Doctoral Dissertation

Micropropagation and transformation of jojoba [*Simmondsia chinensis* (Link.) Schneider], a unique oil-bearing plant

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List of abbreviations

ANOVA: analysis of variance

AS: acetosyringone

BA: 6-benzyladenine

°C: degree celsius

CaMV: cauliflower mosaic virus

cDNA: complementary DNA

CO_2: carbon dioxide

2,4-D: 2,4-dichlorophenoxyacetic acid

GUS: beta-glucuronidase

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric acid

GA3: gibberellic acid

g l: gram per liter

kHz: kilohertz

min: minute

MS: Murashige and Skoog

mg: milligrams

ml: milliliter

mM: millimolar

NAA: α-naphthaleneacetic acid

ng: nanogram

NOS: nopaline synthase

OD: optical density

PCR: polymerase chain reaction

pH: potential hydrogen

PGRs: plant growth regulators

rpm: rotations per minute

RT: reverse transcription

RT-PCR: reverse transcription polymerase chain reaction

SPSS: statistical package for social sciences

TDZ: thidiazuron

T-DNA: transfer DNA

µl: microliter

UV: ultraviolet

w/v: weight per volume

YEB: yeast extract broth

Chapter one

General Introduction

Arid and semi-arid areas are the areas with small amount of rainfall, in which growth of plants are restricted because of the lack of water. In the global surface land area, 41.3% are drylands, including arid and semi-arid areas, and 34.3% of the global population lives there (Millennium Ecosystem Assessment, 2005). Agricultural and industrial development of the areas often lags behind, and most of the lands are not used. Therefore, it is desirable to develop industrial crops that can be cultivated in these areas and can provide cash income to the local people. From the viewpoint of desert afforestation and preventing the world climate change, fruit trees are preferable, because the trees will be sustainably maintained for tens of years with continuous benefits, as well as CO_2 absorption. Among fruit trees suitable to cultivate on arid and semi-arid lands, jojoba, a non-edible oil seed plant with drought and salt tolerance, was found to play significant roles in industrial uses, because its oil content is unique and completely different from other seed oils (Ash *et al.*, 2005). After realization of the industrial value of jojoba oil in 1970th, endeavors have been made worldwide to domesticate it. In order to make this plant an economically feasible for a wide range of industrial uses, biotechnological approaches to achieve high and stable seed production in wide areas of the world are required.

1.1 Jojoba [*Simmondsia chinensis* (Link.) Schneider]: a promising oil crop for sustainable production in arid lands

The industrial value of jojoba oil was firstly recognized in 1970th as a substitute of the whale oil, and then consequent efforts have been done to cultivate it. Jojoba (pronounced as "Hohoba"), *Simmondsia chinensis* (Link.) Schneider, is a perennial green shrub, and is a sole species of the family Simmondisaceae. It is native to the Sonoran Desert of Arizona and arid California of the United States, as well as northern Mexico, and now is domesticated as

an industrial crop in several subtropical countries, such as Peru, Israel or Egypt (Benzioni, 1995). It is 1 to 5 meters high with bushy branching, and has a long life span that might exceed 200 years (Yermanos, 1982). Jojoba plants can grow on wide range of soil types (National academy of Sciences , 1975), can tolerate extreme desert temperatures up to 50°C (Gentry, 1958) and are also drought and salt resistant (Benzioni, 1997). Thus, it thrives in marginal and waste lands not suitable for most crops. The annual rainfall in range 375 to 450 mm has been considered optimum for jojoba growth (Gentry, 1958).

Jojoba is a dioecious plant species. Flowers are unisexual, males and females, carried on separate plants, apetalous and wind-pollinated. Male flowers are small, yellow and borne in the leaf axils in clusters form. Female flowers are small, solitary, inconspicuous, pale green in color, sessile, with five unequal sepals, three styles, superior ovary, three carpels, and borne in axils of the leaves (Thomson, 1982). Moisture and temperature have a key role in synchronization of both sexes to provide abundant of pollens for receptive pistils (Gentry, 1958). The exposure to preconditioning cool temperature for a period of at least 21 days is necessary to induce anthesis in flower buds (Dunstone 1987).

