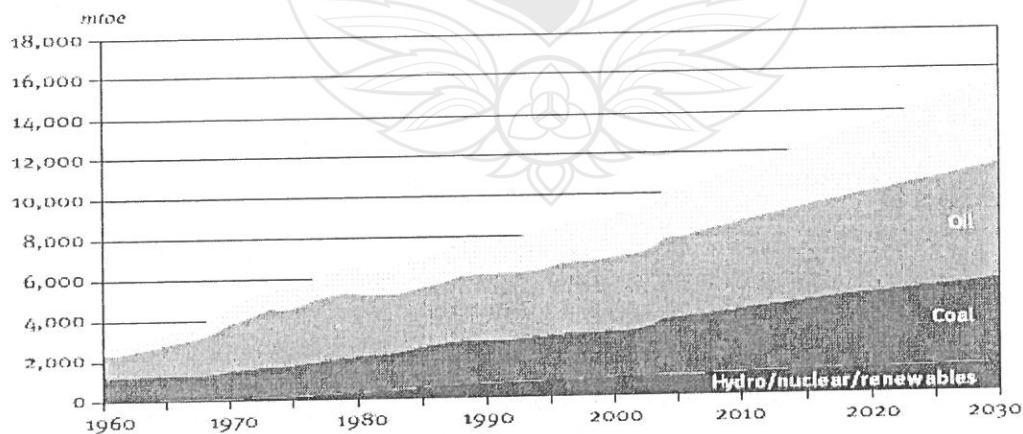


Figure 1 Estimation of energy demand next 20 years (International Energy Agency, 2007)

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 are 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, allow the technology to grow, and invest in improving biofuel technologies (Sexton et al, 2006). The demand for biofuels in USA, Europe and developing nation is partly driven by the need to replace fossil fuels and lessen dependence on high-priced oil (Boddiger, 2007). The demand of biofuel as renewable energy has revealed *Jatropha curcas* as one of 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), that are widely distributed in tropics with a promise for use as an oil crop for biodiesel. *Ricinus comunnis*, or castor bean (English); la-hung (Thai); jarak kepyar (Indonesia), its unique oil chemical properties are suitable for a wide variety of uses make it great industrial importance and medicinal purposes. Countries with tropical and subtropical climates can benefit from renewable energy resources as *Jatropha* cultivation (Lozano,

2007) because *Jatropha* is probably the most highly promoted oilseed crop at present (Gressel, 2007).

Non-edible oil bearing trees like *Jatropha* can be utilized either as biofuel or with processing (Kaushik, et al 2007) and it grow on medium and low fertility soils, and in low and high rainfall areas (Lazano, 2007). Production each hectares reach 4.5-5 tons per year (Prihandana et al, 2007) and Bengue (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 contain more oxygen, with a higher cetane value increasing the combustion quality, 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 communis or castor plants a well-known plants which is produces castor oil since a long time ago. (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 is 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, high molecular weight 298, a low melting point of 5°C and very low solidification point of between -12°C to -18°C 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 is use for human and veterinary medicine used, soap, wax and polishes, insulator, coating, plastic and nylon.

Studies with mutation breeding and interspecific crossing have made little headway in increasing variability in this species (Sujhata and Mukta, 1996; Sakaguchi et al., 1987). Using molecular markers, diversity of *J. curcas* in Thailand and India revealed no genetically differences or low intra-accessional variability (Sujhata, 2007). The limitations in available include; lack of knowledge of the genetic base, poor yields, low genetic diversity and vulnerability to a wide array of insects and disease. In the other hand, high yielding castor has been released during 1990s as a result from many genetic improvement programs in India. The importance of mutations in castor is demonstrated by inducing productive semi-dwarfs with higher yielding potential and earlier maturity (Sujatha et al, 2008). Genetic improvement and breeding program for *J. curcas* x *R. communis* are still needed to combine the traits into a new species with novel characteristics. However, hybridization between these genera could potentially combine the biotic stresses, greater adaptability, utility and genetic diversity of *J. curcas*.

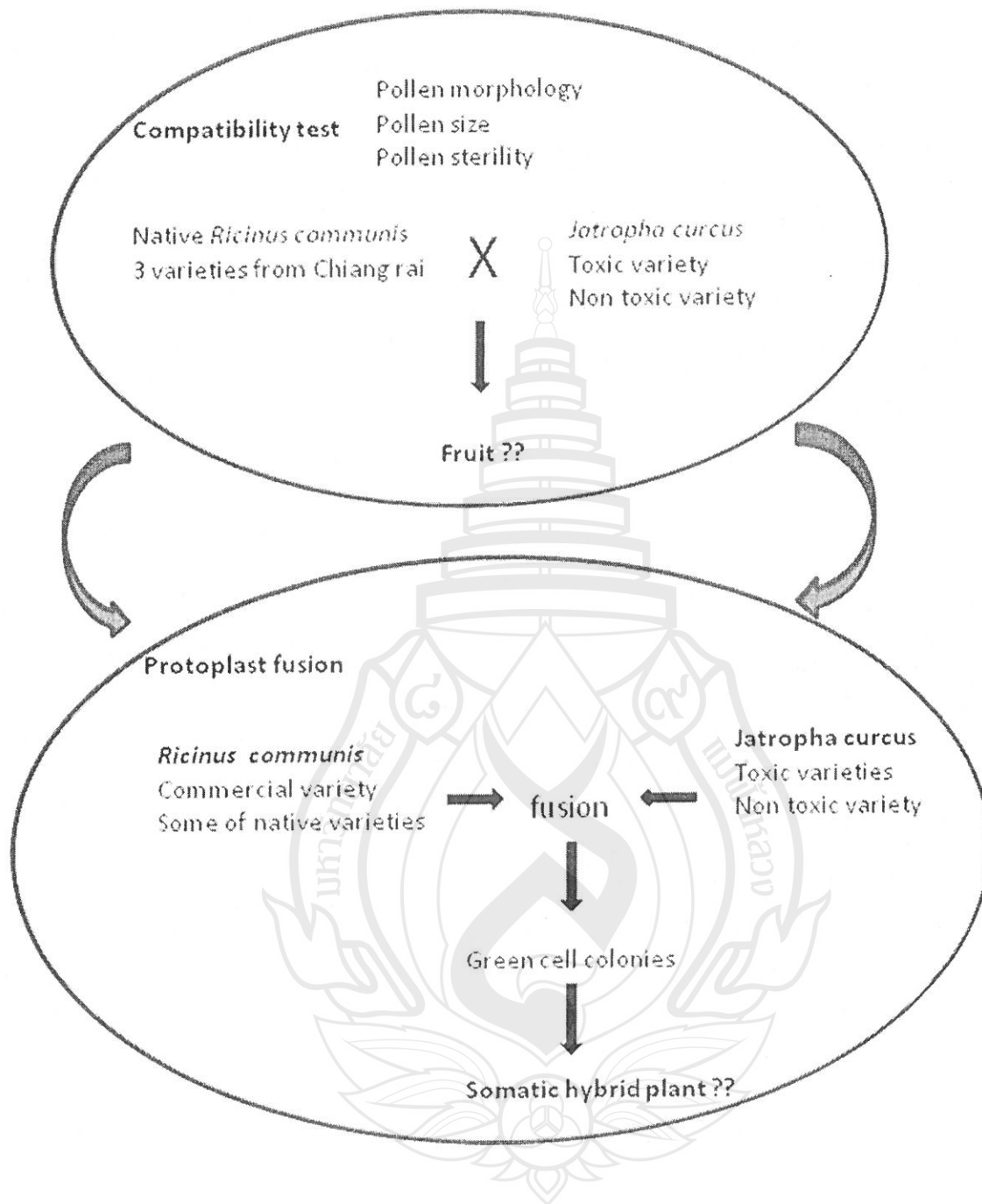
Interspecific or intergeneric hybridization has been undertaken with an objective of combining the desirable traits of these two economically important species. The project aims to investigate the compatibility of these species through *in vivo* pollination, *in vitro* pollination and protoplast fusion, respectively. Pollen morphology and morphology of callus cell were studied to obtain complete information between two genera.

1.2 Objectives

- 1 To study the compatibility of 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 plants.
- 4 To study the compatibility of *Jatropha curcas* cell and *Ricinus communis* cell through protoplast fusion

1.3 Scope of work

This research work on 2 species of *J. curcas* (toxic and nontoxic variety) and 2 varieties of *R. communis* (commercial and wild cultivar), attempted to hybridize through *in vivo* and *in vitro* method based on their pollen size and female flower morphology. The protoplast fusion has been applied to break down the intergeneric barriers. Seed measurement and pollen morphology have been investigated to confirm diversity between two genera.



CHAPTER 2

LITERATURE REVIEW

2.1 *Jatropha curcas* L.

Jatropha curcas is a well known oil seed crop which is widely cultivated in the tropics as a living fence. It is plant with many attributes, multiple uses and considerable potential (Openshaw, 2000). Many parts of the plant are used in traditional medicine. Seeds are used for oil extraction and soap production. *Jatropha curcas* 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	:	<i>Jatropha</i>
Species	:	<i>Jatropha curcas</i>

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, 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 medicinal plant. A non-toxic variety is reported to exist in Mexico and Central America. A species said to be endemic to Madagascar, *J. mahafalensis*, is reported to have equal energetic promise (Benge, 2006).

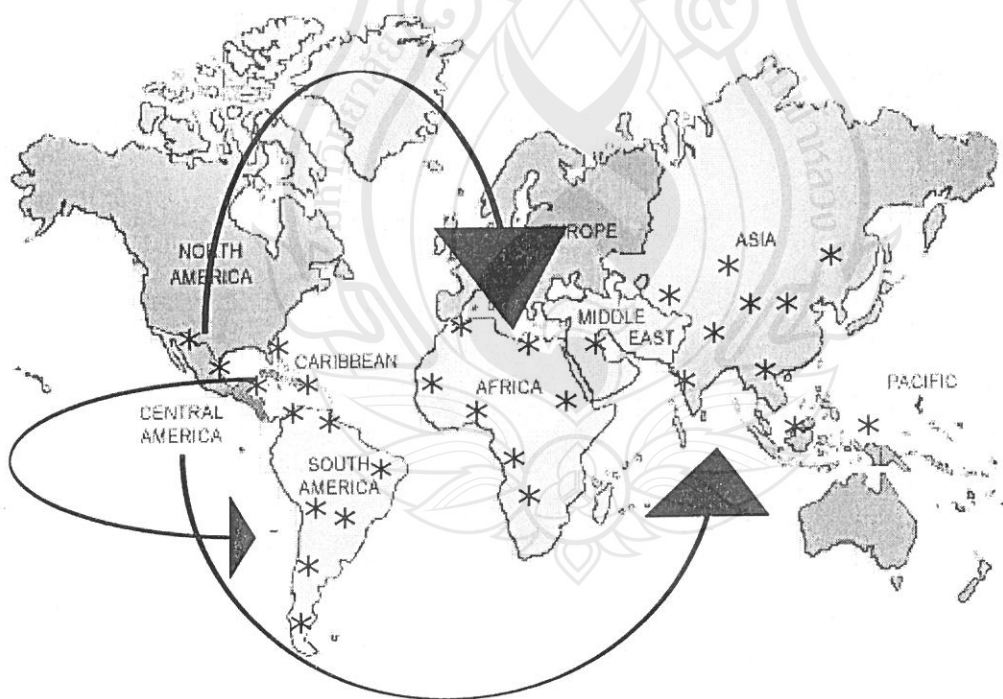


Figure 2: Geographical distribution of *J. curcas*. Central America and Caribbean proposed as centers of origin (source: Heller, 1996).

Physic nut is a small tree or shrub which can reach up to 5 m, has a shallow lobed leaves with length and width of 6 to 15 cm, which are arranged alternately. Inflorescences are formed terminally on branches and are complex. The plant is monoecious and flowers are unisexual; occasionally hermaphrodite flowers occur (Dehgan and Webster, 1979). Raju and Ezradanam (2002) explain that *Jatropha* plant produces flowers in racemose inflorescence, with 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, inaperturate; the exine is semitactate and verrucate. While Gupta (1985) in Heller (1996) investigated its chromosomes, the physic nut is a diploid species with $2n = 22$ chromosomes.



Figure 3 An inflorescence (a), pollinated mature female flower (b) and fruit (c) of *J. curcas*

Physic nut is a cross-pollinated crop. Any genetic improvement has to be based on population. Pollination is by insect, Heller (1992) *cit.* Heller (1996) observed a number of different insects that visited flower and could pollinated. In Senegal, he observed that staminate flowers open later than pistillate flower in the same inflorescence, 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 fleshy 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, with one rainy season per year, there will be only one annual fruiting; for irrigated crops, up to three fruiting can occur each year.



Figure 4. Different component of the physic nut (*J. curcas* L.): 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. Source: Heller (1996).

Seed of *Jatropha* contain curcin, phorbol ester and curcasine which are toxic to goat, sheep and calves. In overseas countries human poisoning is common; especially in children are mistakenly eaten as edible nuts. *Jatropha* seed-cake contain

curcin, a highly toxic protein similar to ricin in Castor (Heller, 1996) 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. In the production of oil inevitably produces a press-cake, *Jatropha* has 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 1).

Table 1 Nutritional analyses of oil seed-cake and manure (percentage)

Property	<i>J.curcas</i> 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, and the kernels must be dried and then oil has to be extracted (Sirisomboon, 2007). Oil content in the *Jatropha* seed is around 30-40% (Sarin et al, 2006), in the kernels 55-60% (Heller, 1996). *Jatropha* oil contains about 14% free fatty acid (FFA), which is far beyond the limit of 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

transesterification reaction with an alkaline catalyst. This procedure yielded more than 95% biodiesel (Tiwari et al, 2007). The 34 % linoleic acid content in kernel oil of possible interest for skin care (Heller, 1996) and 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 with diesel (Sirisomboon, 2007).

Table 2 The chemical analysis of *J. curcas* oil

Property	Value
Acid value	38.2
Saponification value	195.0
Iodine value	101.7
Viscosity (31°C) 'fatty acid'	40.4
Palmitic acid %	4.2
Stearic acid %	6.9
Oleic acid %	43.1
Linoleic acid %	34.3
Other acid %	1.4

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 widely grown for its seeds which yield the much value castor oil (Sujatha and Reddy, 1998). Subsequently, castor seed oil and its derivatives have become important commodities and an increasing number of uses are being found for them in the industrial world (Sujatha and Sailaja, 2005). According BALLITAS (2000) *cit.* (Heyne, 1987):

Kingdom	:	Plantae
Division	:	Spermatophyta
Subdivision	:	Angiospermae
Class	:	Dicotyledonae
Order	:	Euphorbiales
Family	:	Euphorbiaceae
Genus	:	<i>Ricinus</i>
Species	:	<i>Ricinus communis</i>

Castor's contemporary distribution in the warmer regions in worldwide, although its origin is obscured by wide dissemination in ancient times and the ease and rapidity which it becomes established. Russian plant breeders consider there are four large centres 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.

Thailand production castor steadily decreased from 40,000 t in 1970 and to less 10,000 t by 1990, but since then has been given official support with target 50,000 t annually. The castor plants reach 30-40 feet tall tropical perennial usually behaves as an annual in the temperate region where it grows up to 1 feet tall (Francis, 1972), but all types of castor from giant perennials to short internode dwarfs have the same chromosome number $x = 10$, $2n = 20$, and natural polyploidy is probably absent from monotypic genus.

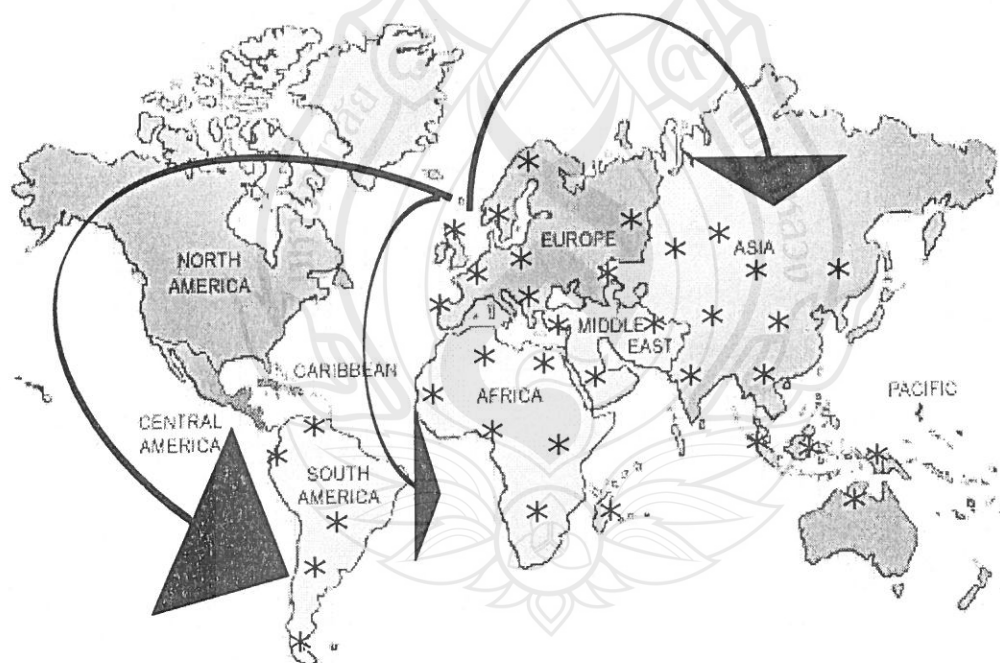


Figure 5. Geographical distribution of *R. communis*. Abyssinia most probably as center of origin and widely grown in tropic and subtropic countries. Africa, South America, and Asia proposed as centre of diversity for this important industrial plant.