Jojoba leaves are simple, opposite, evergreen, leathery thick, oblong-ovate, rounded at both ends; but vary in these characters among plants (Gentry, 1958). Jojoba has a deep tap root, in mature plants it can be researched to 15 to 25 meters long; consequently helping the plant to grow in the arid areas where others plant species cannot (Begg, 1979). The fruit is capsule, greenish at first, becomes brown as it matures after six months, dehiscent, containing up to 3 seeds, and varies in size and shape (Gentry, 1958; Thomson, 1982). The seeds are peanut-sized, brown to black in color, wrinkled; vary in their shape, size and weight. Fruits ripen during the spring and early summer and seeds fall to the ground in late

summer (Benzioni, 1997). The seeds of jojoba contain approximately 50–55% lipid. It is not fat, but liquid wax composed of long-chain fatty alcohol (C20-C22) attached with fatty acid (C20-C22) with a double bond in each side of the ester (Mirov 1952). Miwa (1980) reported the chemical structure and properties of jojoba oil as shown in Tables 1 and 2. Liquid wax of the seeds can be stored for a long term and remarkably resistant to bacterial degradation (Yermanos, 1974). Jojoba is a unique plant that produces and stores liquid wax in seeds, which no other plants accumulate (Benzioni *et al.,* 1999). Due to chemical similarity, jojoba liquid wax has been recognized as a good candidate for sperm whale oil substitution, hence contributes to conservation of whale (Low and Hackett 1981). Jojoba liquid wax can be obtained in high purity by screw pressing of whole seeds (Benzioni, 1997). It has superiority over the sperm whale oil; pleasant odour, and viscosity is not affected by repeated heating to high temperatures. It can be produced in many resource-poor countries as the agricultural product (Committee on Jojoba Utilization, 1975; Yermanos, 1982).

Table 1. Composition and structure of fatty alcohols and fatty acids derived from jojoba oil using GLC analysis (Miwa, 1980).

Alcohols	**%**	**Acids**	**%**
Hexadecanol	0.1	Eicosanoic	0.1
Octadecanol	0.2	Hexadec-7 -enoic	0.1
Octadec-11-enol	0.4	Octadecadienoic	0.1
Octadec-9-enol	0.7	Octadecanoic	0.1
Docosanol	1	Docosanoic	0.2
Tetracos-15-enol	8.9	Hexadec-9-enoic	0.2
Eicos-ll-enol	43.8	Octadec-11-enoic	1.1
Docos-13-enol	44.9	Hexadecanoic	1.2
Eicosanol	Trace	Tetracos-15-enoic	1.3
Hecos-12-enol	Trace	Octadec-9-enoic	10.1
Heptadec-8-enol	Trace	Docos-13-enoie	13.6
Hexacosenol	Trace	Eicos-ll-enoic	71.3
Tetradecanol	Trace	Eicosadienoie	Trace
		Heptadecenoic	Trace
		Nonadecenoic	Trace
		Pentadecanoic	Trace
		Tetracosenoic	Trace
		Dodecanoic	Trace
		Octadecatrienoic	Trace
		Tetradecanoic	Trace
		Tricosenoic	Trace

Table 2. Ester Composition analysis using GLC coupled with HPLC, mass spectrometry and ozonolysis (Miwa, 1980).