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 produce female flowers achieved 95% per inflorescence but average is 30-50% (Wahyu and Sumarsih, 2007). Indeed, number of female flowers reaches 99% and less than 5% in very extreme condition (BALITTAS, 2000).

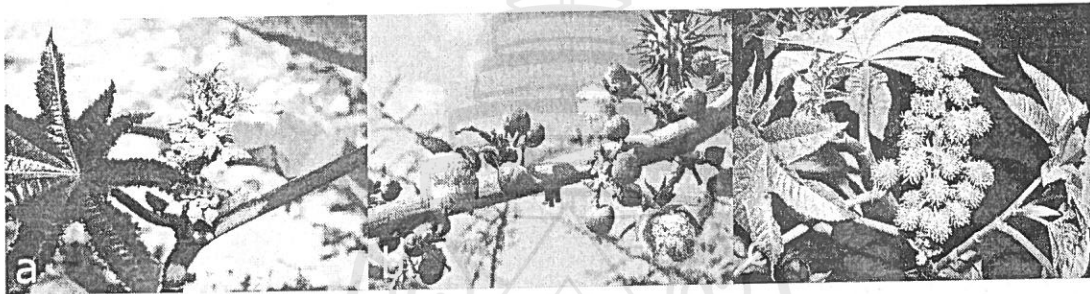


Figure 6. An inflorescence of *Ricinus* flower; upper part is a female and lower part is a male (1), mature male flowers (2) and fruit of *Ricinus* (3).

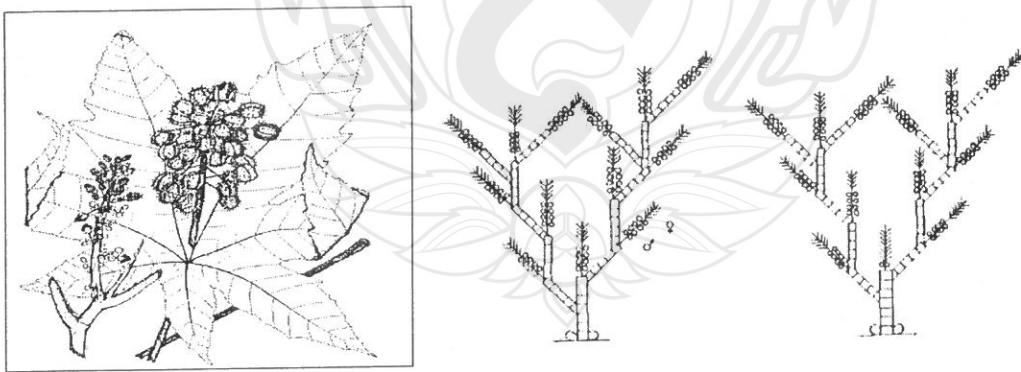


Figure 7. Diagrams of some developmental patterns of sex expression in *Ricinus* (Shifriss, 1955)

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. Shape of stem is round, glabrous and covered with a waxy bloom which gives red or green stems a bluish appearance (Weiss, 2000). Castor plant height reach 1-6 m with diameter 3-5 cm and keep grow and will survive as long as growing factors available, particularly water (Wahyu and Sri, 2007).

Pollen normally shed from 2-3 hours before sunrise until late afternoon, with a temperature of 26-29° 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 along flowering period and in wild giant types may flower year-round; the lowest flowering raceme on the plant is usually the first 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 varies 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 contains three seed (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 growing 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. 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 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. 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. Ricinoleic acid was absent in very young seed, but appeared at 12 days, and at 36 days it represented 90% of fatty acid; fatty acid composition of oil then remained constant (Weiss, 200). Marlina et al (1992) shown the properties of castor oil are:

Table 3. The chemical analysis of *R. communis* oil

Property	Value
Ricinoleic acid %	87
Oleic acid %	7
Saponification number	177-187
Linoleic acid %	2
Palmitic acid %	2
Stearic acid %	1
Viscosity at 20°C	9.5-10.0 dPa.S
Iodine value	82-88

Cerry (1992) found that the seed contents makes up 65-85% of weight of the bean and the oil content can range from 35-52%, depending on variety of seed and environment. Commercially castor oil consists of triglycerides that contain 90% of ricinoleic acid and it has effective hydroxyl functionality (Cunha et al, 2004). It is a fatty acid with 18 carbon atoms, a double bond between the ninth and tenth carbons, and is known as Dodecahydroxyoleic Acid and no other vegetable oil contains such a diverse and high proportion of fatty hydroxyacids.

Table 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

2.3 Pollen morphology

Pollen grains are multicellular structure arising by two mitotic divisions in microspore mother cell, resulting in the formation of a male gametophyte (Batygina, 2002). There are two types of pollen grains in angiosperm; binucleate and trinucleate, and pollen grains of Euphorbiaceae family included in binucleate type. 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. Trinucleate grain is with a vegetative nucleus and two male gametes (sperm cells).

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 envelopes the pollen protoplast and 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).

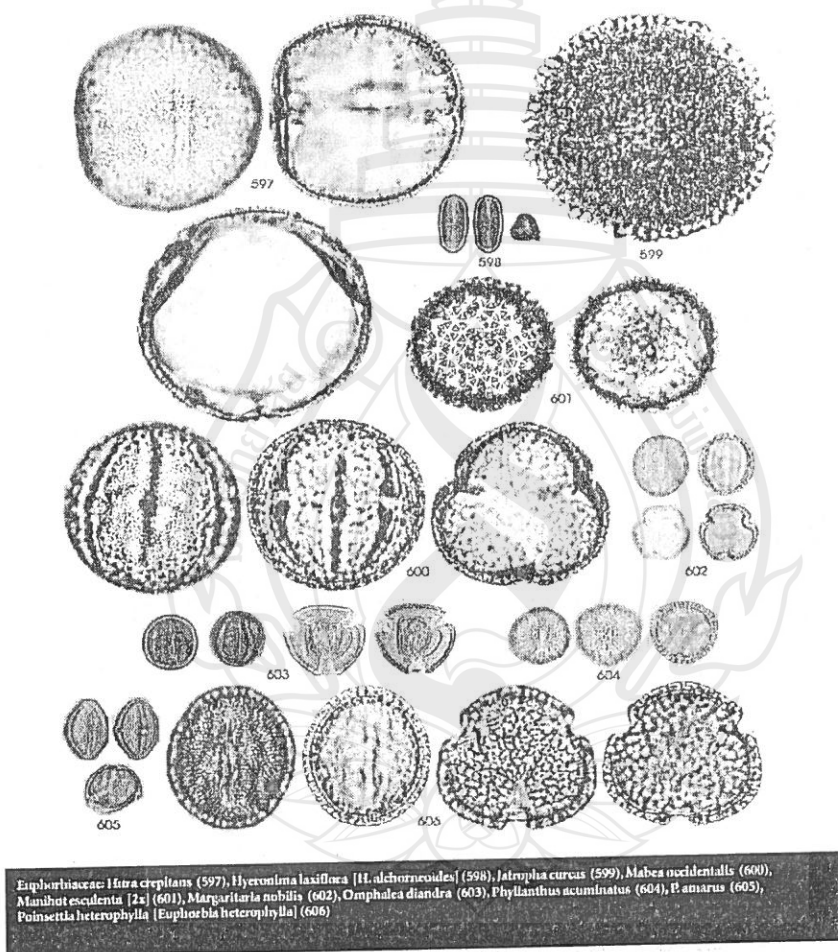


Figure 8. Pollen morphology of some member of Euphorbiaceae family

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 size of pollen is highly variable and ranges from 5 to 200 μm . Smallest grains are recorded in members of Boraginaceae and larger pollen grains are produced in members of Cucurbitaceae, Malvaceae and Nyctaginaceae. Walker (1971) in Shivanna (2003) was reported 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 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 depend on whether it is observed in polar view or equatorial view. Pollen grains of a large number of species in polar view are circular or elliptic. However, triangular, quadrangular and rectangular pollen are also present.

2.3.1 Pollen and pollination

Pollen-pistil plays an important role in sexual reproduction. The pollen grain is screened during pollen-pistil interaction; the pistil facilitates germination of compatible pollen and growth of the resultant pollen tube until it reaches the embryo sac while incompatible pollen is inhibited before germination or during the growth of pollen tube before its entry into the embryo sac (Shivanna, 2003).

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 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.

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 pistil (Wheeler et al, 2001). The coat is full of proteins that could act as ligands for receptor in the stigma, preparing the way 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 hydrated, the tube cell cytoplasm becomes activated and some genes, such 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 the to the ovary.

It is known that pollen 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).

A large number of genetically controlled physiological, biochemical and morphological processes within the flower are involved in the production of fertile pollen. Any disruption during development of stamen, differentiation of the sporogenous cells, meiosis, development of the free microspore, microspore mitosis, pollen differentiation or anthesis can result in male-sterile plants (Glover et al, 1998).

2.4 *In vivo* and *in vitro* pollination

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 lands on 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).

The past several years have seen a resurgence of *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 direct observation of 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. According 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.

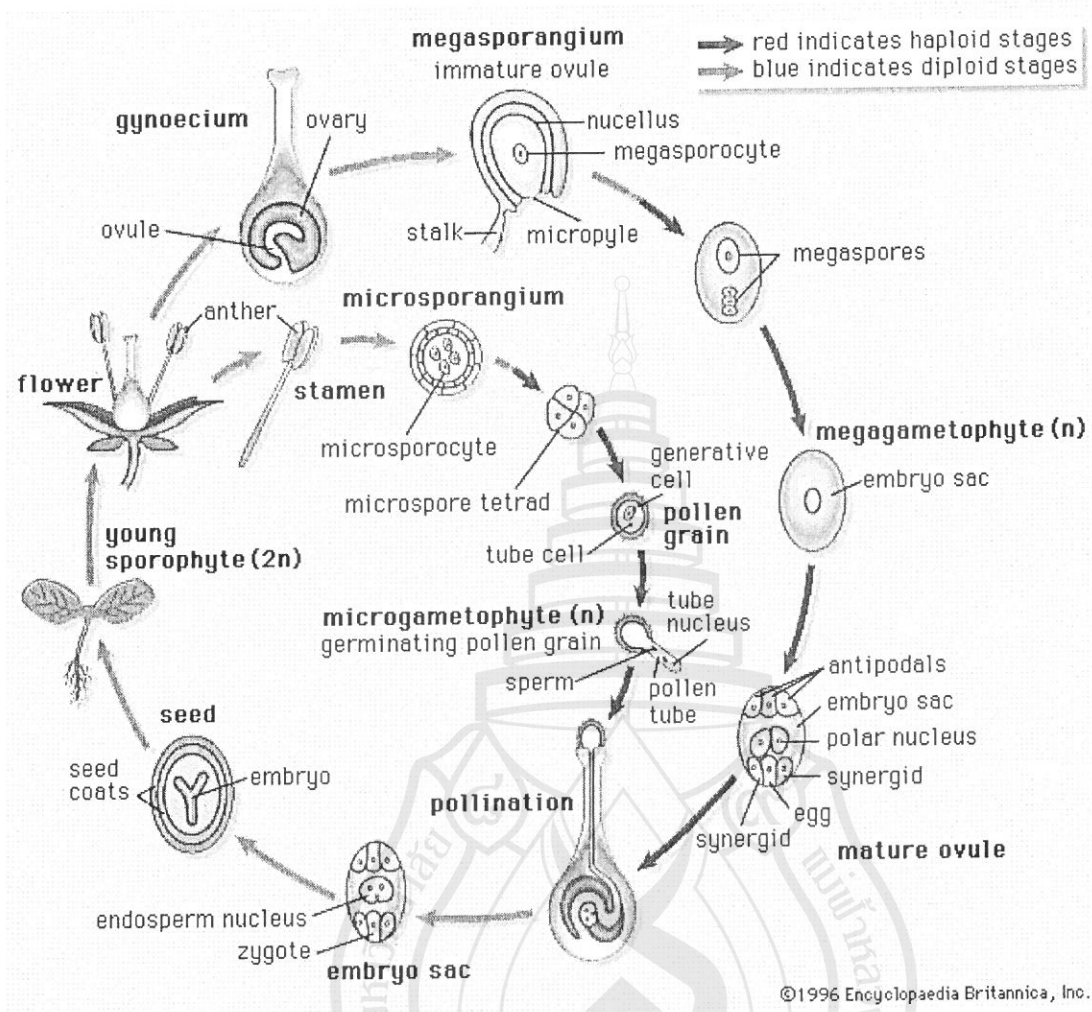


Figure 9 The cell cycle and fertilization in flowering plant.

Early results in angiosperm, however, provided a baffling variety of data and DNA complements. Recent evidence suggests three major patterns of pollen dissemination in the bicellular (generative cell-containing) and tricellular (sperm cell-containing) pollen in the flowering plants: 1C, 2C and intermediate values (Friedman, 1999). Furthermore, fusion occurs in two conditions: 1C (G1 fusion) and 2C (G2 fusion). Only in *Arabidopsis* and the gnetophytes have data on both the egg cell and sperm cell been followed through development to fusion, although these may be inferred in others by zygotic measurements.

Evidence for pollen tube attraction has been elusive target recently, and efforts to demonstrate it using living male and female gametophytes have been inconclusive. Pollen tubes of in *Arabidopsis* were attracted over distance of 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.”

In nature, intergeneric or interspecific hybridization occurs less frequently and 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. Interspecific hybridization has allowed creation of new plants and the transfer desirable's features from one species into another, e.g., via introgression from wild into related cultivated species (Kobayashi, 2003).

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 subsequent development of embryo. Pollination of cultured pistils and their growth in vitro mature frits provide a very convenient technique for experimental studies on pollen-pistil interaction, fertilization and development of fruits and seed. This technique has been successfully achieved in 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, 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, which 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 species Papaveraceae and Solanaceae, or excised ovules together with placenta called *in vitro placenta* pollination, which has been successfully applied to overcome self-incompatibility in *Petunia axillaris*.

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 of ovule physiology before, during and after fertilization than *in vivo* systems.

2.5 Reproductive incompatibility

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 to selection on mating process (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 interspecific or intergeneric incompatibility.

Interspecific incompatibility as a reproductive barrier resulting in failure of seed-set between two reproductively isolated species. Interspecific incompatibility may therefore operate before fertilization (prefertilization) and/or after fertilization (post-fertilization). This system prevents gene flow between species and maintains species identity. Although interspecific 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.

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. Reproductive isolating mechanisms can be divided into two categories: prezygotic and postzygotic barriers.

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 behavioral isolation, reproduction between similar species is prevented because each group possesses its own characteristic courtship behaviors. Wood and leopard frogs exhibit behavioral isolation because the males of each species have vocalizations that only attract females of their species. Mechanical isolation occurs because the genital organs of different species are incompatible. Even if members of two species court and attempt copulation, mating is not successful. 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. Gametic isolation is particularly important in aquatic environments because many aquatic animals release their gametes into the water, where fertilization takes place.

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. Mules are sterile hybrids formed by mating a female horse with a male donkey. 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.

2.6 Protoplast fusion

Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells. Protoplast can be obtained by specific lytic enzymes to remove cell wall. Protoplast fusion is a physical phenomenon, during fusion two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents. By protoplast fusion it is possible to transfer some useful genes such as disease resistance, nitrogen fixation, rapid growth rate, more product formation rate, protein quality, frost hardiness, drought resistance, herbicide resistance, heat and cold resistance from one species to another. Protoplast fusion an important tools in strain improvement for bringing genetic recombinations and developing hybrid strains in filamentous fungi. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. These are the powerful techniques for engineering of microbial strains for desirable industrial properties. Protoplast fusion would continued to be an existing area of research in modern biotechnology. This technique in the future will be one of the most frequently used research tools for tissue culturists, molecular biologists, biochemical engineers and biotechnologists. In this study, we report the first results on protoplast fusion between *J. curcas* and *R. communis* using an enzymatic fusion protocol.