Wax ester chain length	Alcohol/acid combination	% by GLC & GC-MS
34	18/16	0.1
36	18/18	0.1
	20/16	1.8
38	16/22	0.2
	18/20	1
	20/18	5.4
	22/16	0.2
40	16/24	0.6
	18/22	1.5
	20/20	24.3
	22/18	3.6
	24/16	0.3
42	18/24	1.5
	20/22	10.5
	22/20	37
	24/18	1
44	20/24	0.9
	22/22	2.1
	24/20	7
46	24/20	0.8
48	24/24	0.1
50	26/24	0.02

1.2 Potential values of the jojoba cultivation

Potential values of the jojoba cultivation have been described previously (Yermanos, 1982). The jojoba shrub can be used as an ornamental plant and to protect soil erosion by revegetation of dry lands. Cultivation of jojoba plants in arid and semi-arid regions can contribute to the reduction of greenhouse gases. Other crops that can withstand harsh environment conditions can be intercropped. Jojoba oil has diverse industrial uses including cosmetics, lubricants, factices and adhesives, medicines, pharmaceuticals, source of acids and alcohols, electric insulators, foam control agents, and plasticizers. Residual meal after oil extraction contains 30-35% protein, and can be used as a supplement in feedstock after detoxification (Forster and Wright, 2002).

1.3 Propagation of jojoba

Jojoba is a dioecious plant species, and only female plants can produce seeds containing the jojoba oil. Jojoba can be planted through seeds or vegetative methods such as air-layering, grafting, stem cuttings and micropropagation. Direct seed plantation is easy and simple, but has some disadvantages. Number of male plants predominate female plants, and it is not possible to distinguish between male and female plants phenotypically before flowering, which takes 3 to 4 years. Even within female plants, there is remarkable genetic heterogeneity, and as a result, they show low average yields (Benzioni, 1997). An alternative is to select superior clones with the desired quality of male to female plants, and to develop efficient asexual method to propagate them (Benzioni, 1997). Several vegetative methods have been used to propagate jojoba (Reddy, 2003; Bashir *et al.*, 2009), however, they are inefficient and time-consuming for the large-scale propagation. Phenotypic heterogeneity in jojoba plants suggests variable genotypes, and it was recommended to optimize protocol for each genotype because this might affect *in vitro* regeneration efficiency (Llorente and Apóstolo, 2013). In order to propagate jojoba elite clones from the selected stock plants in a short time and independent of the season, efficient shoot multiplication methods via *in vitro* regeneration would be beneficial.

1.4 Genetic transformation as an approach to improve jojoba

Although jojoba has advantages for the cultivation in arid and semi-arid lands, it still does not have any programmed breeding for its improvement. Fluctuation of jojoba production is severely affected by bud dormancy and frost (Dunstone, 1980). *Agrobacterium*-mediated transformation has been used for improvement in various crops, because it has several advantages such as the defined integration of transgenes, potentially low copy number, and

preferential integration into transcriptionally active regions of the chromosome (Koncz *et al.*, 1989; Hiei *et al.*, 2000). Genetic transformation of jojoba, however, has not been established yet.

1.5 Mechanism of *Agrobacterium*-mediated transformation

Plant transformation mediated by *Agrobacterium tumefaciens*, a gram negative pathogenic plant bacterium, has been widely used to introduce foreign genes into plant cells. Its naturally induces tumors at wound sites in dicotyledonous plant causing crown gall disease (Smith and Townsend, 1907). The molecular mechanism of *Agrobacterium*-mediated transformation is a transfer of particular DNA fragment (T-DNA) of tumor–inducing (Ti) plasmid into the plant nuclear genome, following to the stable integration into the plant genome (Nester *et al*., 1984; Binns and Thomashow, 1988). T-DNA (transferred DNA) originally encodes two types of genes, the oncogenes which are involved in auxin and cytokinins synthesis and responsible for tumor formation; and others encoding for opine synthesis genes to serve opine as carbon and nitrogen resource for *Agrobacterium* growth. In addition to T-DNA, Ti plasmid contains virulence (*vir*) genes involved in the processing of T-DNA transfer from *Agrobacterium* into the plant cells (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995). T-DNA can be engineered by removing oncogenic region to produce a non-oncogenic *Agrobacterium* strain for plant transformation (Zambryski *et al.*, 1983). *Agrobacterium* strains have been developed by introducing two distinct plasmids, a binary vector and a vir helper plasmid (disarmed plasmids) after removing tumor formation and opine synthesis genes (Hoekema *et al.*, 1983). Genetic engineering of plants can be achieved by cloning of a DNA fragment into T-DNA region of disarmed plasmid, instead of tumor-inducing genes or opine synthesis genes, introducing

into *Agrobacterium*, transferring into plant cells, and ultimately integrating into the plant genome.