CHAPTER 3

MATERIALS AND METHODS

This experiment had three parts; pollen study, *In vivo* and *In vitro* pollination, and protoplast fusion. All plants materials were planted in Mae Fah Luang University field, and the seed of *R. communis* commercial cultivar (dwarf-internode) for planting and oil extraction was introduced by Prof. Dr. Sujin Jinahyon from East West Seed International Ltd., Nonthaburi, Thailand.

Table 5. Plant material for each experiment

Species	Fresh pollen analysis	SEM analysis	<i>in vivo</i> pollination	<i>in vitro</i> pollination	Seed diversity	Protoplast fusion
<i>J. curcas</i> toxic (Thailand)	✓	✓	✓	✓	✓	
<i>J. curcas</i> nontoxic (Mexico)	✓		✓	✓	✓	✓
<i>R. communis</i> commercial cultivar	✓	✓	✓	✓	✓	✓

3.1 Pollen study

3.1.1. Fresh pollen measurement

This method has been used to study the pollen grains morphology, shape and size of *J. curcas* and *R. communis* in the fresh condition. Observation fresh pollen grains aims to study the real shape of grains before remove the lipid, carbohydrate and some protein in the exine layer. More than 100 pollen grains of each species have been studied. Fresh pollen grains were taken from the field in separate bags. Put pollen grains on clean Petri dish and take some with tooth stick, transfer to object glass and put a drop of water. Pictures of pollen grains were taken and measured by Motic camera program. Sizes of fresh pollen grains were counted and record as a basic information to determine of parent plant in the hybridization experiment. Statistical analysis of pollen grains data was performed by Univariate ANOVA using SPSS (ver. 16.0 for Windows).

3.1.2. Pollen acetolysis and SEM investigation

Acetolysis method introduced by Erdtman (1954, with minor modification) is still highly successful technique to destroy al pollen material with the exception of sporopollenin that forms in the exin (Hesse and Waha, 1989). A hundred pollen grains of *Jatropha* and castor were counted to compare under Light Microscope with 40x lens. Pollens grains were poured into graduated clean centrifugal tubes and rinsed with 70% Ethyl alcohol, centrifuged for 5 min (14,000 rpm) than changed with Acetic and Sulphuric Acid (9:1). Shake and mixed pollen-solution well, boiled in water bath in 94-100°C (10 minutes) and centrifuged (14,000 rpm) for 5 min. Repeated once to ensure pollen grains are clean. Acetic and Sulphuric Acid (1:1) were used for

next solution and centrifuged (14,000 rpm) for 5 min and were repeated three times. By placing these droplets on a cover slip and dry overnight, size and shape of pollen grains can be followed under Scanning Electron Microscope for observation.

3.2. Hybridization

3.2.1. *In vivo* pollination

Sixty five of *J. curcas* toxic and sixty non toxic female flowers were pollinated with castor pollen grains. Pollinations were performed by emasculating male flowers before the female flowers open. Removing fresh castor pollen grains with paintbrush and then brushing on to stigma of the female parent. Flowers were labeled and numbered then bagged in order to prevent undesired pollination. Pollinated pistils were extracted at 4h, 48 h, 3 days, 5 days, and 7 day after pollination and fixed in acetic:alcohol (1:3) v/v for 12 h. Fixed pistils were rinsed thoroughly in tap water and treated with aceto archein. The pistils were section by razor blade and kept in slide and cover slip to observe the pollen tube germination and hybridization. The zygote growth step was observed following the microtome section technique (Ruzin, 1999) using the fixed pistils in formalin: alcohol:acetic acid:water (10:50:5:35).

Investigation by microtome section was the further method to investigate the intergeneric compatibility, and the steps of Microtome Section are: female flower of *Jatropha* were fixed overnight at 4°C-Room Temperature in FAA (50% EtOH, 5% glacial acetic acid, 10% formalin, 35% water) solution. The tissues were killed and hardened within 18-24 h when treated at room temperature and then were washed in 50% EtOH, 15 min. The tissues which have already fixated were dehydrated and transferred into paraffin using TBA (tert-butyl alcohol) as the intermediate solvent which is 2 hours for each step as the following order:

- a. EtOH 95%:H₂O:TBA= 50:40:10
- b. EtOH 95%:H₂O:TBA= 50:30:20
- c. EtOH 95%:H₂O: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 replace 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 4 hours intervals, ½ 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 3 times at 1 h 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 revealed. Mayer's adhesive preparation as the section adhesive. Mix 1:1 fresh egg white and glycerol plus 1 g sodium salicylate. The glass microscope slides were coated very lightly and let it dry.

- The paraffin embedded tissue was section at 8 µm on a rotary microtome. The ribbon was cut into length about 75% of the available slide length to allow for expansion. The ribbon was floated on the warm water at 58°C.
- The ribbons were attached onto slide surface which has already mounted by Mayer's adhesive. The slides were placed horizontally for overnight in a 42°C oven.
- At the staining step the ribbon was soaked into Coplin jars which each contain:
 - a. Xylene, 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 (1% Safranin O in distilled 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 (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.
- coverslip was mounted with permount and the object ready to observe.

3.2.2. *In vitro* pollination

3.2.2.1 Equipment sterilization

Glassware, Petri dishes, forceps, Bunsen burner, dissecting needle, spatulas, scalpel were autoclaved for 60 min and laminar chamber was sterilized by running the UV light for at least 3 h. Laminar chamber was sprayed and cleaned by alcohol 95% before experiment was conducted.

3.2.2.2 Media preparation

Basal medium (see more detail on APPENDIX A) consisted of MS (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 NAA (auxin) singly or combination. Subculture medium callus induced supplemented with 0.5 mg L⁻¹ of BA and NAA singly or combination. The pH of each medium was adjusted to 5.7 ± 0.5 and autoclaved at 121°C for 15 min. All culture was incubated at $24 \pm 2^\circ\text{C}$ under a 16-hours photoperiod using cool, white fluorescent light.

3.2.2.3 Sample preparation and pollination

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

3.3 Protoplast fusion

The protoplast fusion technique was hybrids of two or more species, which could not be obtained by classical mating. Polyethylene glycol (PEG) was an effective and powerful fusogenic agent. Inter-generic or inter-specific fusion provides a method for the introduction and transfer of desirable genes. The procedure involves two discreet stages.

- Protoplast isolation
- Protoplast fusion

Protoplast isolation

The whole process of protoplast isolation is performed under sterile conditions. Detach fully expanded leaves (from 10 plantlets, 0.5 – 1 g). Cut them into small pieces with a sharp scalpel in one Petri dish ($\varnothing = 5$ cm) containing 5 – 6 ml of enzymatic solution (0.5% pectinase, 2% cellulose and 13% manitol at pH 5.4), pack the Petri dish with Parafilm. Incubate Petri dishes overnight in dark (14 – 16 h) in a thermostat at 25 °C. After this period, investigate the degree of plant tissue digestion under microscope. Transfer enzymatic solution with the plant tissue by a disposable serological pipette with open end onto a small sieve (75 μ m pore size) placed on Petri dish ($\varnothing = 5$ cm). Rinse the sieve with a sterile solution of 0.5 M sucrose while gentle stirring. By this step released protoplasts are separated from plant tissue debris. Divide protoplast suspension derived in this way into centrifugation tubes (8 ml) and overlay with 1 ml of buffer solution. Centrifuge (700 rpm, 12 min). Carefully take away floating protoplasts forming a ring on the interface of applied solutions by a Pasteur pipette and put into a new centrifugation tube, add required amount of buffer (5 ml)

and resuspend. Centrifuge (500 rpm, 6 min). Remove the supernatant with a Pasteur pipette, add buffer (2 – 5 ml) and resuspend the pellet.

Centrifuge (500 rpm, 6 min) and remove the supernatant with a Pasteur pipette.

Add culture medium to obtain required density 6×10^5 /ml. The number of protoplast was measured under light microscope.

The callus tissues used in these studies were routinely grown on agar media and were transferred to fresh media two weeks before use. One gram of callus was suspended in 6 ml of an enzyme mixture (0.5% pectinase, 2% cellulose and 13% manitol at pH 5.4). The reaction medium was incubated in 25 ml flask at 37 C for 15 hours and occasionally shaken by hand in order to free protoplasts.

Inter-generic protoplast fusion

The protoplast fusion by PEG requires sufficient amount of suitable plant material for protoplast isolation and their culture after fusion for obtaining plant regeneration. Protoplasts of the two fusion partners, at a density of 5×10^5 protoplasts per ml were mixed in equal proportion in a fusion solution containing 0.5 M mannitol and 0.5 mM CaCl_2 . Drops of 300 μl suspension containing 1.5×10^5 protoplasts were transferred into each Petri dish (5.5 cm diameter). For each fusion experience, 12×10^5 protoplasts were used (i.e. eight Petri dishes). After the protoplasts had settled, 4–6 drops of 50 μl each of PEG solution (10%, 20%, 30% and 50% PEG, 0.5 M mannitol and 0.5 mM CaCl_2) were slowly added. The protoplasts adhered to the Petri dish (15, 20 and 30 min) after polyethylene glycol treatment. The PEG-solution was gently diluted with 3 ml fusion solution. Ten minutes after dilution, the diluted PEG-solution were gently removed and replaced with liquid culture medium as described above. The fusion

process was observed microscopically to estimate the number of binary fusions. The fusions cells were culture in the culture medium.



CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Pollen Study

4.1.1 Fresh pollen and light microscopic observation

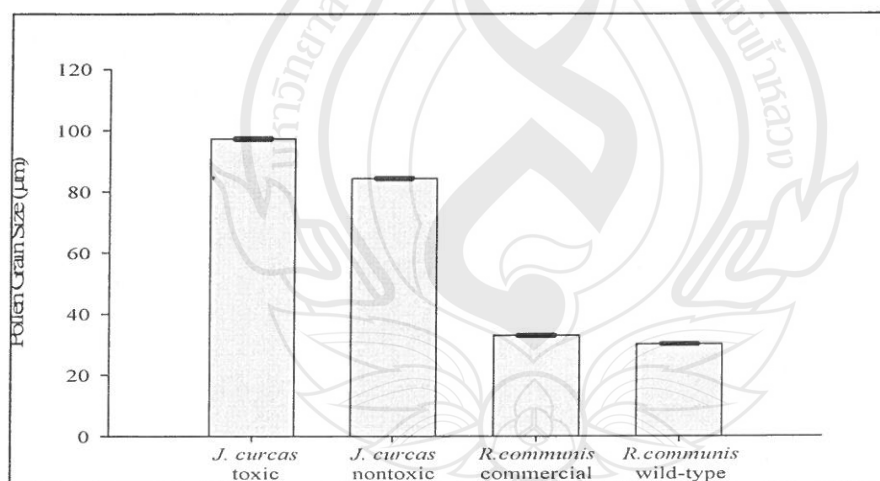
Observation under light microscope had shown some variation in *J. curcas* pollen grain size. The pollen grain of *J. curcas* toxic variety was the biggest among the four accessions. The diameter of pollen grains of toxic *J. curcas* and nontoxic *J. curcas* averages 96.01 μm (SD= 0.1620) and 84.26 μm (SD= 0.0075), respectively. There was significant difference between toxic varieties and non toxic varieties ($P>0.05$). 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 (Bhattacharya *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 size was bigger than Raju and Ezradanam (2002) have reported; the grain of *J. curcas* pollen is 89 μm , but the nontoxic variety was smaller.

Moreover, the presence of small grains (35-40 μm) compared to the average size was assumed 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 6. Pollen grains size

Plant origin	N	Average		
		1	2	3
<i>R. communis</i> wild-type	100	30.03 ^a		
<i>R. communis</i> commercial variety	100	32.84 ^a		
<i>J. curcas</i> nontoxic	100		84.26 ^b	
<i>J. curcas</i> toxic	100			96.01 ^c

Note: different letter indicate significant difference between accessions

**Figure 10.** Comparative size hydrated pollen grains.

The average of *R. communis* commercial varietie 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. There was no reciprocal cross because of incompatibles in *Jatropha* pollen graine size and female *Ricinus*.

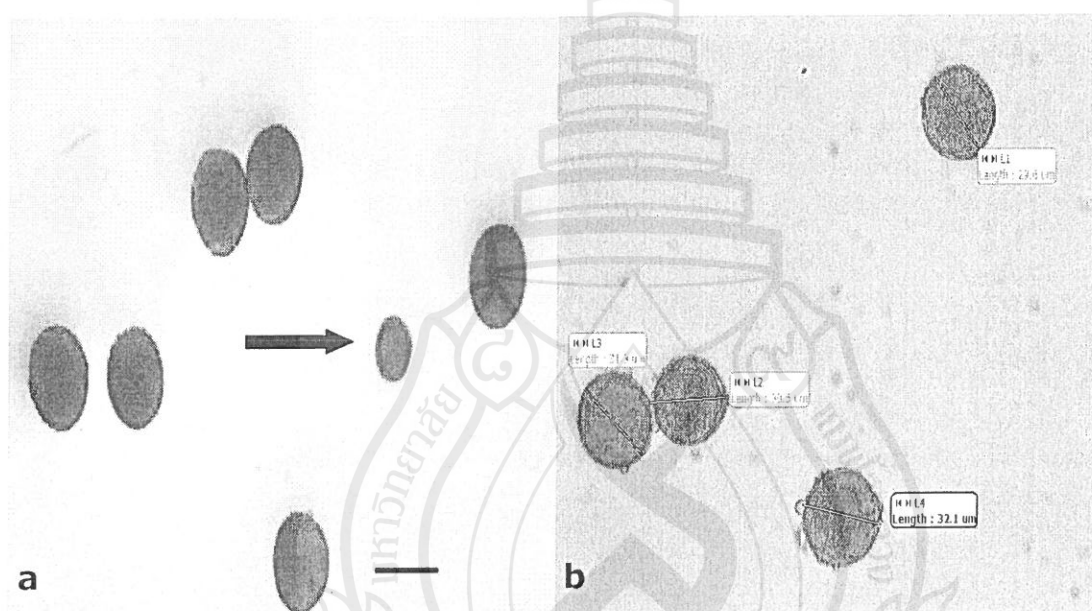


Figure 11. Hydrated pollen grains of *J. curcas* and *R. communis*; the arrow indicate sterile pollen in *J. curcas* (a), bar 100 μm and *Ricinus communis* commercial-type hydrated pollen grains (b), bar 50 μm .

Observation under light microscopy (LM) pollen grains of *J. curcas* and *R. communis* (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 like *R. communis*. *Jatropha 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 apertures, 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 aperture part both genera is also distinguishable from the pictures.

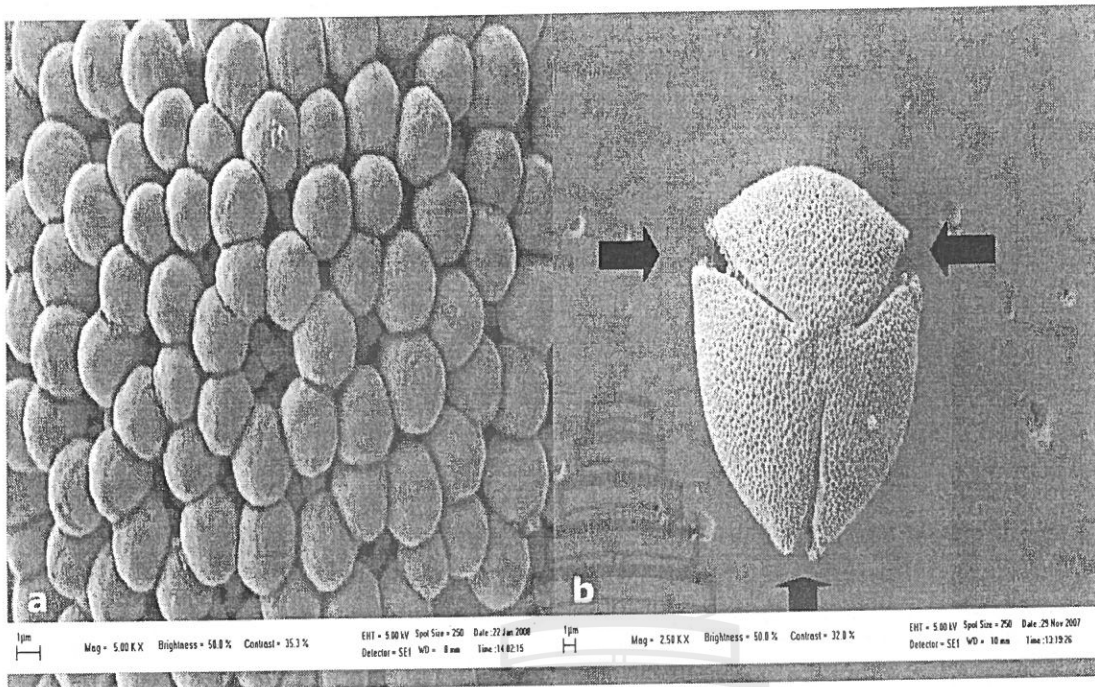


Figure 12 Apertures in the pollen exine layer. *Jatropha curcas* inaperturate grain (a) an three arrows show of *R. communis* tricolporate or posses three apertures (b).