T-DNA has short repeat border sequences, right and left borders, 25 bp in length and highly homologous sequences (Yadav *et al*., 1982; Jouanin *et al*., 1989). These sequences are targets of the VirD1/VirD2 border-specific endonuclease that facilitates the T-DNA transferring from the Ti plasmid (Filichkin *et al.*, 1993). Several steps are involved in the T-DNA transfer from *Agrobacterium* to plant cells, as described below (Pitzschke and Hirt, 2010; Fig. 1).

1.5.1 Recognition of plant cells

Wounded plant cells secret low molecular weight phenolic compounds such as acetosyringone that act as signals to attract *Agrobacterium* attachment to the host cell and to activate *vir* genes.

1.5.2 Virulence genes expression

Plant signals induce expression of *vir* genes through activation of two regulatory genes, *virA* and *virG*, which encode proteins of a two-component phospho-relay system, a membrane-bound sensor (VirA) and the intracellular response regulator (VirG) (Wolanin *et al.*, 2002). After virA receiving the signals activates virG to bind at specific DNA sequences of the vir gene promoters and function as transcription factors to induce their expression (Brencic and Winans, 2005).

1.5.3 Generation of T-DNA transfer complex

After *vir* genes are expressed, VirD1 and VirD2 proteins cleave a single-stranded T-DNA border sequences to produce a copy of the bottom T-DNA strand called T-strand which is transferred into plant cells (Jasper *et al.*, 1994). VirD2 remains covalently attached to the 5´

end of the single-stranded T-strand to protect the T-strand from nucleases and to serve as a target signal for T-DNA transfer complex (Dürrenberger *et al.*, 1989).

1.5.4 Translocation of T-DNA complex

To prevent from nucleases and translocation, the T-DNA complex is coated by VirE2 protein. T-DNA complex was transferred into the plant cell via type IV secretion systems (T4SS) which consisted of 12 proteins, VirB1-VirB11 and VirD4 (Christie, 1997).

1.5.5 Integration of T-DNA into plant nuclear genome

VirD2 and VirE2 play an important role in this step (Hooykaas and Schilperoort, 1992). T-DNA is stably integrated into the plant genome by illegitimate recombination (Mayerhofer *et al*., 1991).

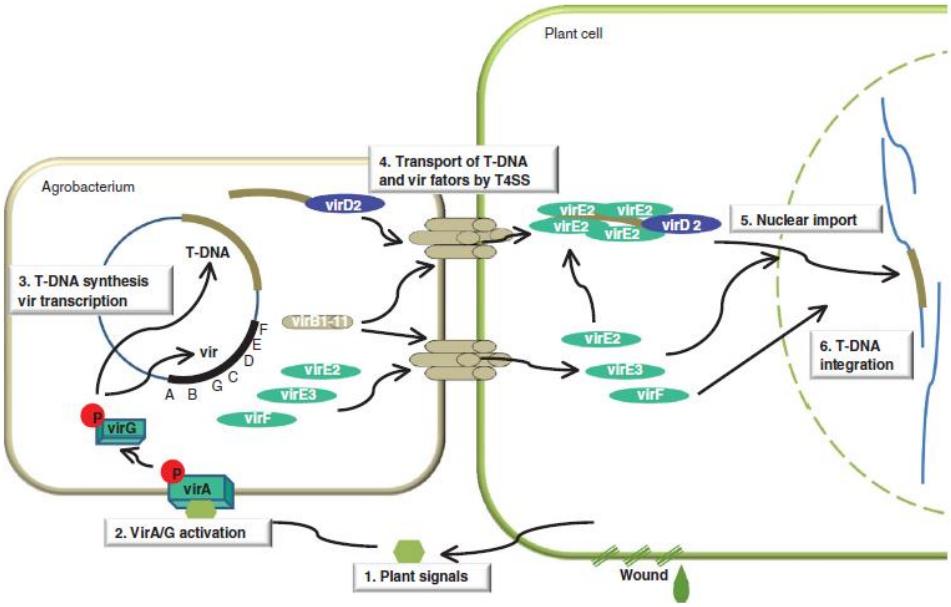

Fig. 1 Overview of the *Agrobacterium*–plant interaction (Pitzschke and Hirt, 2010).

1.6 Objectives of this study

The main objectives of this study are:

1- To establish an effective *in vitro* regeneration system to multiply elite jojoba plants, by using *in vitro* shoot multiplication from nodal segments, as well as a plant growth regulator, Thidiazuron (TDZ).

2- To establish the *Agrobacterium*-mediated genetic transformation system of jojoba.

Chapter two

High-frequency shoot regeneration of jojoba

2.1 Introduction

Jojoba has been gained attention worldwide due to its adaptability to grow in arid lands and uniqueness of its seed lipids (Mirov 1952; Gentry, 1958). A commercial-scale jojoba plantation is restricted due to the lack of high-yielding varieties. To achieve successful commercial-scale jojoba plantations, there are several problems need to be solved. Jojoba is dioecious, wind-pollinated plant species, and shows high variability in phenotypes, sex bias and yield when raised through seeds (Benzioni, 1997). The sex of plants is important in plantings of jojoba, but it is impossible to distinguish between male and female plants phenotypically until it flowers (Sharma *et al*., 2008; Kumar *et al*., 2011). One way to overcome this problem is overplanting of seeds followed by roguing out excess male plants to a level compatible with their function as pollinators, but it is time, labour and cost consuming. Also, genetic heterogeneity among female plants is a serious problem in seed propagation of jojoba.

Therefore, vegetative propagation of the plants with known sex and phenotypes would be the best way for jojoba propagation instead of seed propagation. Vegetative propagation in jojoba has been received much interest due to its ability to provide fruits in a short time, as well as to ensure the uniform and predictable plant growth and yield (Birnnbaum *et al.*, 1984; Lee, 1988). Several vegetative propagation methods have been used for jojoba propagation such as air-layering (Reddy, 2003), grafting (Shah and Bashir 2000) and stem cuttings (Bashir *et al.*, 2009). Although these methods showed advantages over seed plantation, but the rooting rate is low and plant growth is slow (Lee *et al.*, 1985; Benzioni, 1995). Moreover, the maximum number of the propagules is limited by the size of elite plants and time of year (Mills *et al.*, 1997; Reddy and Chikara, 2010).

Micropropagation has advantages over conventional vegetative methods, to produce huge numbers of pathogen-free and true-to-type plants in a short time. *In vitro* propagation has immense potential for mass production of genetically selected jojoba elite clones in a short time and independent of the season (Tyagi and Prakash, 2004; Meyghani *et al.*, 2005). Furthermore, *in vitro* regenerated jojoba plants exhibited vigorous growth compared with seedlings and rooted cuttings and significantly larger after one year of growth (Birnnbaum *et al.,* 1984). Regeneration of entire jojoba plants *in vitro* can be obtained through callus or organ cultures. Recently few reports successfully regenerated shoots from callus of jojoba (Kumar *et al*., 2013). However, these results were not reproducible. Callus culture takes more time than organ culture and it may show somaclonal variation among the newly regenerated plantlets, hence, should be avoided when mass clonal propagation is desired (Kenny, 1988). Rooting ability of the regenerated shoots from callus culture can also vary (John, 1983).