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).

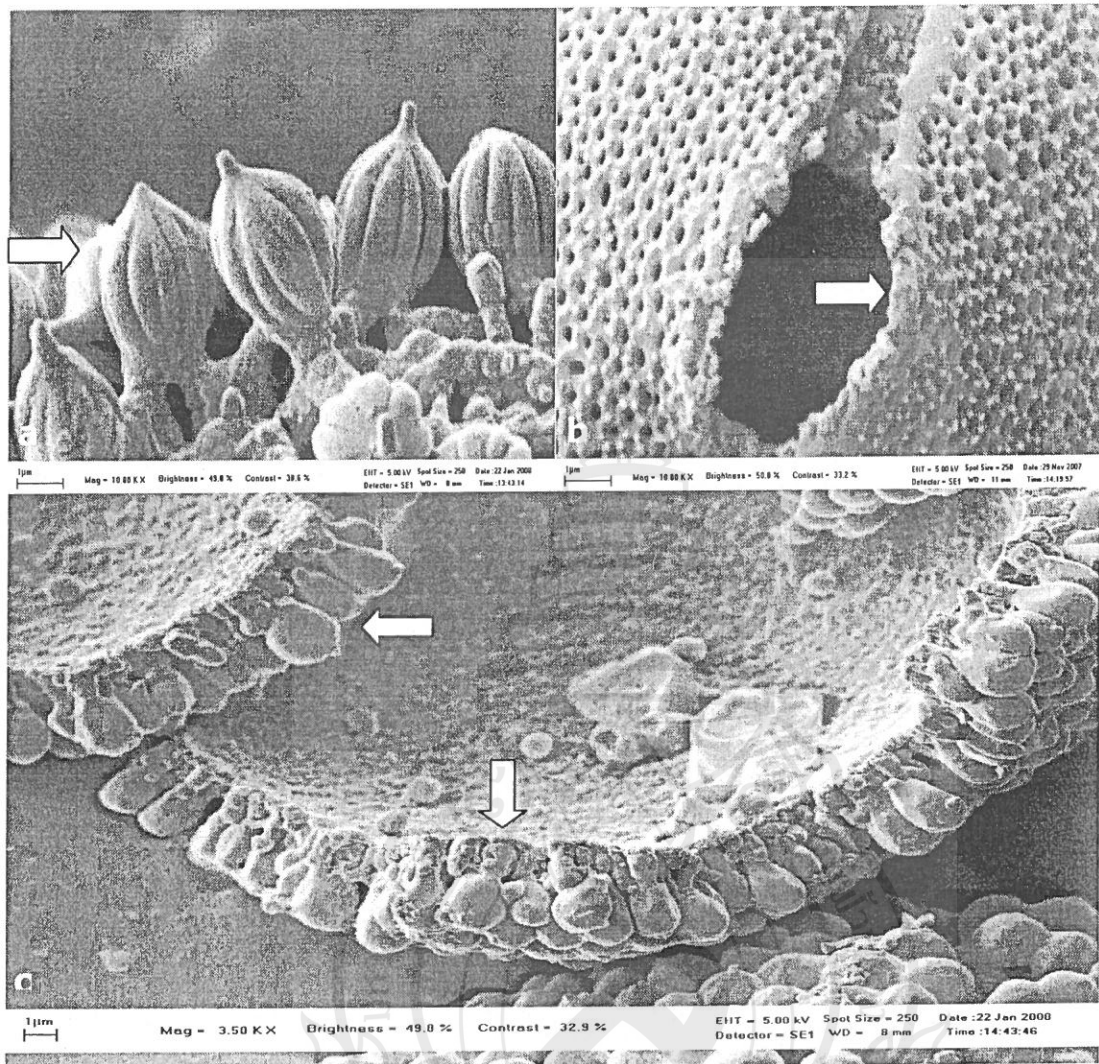


Figure 13 Small spinules in exine layers of *J. curcas* (a) and small dots in exine of *R. communis* (b). Fig. c top arrows show the exine part and bottom arrow show intine part of *J. curcas* pollen.

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 aperture 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 was 1.4-1.9 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).

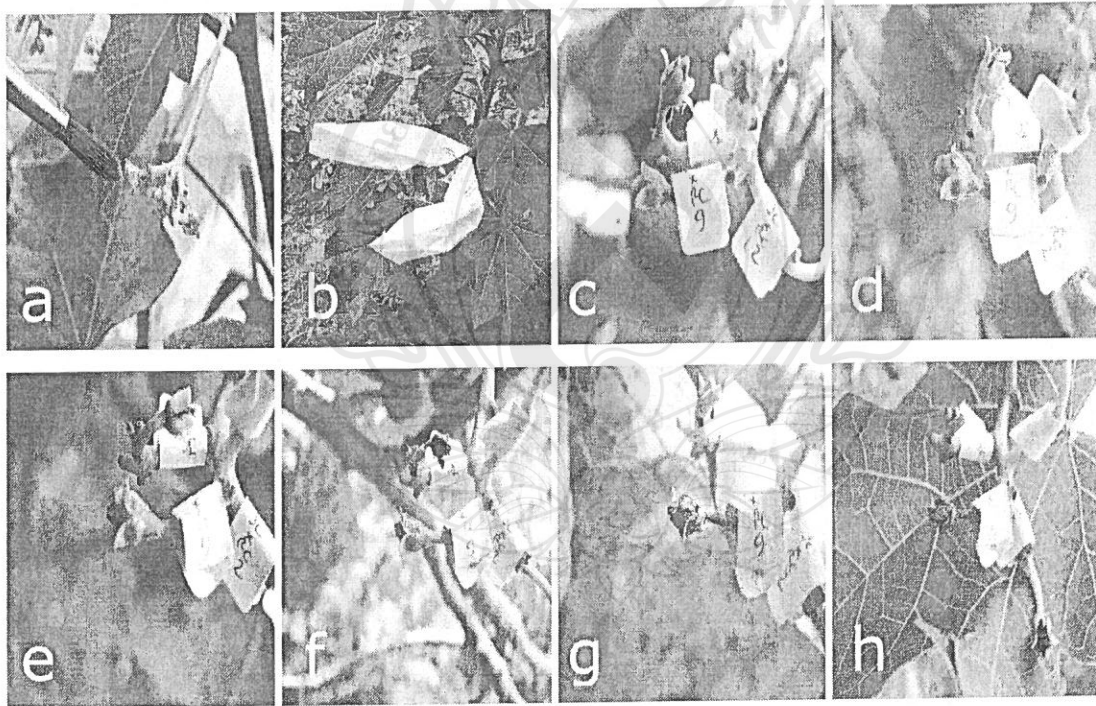


Figure 14 *Jatropha curcas* female flowers after pollination. Flower still remained healthy for 3 days (c), 5 days (d) and 7 days (e). Flower turned yellow in 9 days (f), brown in 11 days (g) and was dry in 13 days after pollination (h).

Table 7 Hand-pollinate field *Jatropha curcas*

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

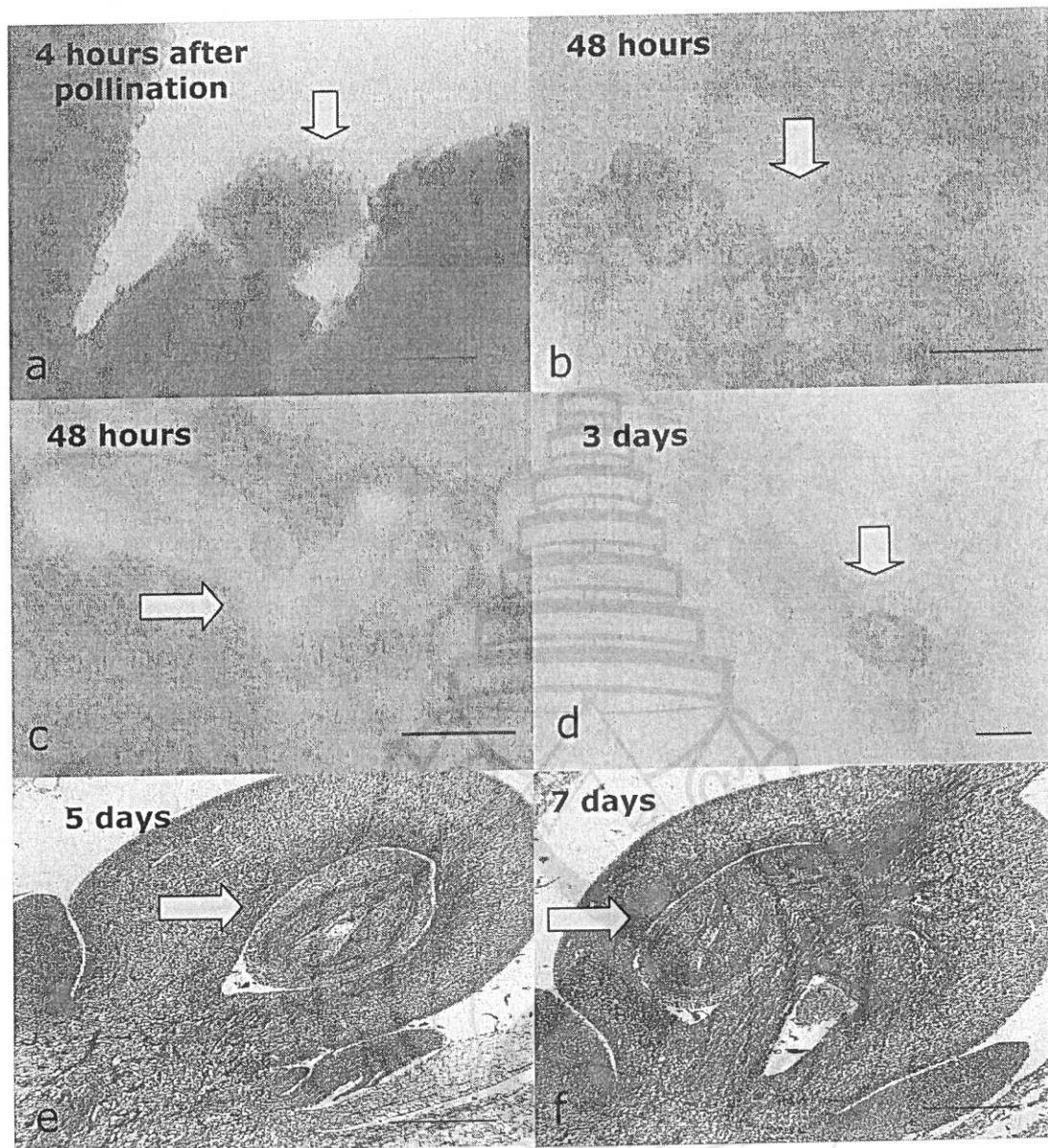


Figure 15 Pollen tube growth and hybrid zygote development. *Ricinus* pollen grains on *Jatropha* stigma 4 hr after pollination, bar 150 μm (a). Pollen tube germinating in the placenta 48 hours after pollination, bar 60 μm (b&c). Hybrid zygote assumed developed in embryo sac 3rd day after pollination, 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.

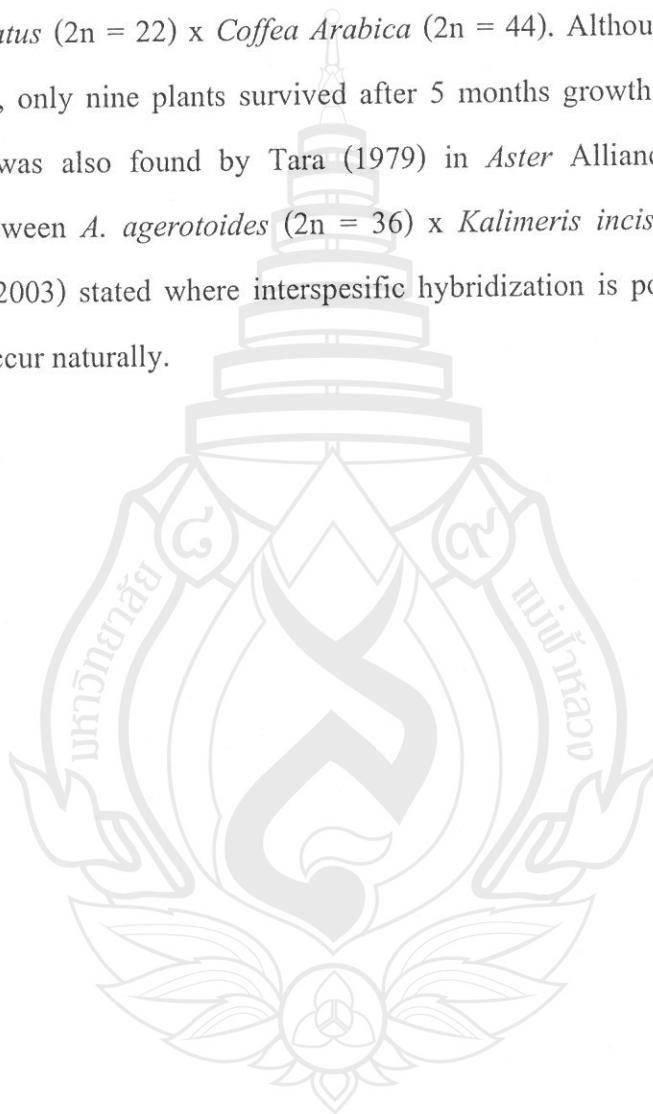
Even though the zygote presented (figure 4.6 d, e and f) in 3 days and developed in 7 days after pollination, but it seemed failed to set the seed more than 9-10 days. The endosperm development retarded more than 10 days is often the cause of spontaneous abortion of hybrid embryos after interspecific 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 interspecific 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 interspecific 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* interspecific 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 interspecific 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 interspecific 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).

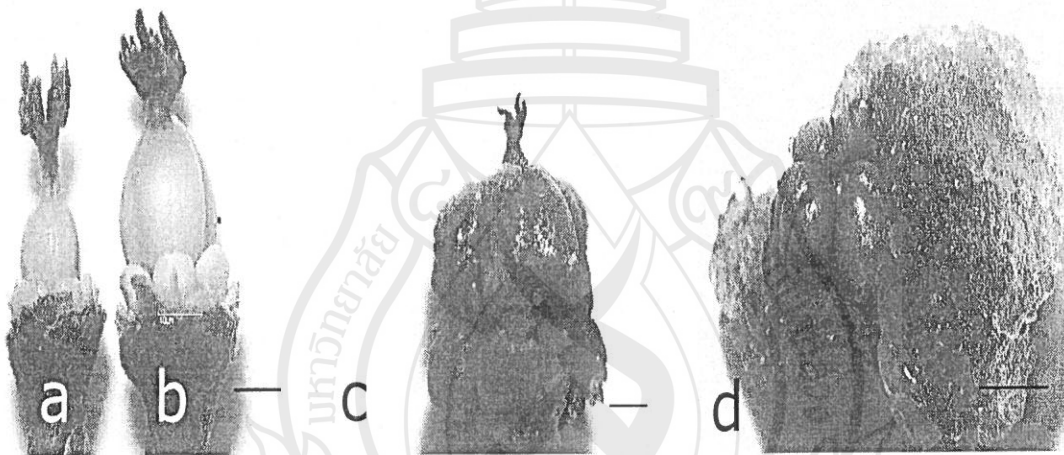


Figure 16 *Jatropha curcas* nontoxic (a) flower and toxic (b) varieties, bar 10 μm . *In vitro* pollinated toxic flower (c) in 57 days in media culture 0.125 mg/L BA, bar 5 μm . *Jatropha curcas* flower induced to callus (0.5 mg/L BA+NAA) after 3 week after *in vitro* pollination (d) bar 10 μm .

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

reputed yet. Two varieties of *J. curcas* in MFU germplasm 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 from the somatic cell of *Jatropha*. Richard and Rupert (1980) noted fertilization and partial embryo development occurred in interspecific 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 Seed physical characteristics and oil analysis

4.3.1 Seeds physical characteristics

Table 4.3 shows the maximum of seed length (18.64 mm) obtained from *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 because both *Jatropha* species has grown in the same environment.

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. communis* 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%.

Table 8 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* (%)
<i>J. curcas</i> toxic	17.49 ^a	11.21 ^a	8.41 ^a	73.17 ^b	5.9	53.03 ^c
<i>J. curcas</i> nontoxic	18.64 ^a	10.99 ^a	8.72 ^a	65.43 ^a	5.2	39.47 ^b
<i>R. communis</i> commercial	14.30 ^b	8.95 ^b	6.01 ^b	50.83 ^c	3.4	27.03 ^a
<i>R. communis</i> 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

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.

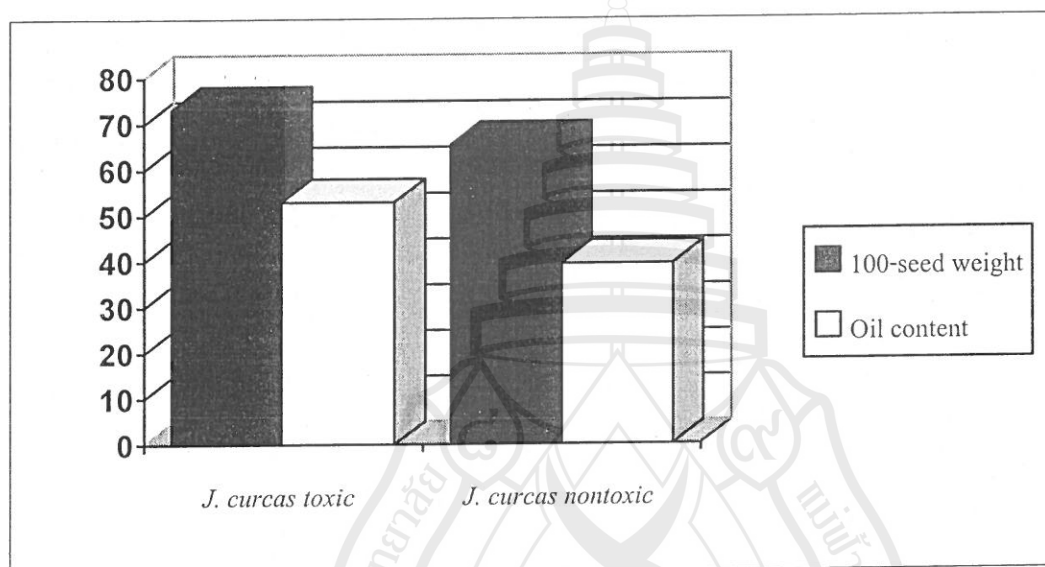


Figure 17. Average of 100-seed weight (g) and oil content (%) of *J. curcas*.

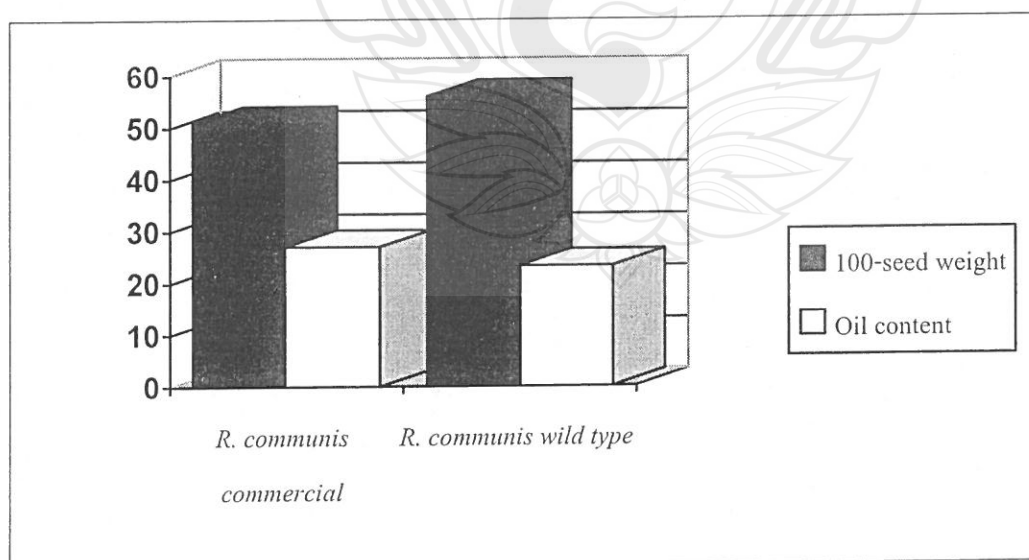


Figure 18. Average of 100-seed weight (g) and oil content (%) of *R. communis*.

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).

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 hydroxylate 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 hydroxylated 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°C, higher compressibility than other vegetables oils and hygroscopicity. 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 biodiesel 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 due 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.

4.3.3 Protoplast fusion

Protoplast Isolation

Protoplast isolation is influenced by several parameters, particularly the plant genotype, the source tissue and its physiological status, together with environmental factors (Davey, 2004). The result show a greater yield of protoplast isolating from a young leaf is than the isolation of protoplasts from the young callus cell (Table 9). By observing under the light microscope, protoplast cell is noticed by the round shape of the cell. As shown in Figure 4.8, the protoplast of *Jatropha* (a) and *Ricinus* (b) was obtained from standard extraction protocol with minor modification.

Table 9. Isolation of protoplast from young leaves and callus tissues of *J. curcas* and *R. communis*.

Species	Time of incubation (hr)	No. of released protoplast (ml)
<i>J. curcas</i> expanding leaves	Overnight (18 hr)	2.3×10^4
<i>J. curcas</i> mature leaves	Overnight (18 hr)	1.2×10^4
<i>J. curcas</i> callus	Overnight (18 hr)	0.6×10^4
<i>R. communis</i> expanding leaves	Overnight (18 hr)	3.3×10^4
<i>R. communis</i> mature leaves	Overnight (18 hr)	1.3×10^4
<i>R. communis</i> callus	Overnight (18 hr)	0.5×10^4

The isolated protoplasts usually show vigorous cyclosis. The stability of isolated protoplasts varies with plant species. Some of *Jatropha* protoplasts readily survived overnight, while the *Ricinus* protoplasts often survived a few days or more.

However the results of these studies do not represent the best obtainable results for each species, since the proper age and physiological stage of the plant materials are scarcely controlled during the experiment. On the other hand, the conditions of enzyme mixture optimal for isolation of protoplasts may be diverse among species. Therefore, more trialed examination in this respect of individual species should lead to improve in the yield of protoplasts.

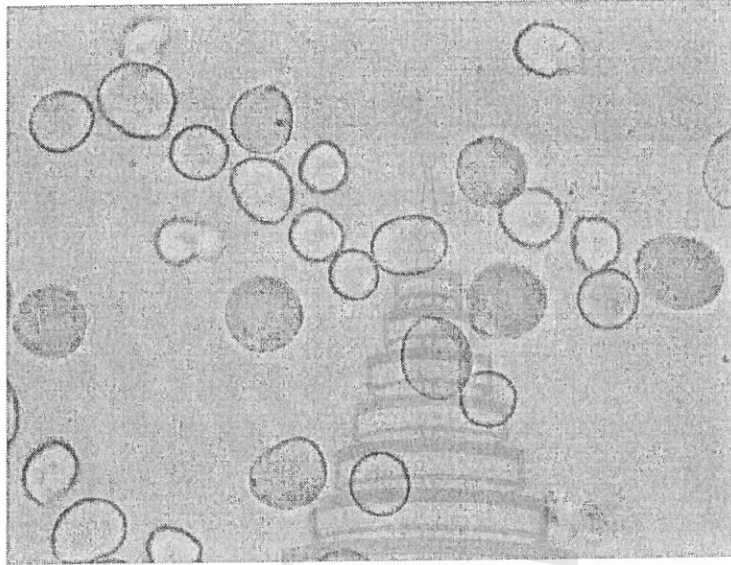


Figure 19 (a) Protoplast cell of *Jatropha curcas*

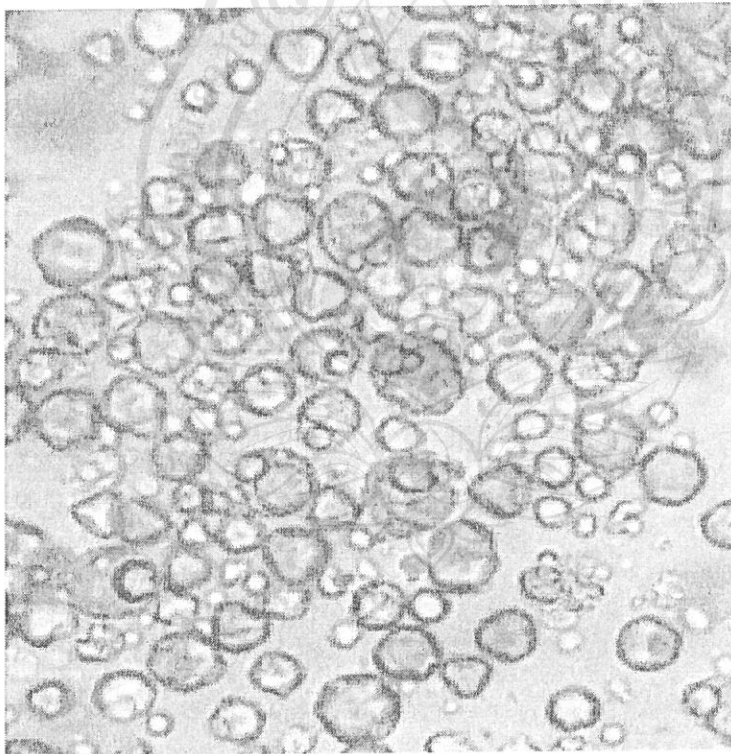


Figure 19 (b) Protoplast cells of *Ricinus communis*

Protoplast fusion results

As shown in Figure 20a and 20b, the multiple fusion and binary fusion were obtained. With the variation of 10%, 20%, 30% and 50% of PEG solution, 10% PEG shows the best result of binary fusion. Regarding the fusion efficiency the average of binary fusion was 60% of total cell. However, the rate of fusion is low (less than 50%). This is due to several factors such as the enzyme mixture, the fusion agents, the ratio between protoplasts belonging to each partner and the culture media could all have an influence on the results. Due to its toxicity, PEG is shown to affect protoplast viability during fusion period. A PEG concentration higher than 30% led some protoplast damage. The protoplast fusion experiment needs further investigation.

Somatic hybridization could be an excellent tool for the intergeneric breeding. There are very few reports of the success intergeneric protoplast fusion. This work is the first attempt for the cybridization between *Jatropha* and *Ricinus*. Unfortunately, PEG treatment seemed to affect the regeneration of hybrid cells. The electro fusion should be investigate in the next experiment.

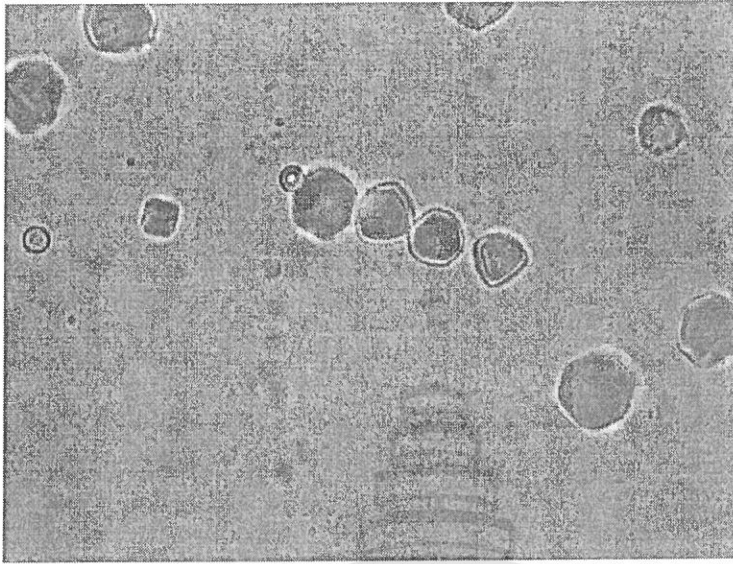


Figure 20 (a) a multiple fusion of *Jatropha curcus* and *Ricinus communis*

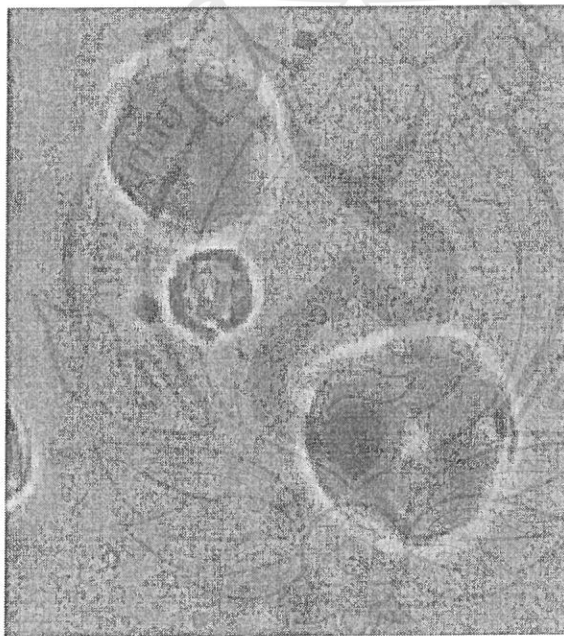


Figure 20 (b) a binary fusion of *Jatropha curcus* and *Ricinus communis*

CHAPTER 5

CONCLUSIONS

This is the first report on intergeneric hybridization between *J. curcas* x *R. communis* through *in vivo* and protoplast fusion techniques. 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. *In vivo* pollination test shows the abortion in all pollinated flowers in two weeks indicating their intergeneric incompatibility. *In vitro* pollination test shows that the flowers can survive four weeks longer than *in vivo* test. Incompatibility between *J. curcas* and *R. communis* shows lack of pre zygotic barriers. The incompatibility caused by post zygotic barriers. A yield of protoplast cell of *Ricinus* and *Jatropha* isolating from young leave is higher than the isolation from young callus cell. The best number of binary fusion was obtained from 10% PEG.

Further work relevant to investigation intergeneric cross include: Embryo rescue techniques need to be established before the zygote abortion. The fusion technique still needs a number of adjustments to increase the viability of heterokaryons and their further evolution to microcalli. Somatic embryogenesis techniques need to be established.

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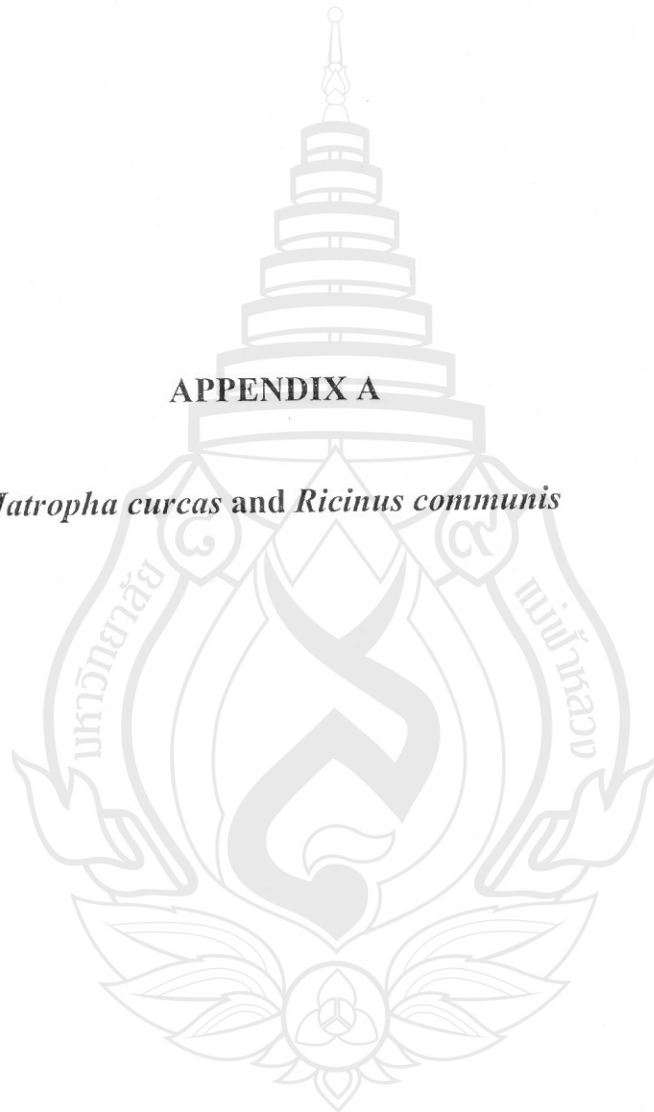
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APPENDIX A

Jatropha curcas and *Ricinus communis*



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

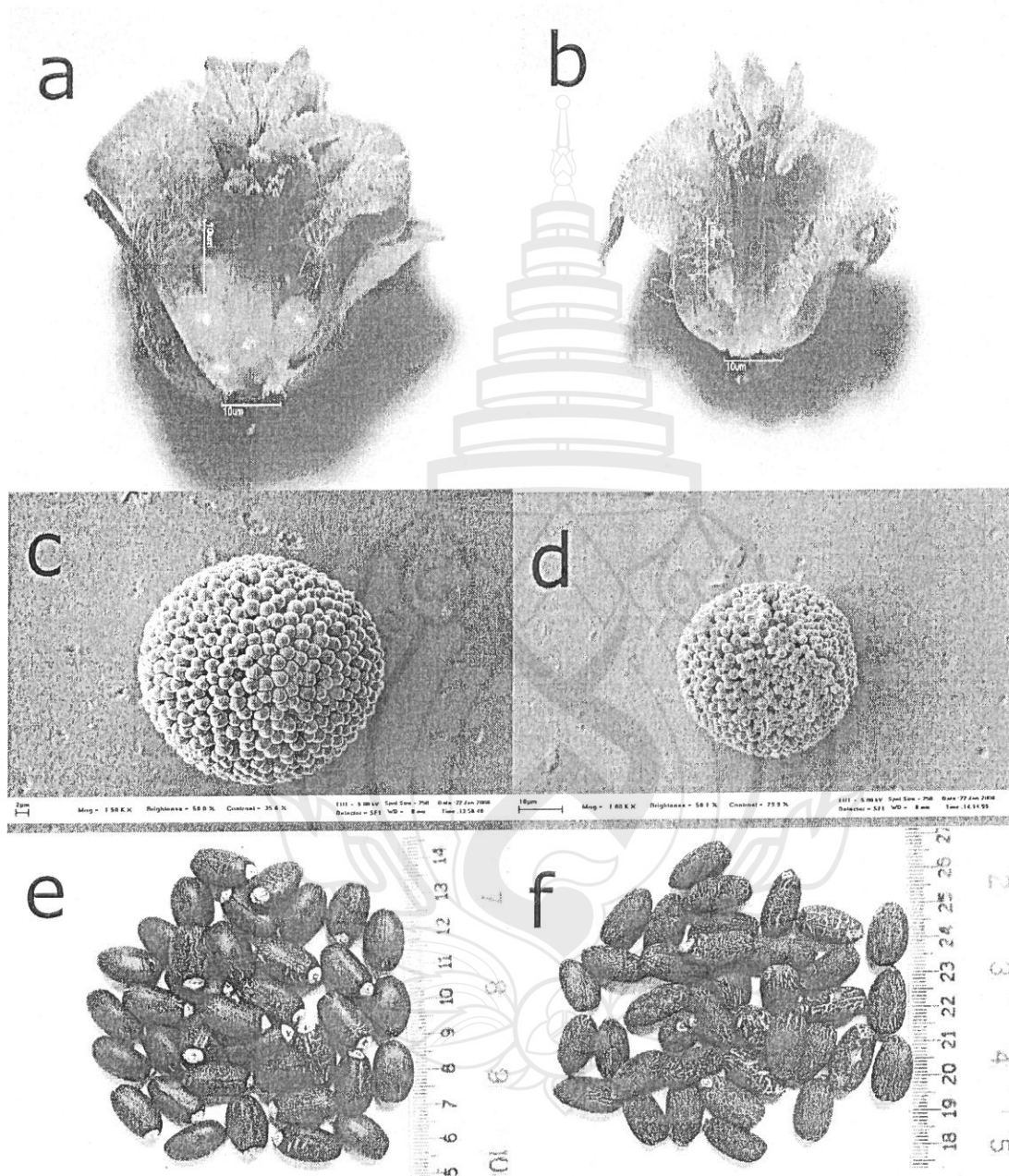


Figure A1. *Jatropha curcas* toxic variety; a. male flower c. pollen (inaperture) d. seed (smooth skin). *J. curcas* nontoxic variety; b. male flower d. pollen (inaperture) f. seed (rough skin)

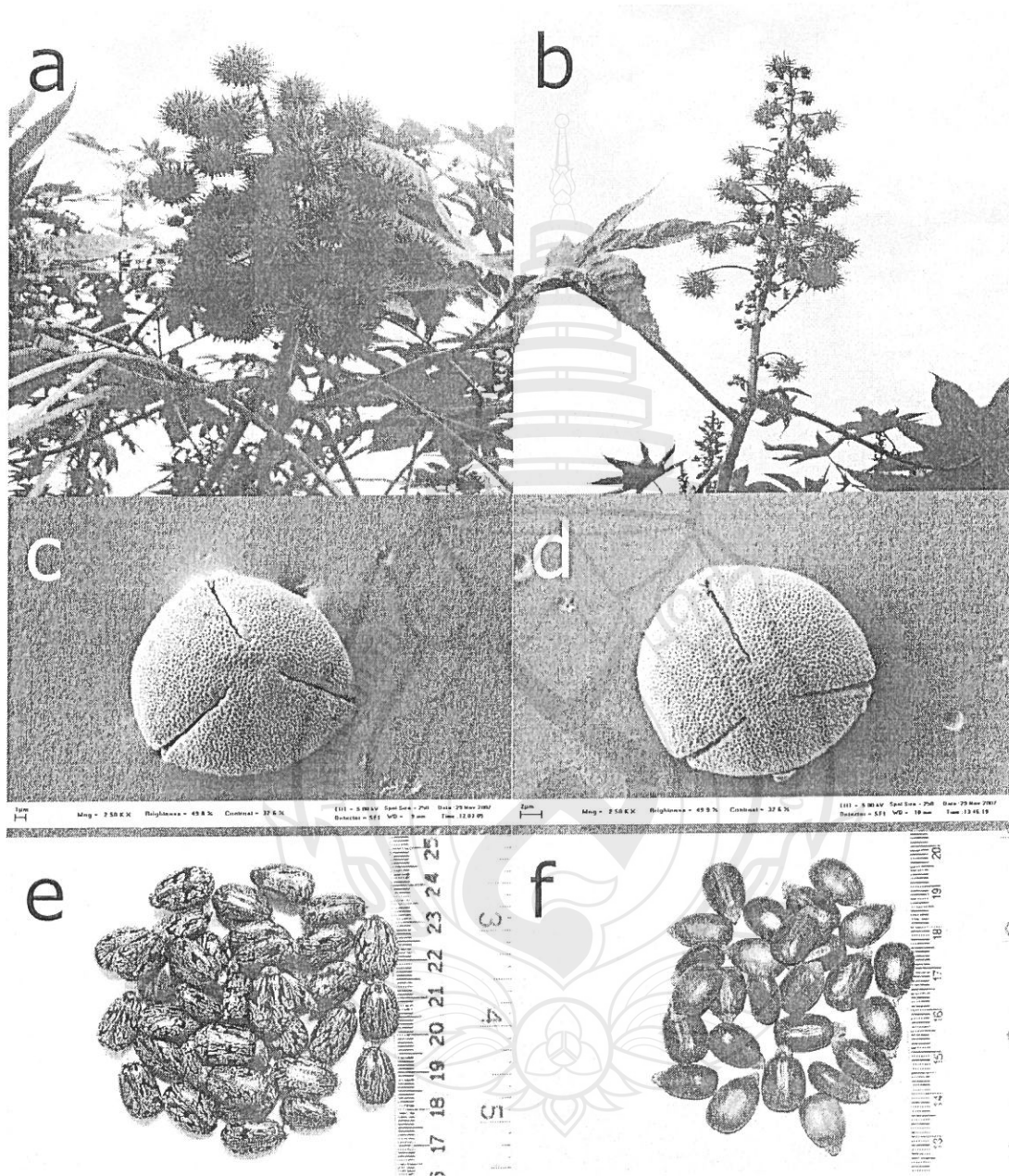
A2. *Ricinus communis* wild-type in MFU germplasm

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

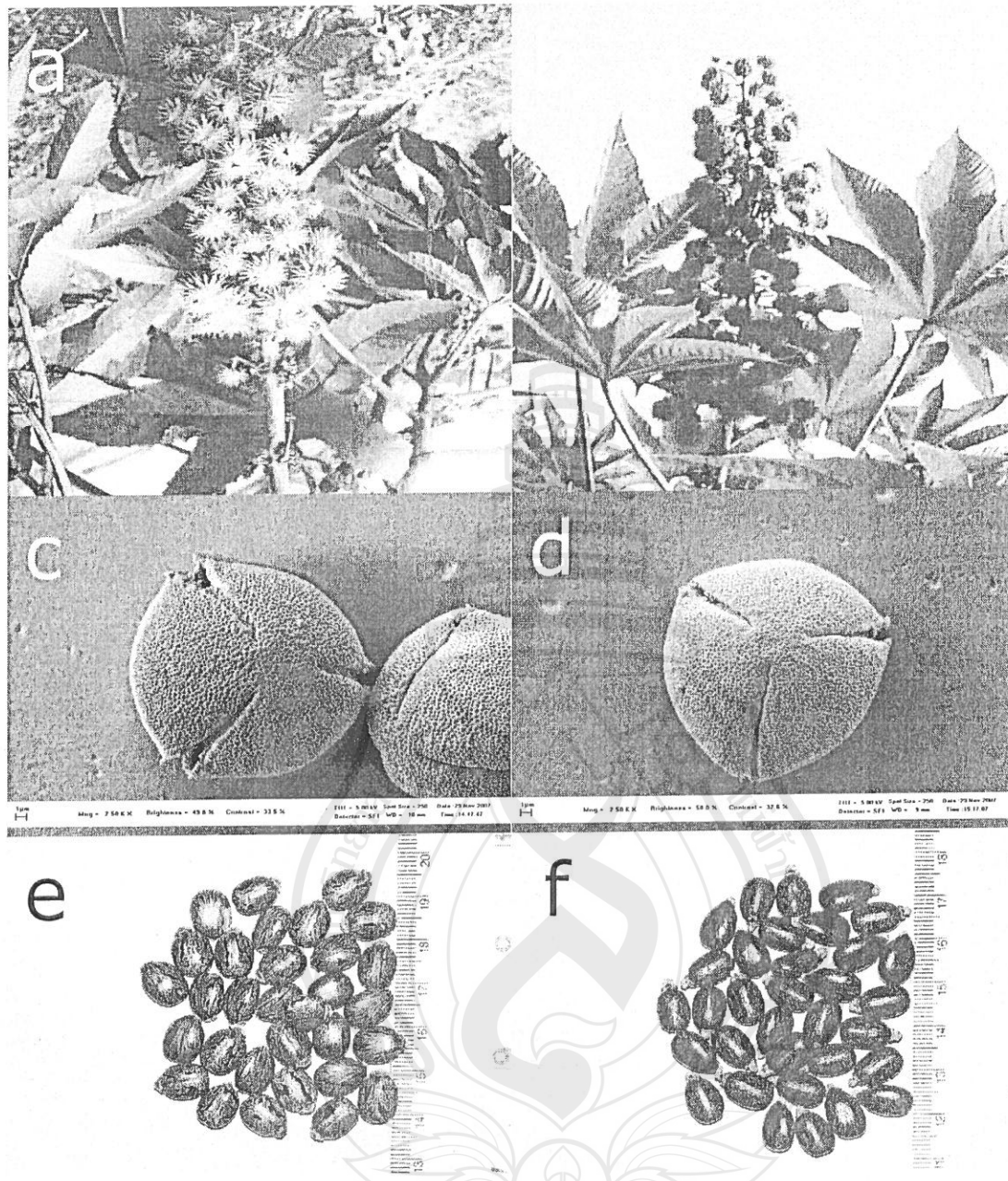


Figure A3. *Ricinus communis* wild-type C; a. cylindrical spike and very compact c. tricolporate pollen e. seed: small size and brown. *Ricinus communis* wild-type D; a. cylindrical spike and semi compact c. tricolporate pollen e. seed: medium size and brown.

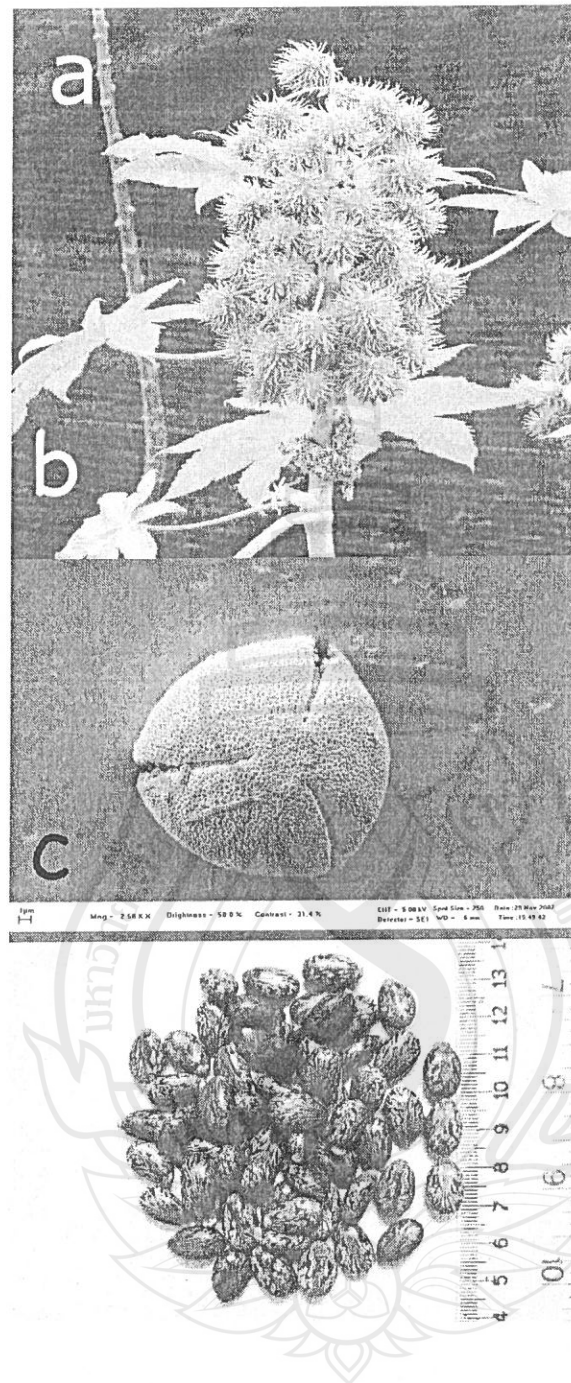


Figure A3. *Ricinus communis* commercial-type; a. umbrella spike and compact c. tricoloporate pollen e. seed: medium size and brown.

APPENDIX B

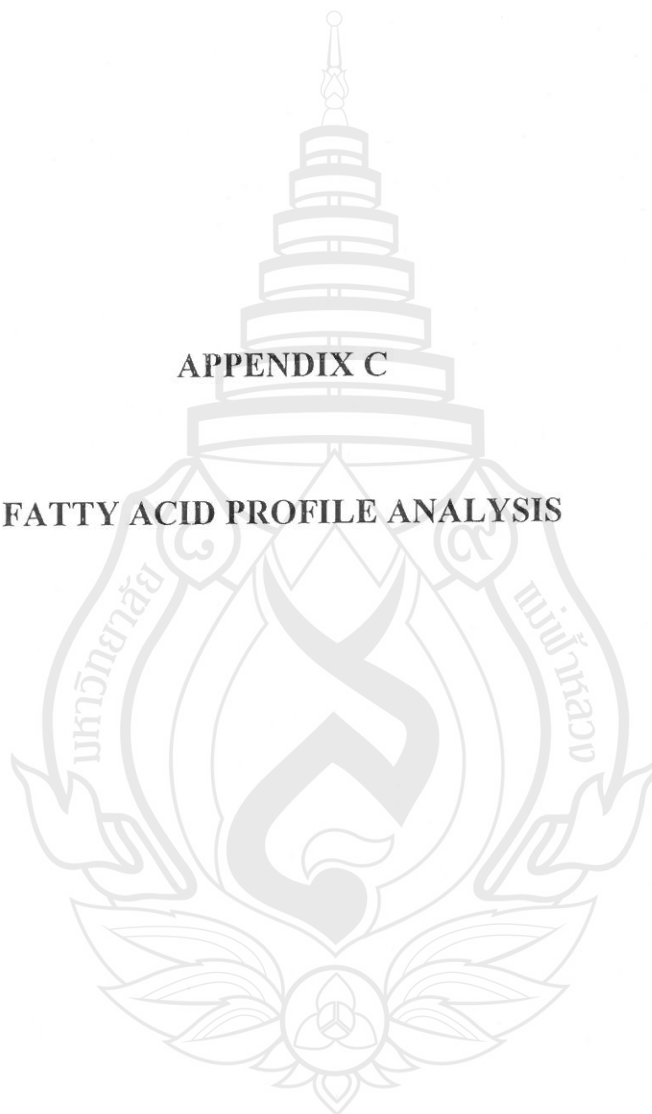
MEDIA COMPOSITION

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

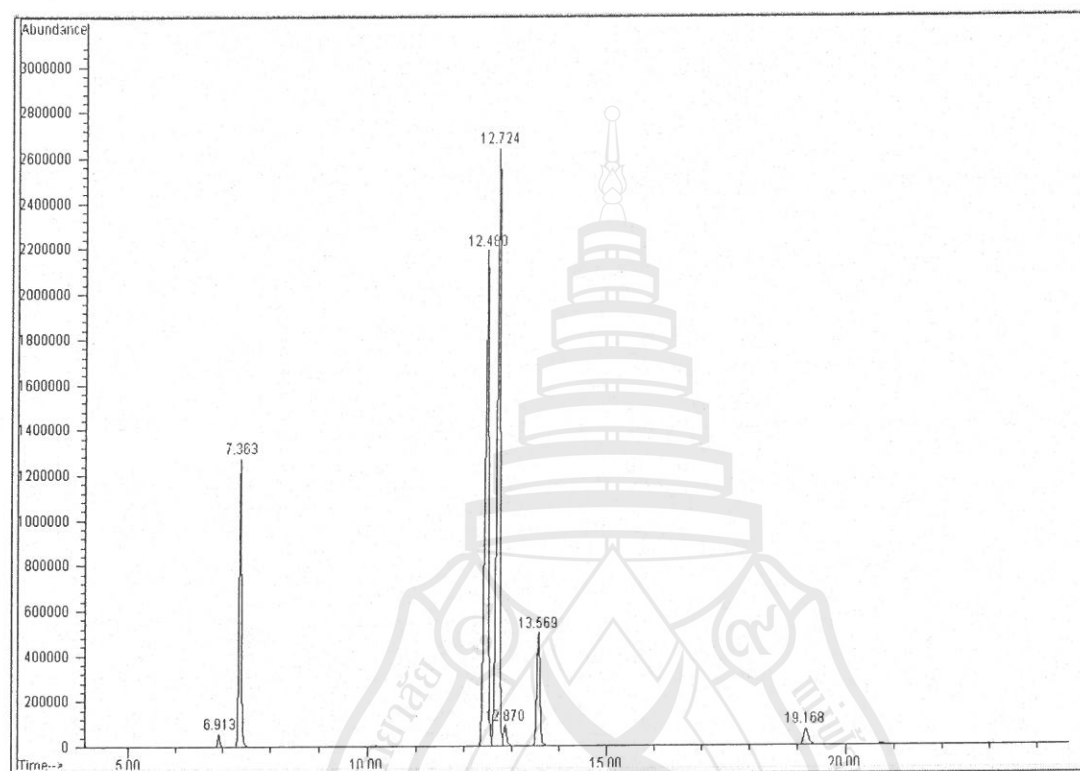
Chemical compounds	Supplier	Concentration in stock solution (mg/l)	Concentration in medium (ml/l)
MS Major Salts			20
Potassium nitrate (KNO_3)	UNIVAR, Australia	50	
Ammonium nitrate (NH_4NO_3)	UNIVAR, Australia	82.5	
Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	UNIVAR, Australia	22.0	
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 ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$)	UNIVAR, New Zealand	0.025	
Cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$)	UNIVAR, New Zealand	0.0025	
Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	UNIVAR, New Zealand	37.0	
Manganese sulfate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$)	UNIVAR, New Zealand	2.23	
Zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	UNIVAR, New Zealand	0.86	
Cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	UNIVAR, New Zealand	0.0025	
EDTA sodium ferric salt			5
EDTA	FLUKA, Switzerland	7.45	
Ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	FLUKA, Switzerland	5.57	
MS vitamins			10
Glycine (recrystallized)	Fisher scientific, USA	0.2	
Nicotinic 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

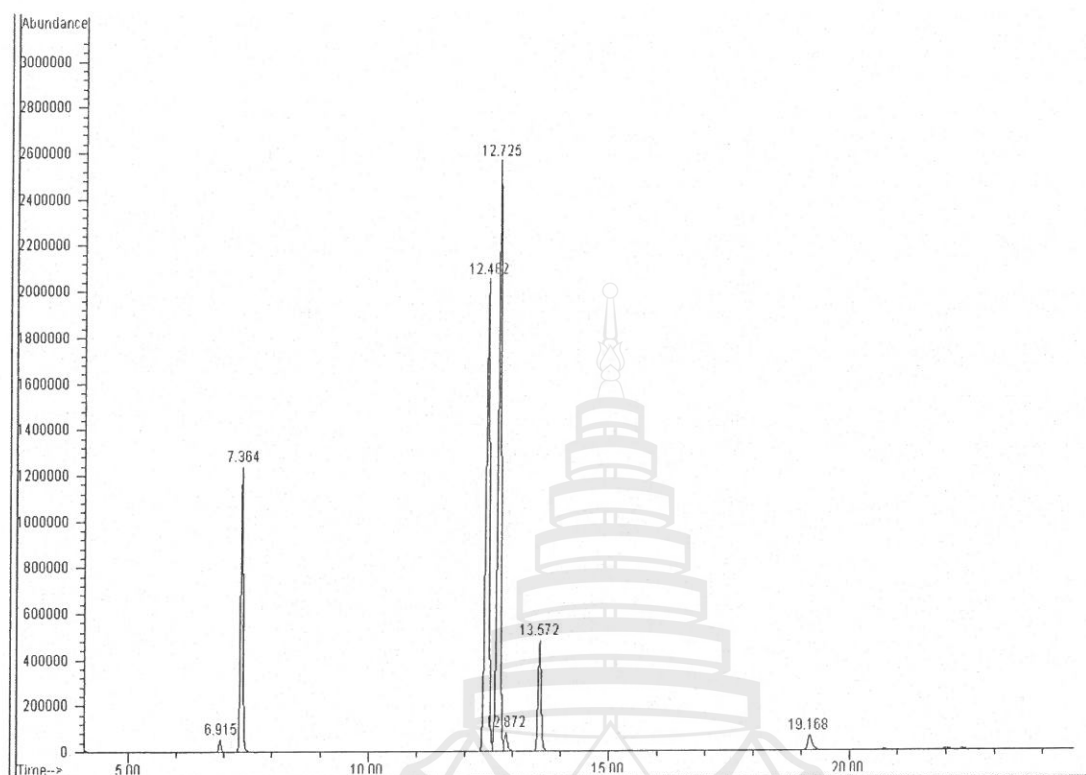


Figure C1.2. Chromatogram of fatty acid from *J. curcas* toxic variety (replication 2)

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

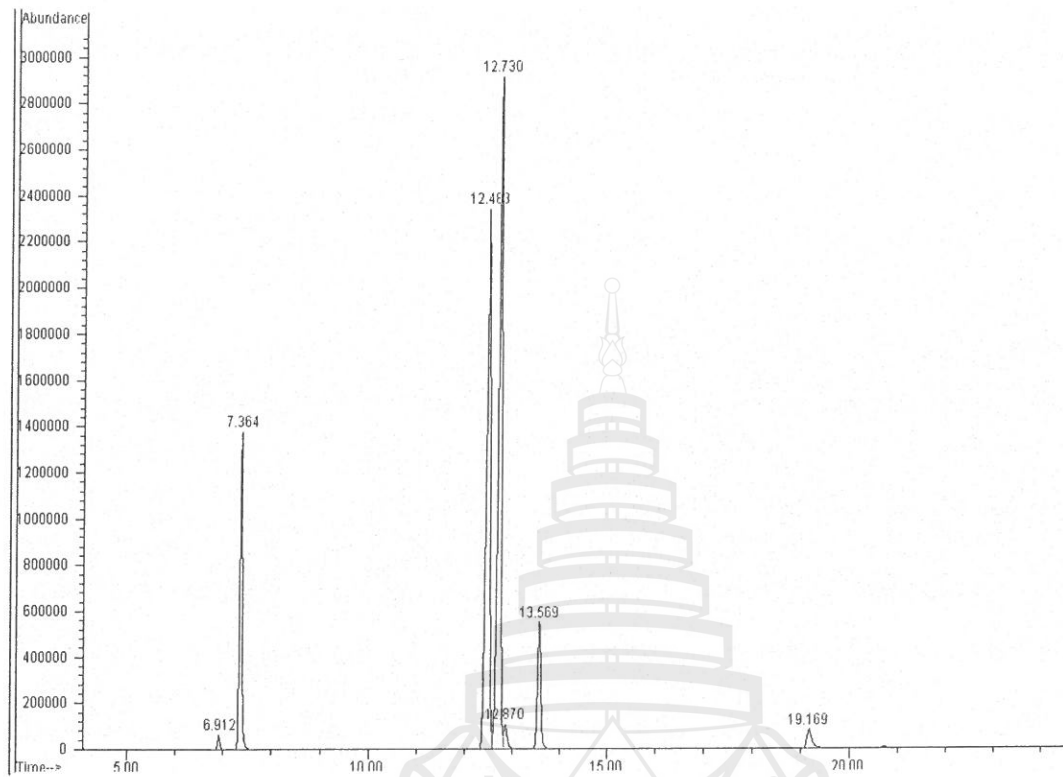


Figure C1.3. Chromatogram of fatty acid from *J. curcas* toxic variety (replication 3)

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

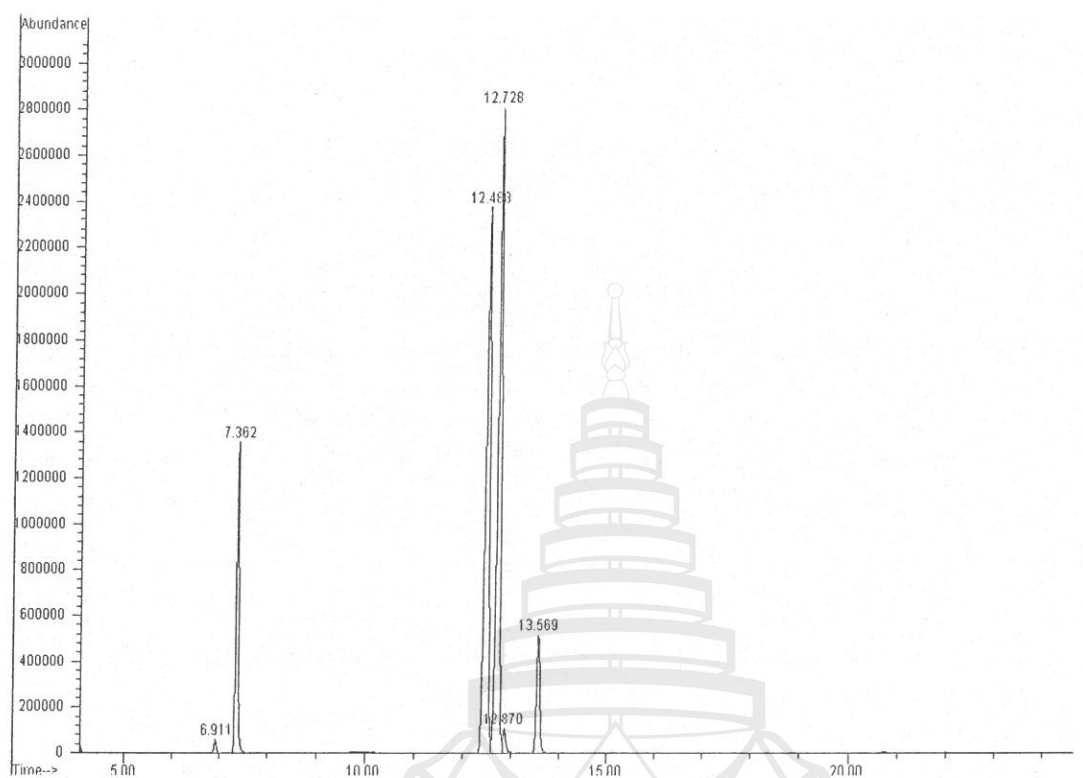


Figure C1.4 Chromatogram of fatty acid from *J. curcas* nontoxic variety (replication 1)

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

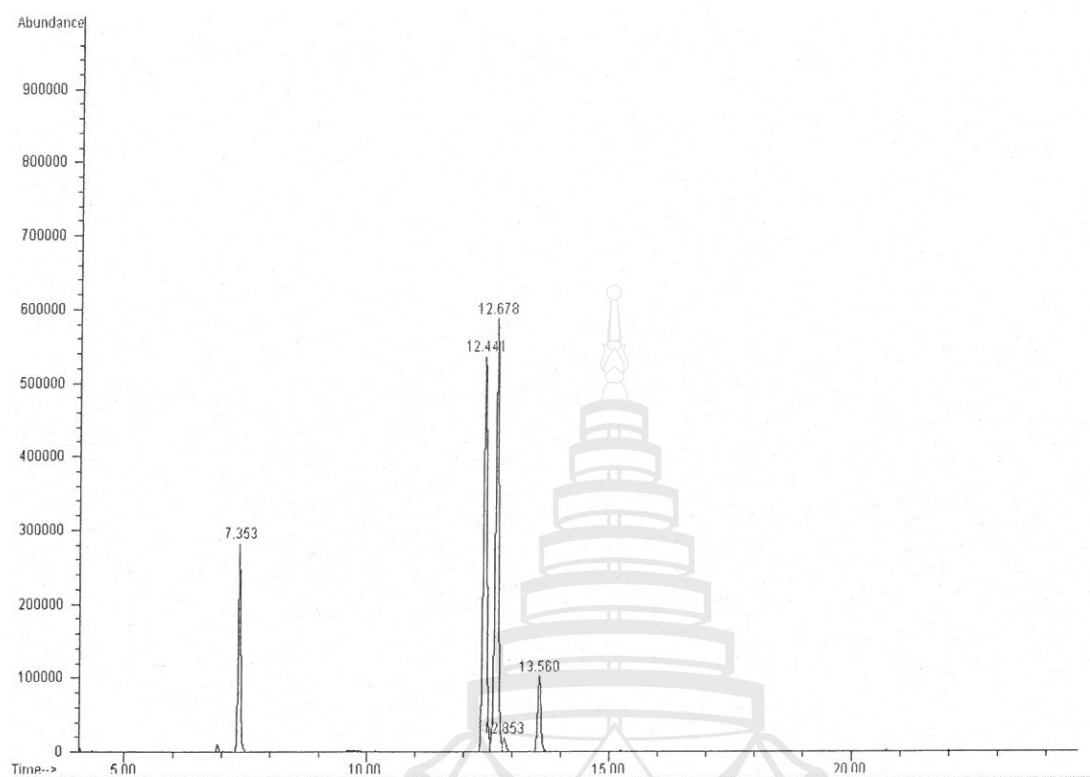


Figure C1.5 Chromatogram of fatty acid from *J. curcas* nontoxic variety (replication 2).

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

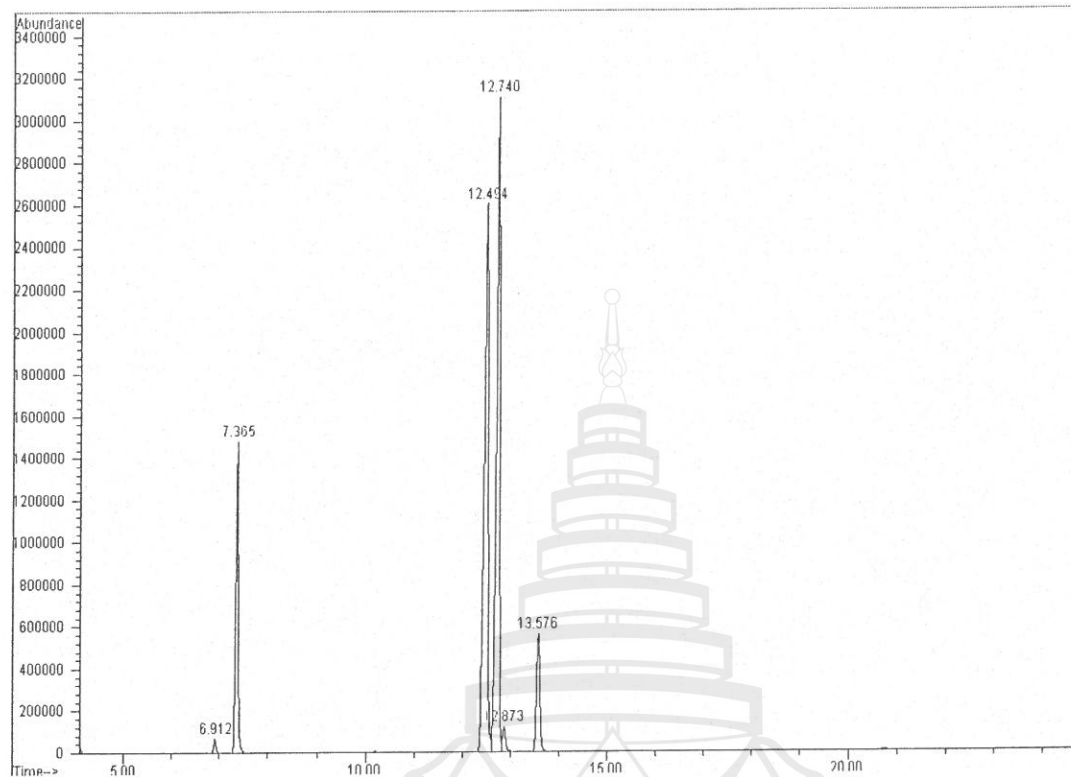


Figure C1.6 Chromatogram of fatty acid from *J. curcas* nontoxic variety (replication 3).

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

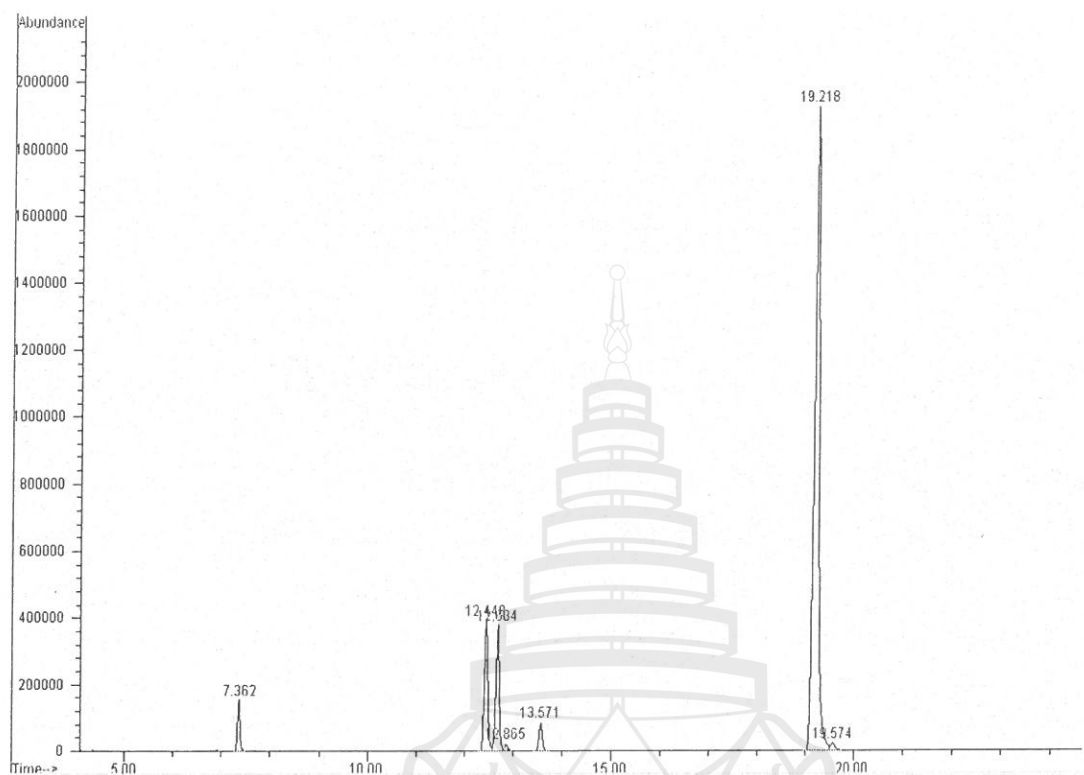


Figure C1.7 Chromatogram of fatty acid from *R. communis* commercial-type (replication 1).

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

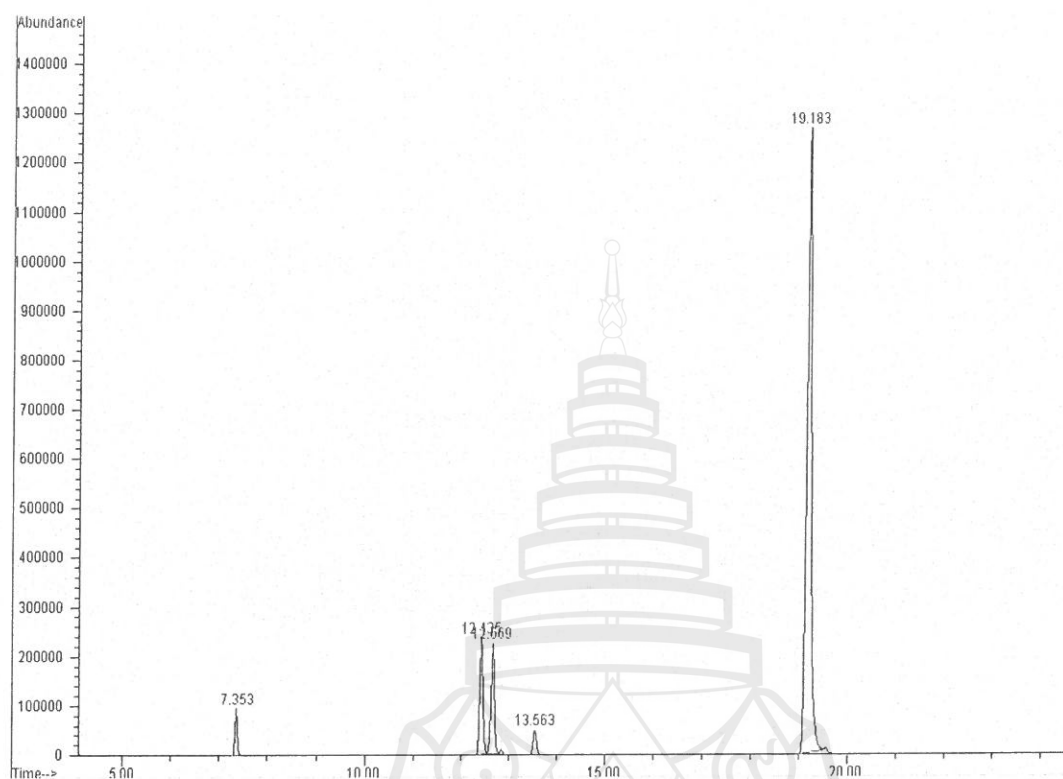


Figure C1.8 Chromatogram of fatty acid from *R. communis* commercial-type (replication 2).

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

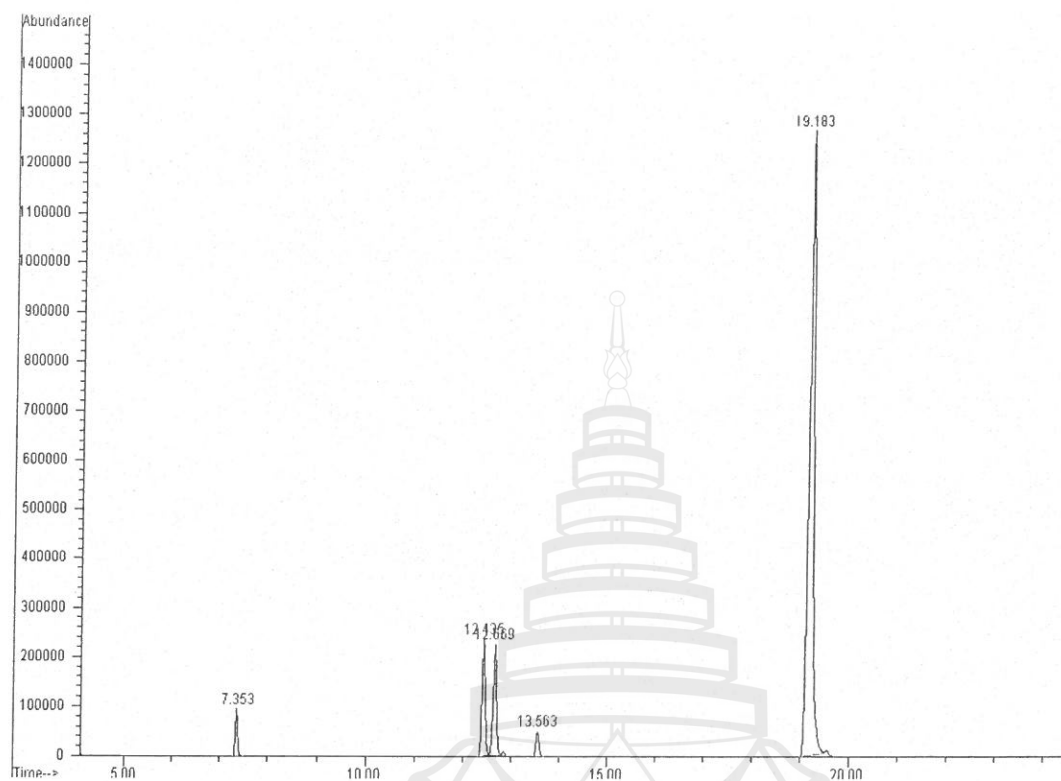


Figure C1.9 Chromatogram of fatty acid from *R. communis* commercial-type (replication 3).

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

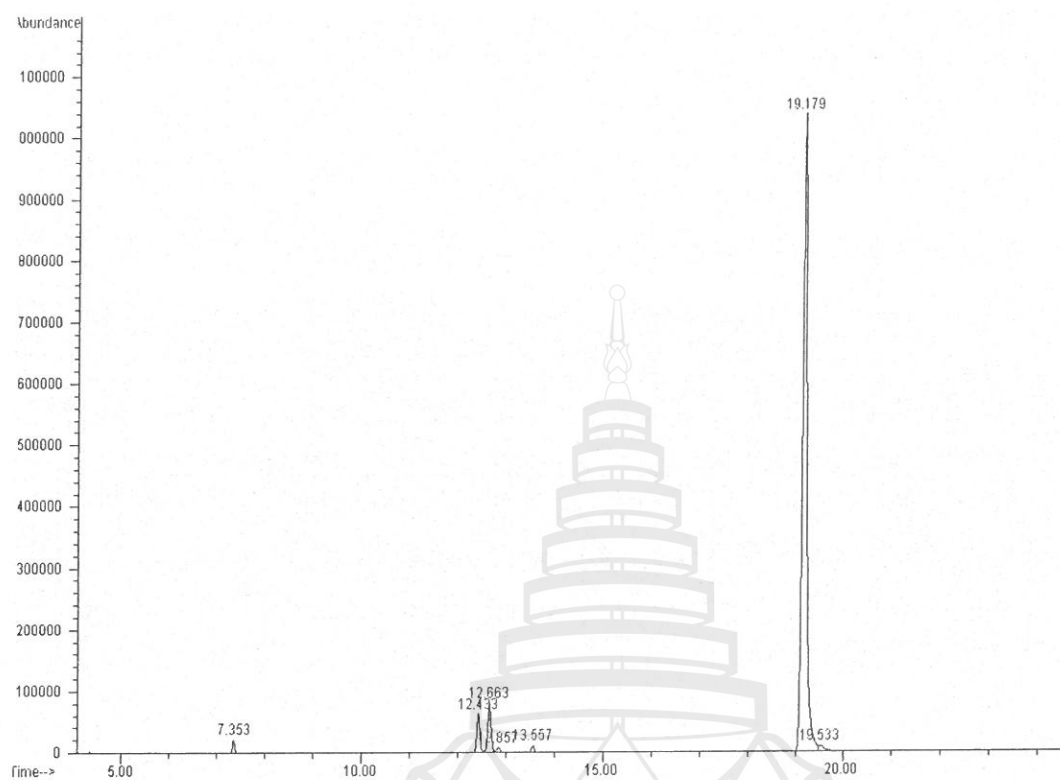


Figure C1.10 Chromatogram of fatty acid from *R. communis* wild-type A (replication 1).

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

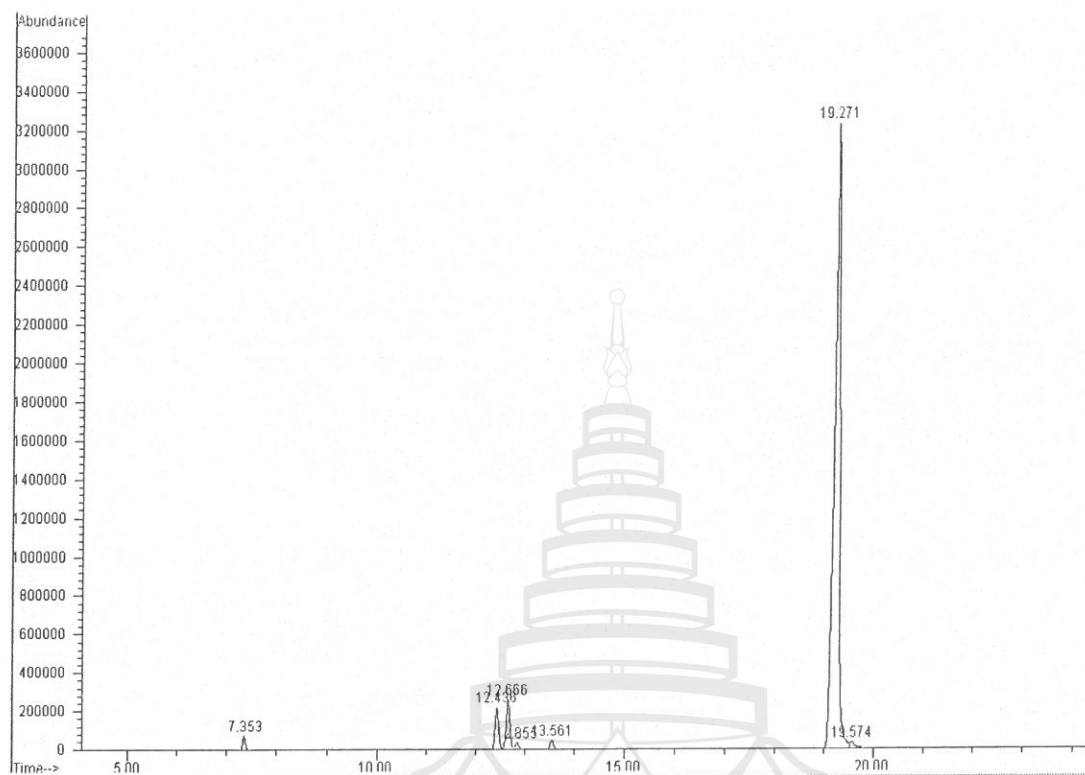


Figure C1.11 Chromatogram of fatty acid from *R. communis* wild-type (replication 2).

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

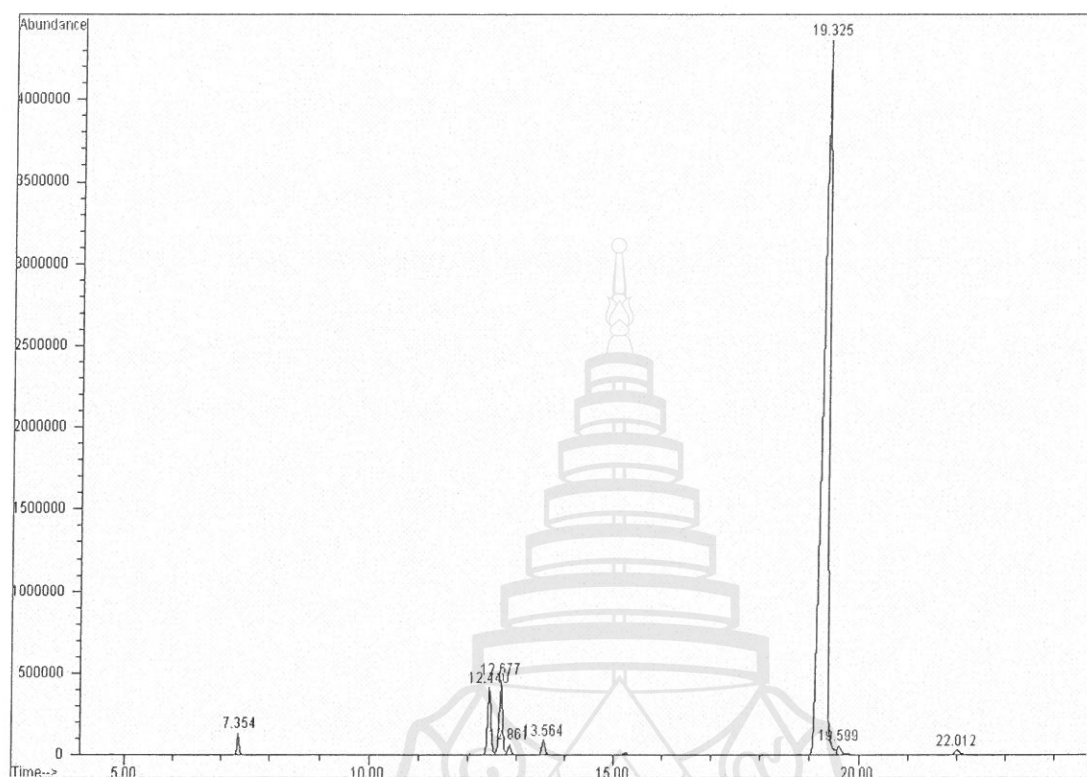


Figure C1.12 Chromatogram of fatty acid from *R. communis* wild-type (replication 3).

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