In contrast, micropragation via apical and axillary shoot proliferation is more reliable method for mass multiplication since it conserves genetic identity of clones (Kumar and Reddy, 2011). It can be used to propagate recalcitrant species (Giri *et al*., 2004). For a large-scale multiplication of jojoba, varying levels of success have been achieved by using various culture media containing different concentrations of plant growth regulators (PGRs) (Gao and Cao 2001; Prakash *et al.,* 2003; Tyagi and Prakash 2004; Bashir *et al.,* 2007b). In most of these studies, cytokinins [6-benzyladenine (BA), Kinetin and Zeatin] have been used in combination with auxins [Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA)] or Gibberellic acid (GA3). Botti and Zunino (1988) used Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing different

concentrations and combinations of BA, NAA and GA3, and found that the best response was obtained from BA in the medium. In consistent with it, Llorente *et al.* (1996) reported that the shoot growth was significantly greater in BA than Kinetin treatments, and Llorente and Apostolo (1998) found that the multiplication rate was significantly greater on MS medium containing BA than those containing other cytokinins and/or auxins. There are also reports showing significant effects of BA on the *in vitro* shoot multiplication of jojoba (Agrawal *et al.,* 2002; Prakash *et al.,* 2003; Tyagi and Prakash, 2004). Furthermore, Magdi and Ahmed (2014) found that shoot formation and multiplication were more significantly enhanced in MS containing BA than kinetin. Recently, Bakheet *et al.* (2015) showed the results consistent with those reported by Llorente and Apostolo (1998). From these reports, we can conclude that BA has a potential role in shoots multiplication of jojoba plant over any other cytokinin.

Consequently, many previous reports have described *in vitro* propagation of jojoba using nodal segment as explants (Kacker *et al.*, 1993; Gabr, 1993; Agrawal *et al.*, 2002; Tyagi and Prakash, 2004; Bashir *et al.*, 2008; Singh *et al.*, 2008; Mohasseb *et al.*, 2009; Llorente and Apóstolo, 2013; Hegazi *et al.*, 2014; Bakheet *et al.* 2015). However, number of shoots per explant was low (between 2 to 4 shoots per explant). Mills *et al.* (1997) described *in vitro* plant regeneration from nodal explants of mature jojoba shrubs, but the multiplication rate was low. Later, five shoots per explant were produced on the MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg L^{-1} NAA (Meyghani *et al.*, 2005), and Bashir *et al.* (2007a) achieved maximum number of shoots per explant (19.0 shoots/explant in average) in MS medium containing 5.0 mg L^{-1} BA. Recently, Bakheet *et al.* (2015) reported an average of 7.90 ± 0.04 shoots per explant in MS media containing 1.0 mg L^{-1}

BA. However, rates of survival, multiplication rate, rooting and establishment were still not sufficient for efficient micropropagation of jojoba. Therefore, improvement of efficient mass propagation method for elite jojoba genotypes is desirable.

Thidiazuron [TDZ: N-phenyl-N-(1,2,3-thidiazol-5-yl)urea] has been found to exhibit more effect than BA on *in vitro* morphogenesis of a wide number of plants (Malik and Saxena, 1992; Vinocur *et al.*, 2000; Park *et al.*, 2003; Sujatha and Kumari, 2007; Lata *et al.*,2009). It has a stimulating effect on the multiple shoot formation in a wide variety of plant species that are considered to be recalcitrant to shoot organogenesis, including *Phaseolus vulgaris* L. (Malik and Saxena, 1992), the medicinal "Cancer bush" *Sutherlandia frutescens* L. (Dewir et al., 2010), and potatoes, *Solanum tuberosum* L. (Sajid and Aftab, 2009). TDZ is more effective in most of species that has been tested, particularly in woody species (Faisal *et al.*, 2005; Khurana *et al.*, 2005; Ahmad and Anis, 2007). In spite that mechanism of TDZ action was not clearly determined, recent study showed that it can regulate the metabolism of endogenous plant growth regulators, and can promote accumulation of mineral ions and antioxidant enzymes (Guo *et al.*, 2011). Although TDZ has been used widely for shoot induction and multiplication, prolonged exposure to TDZ should be avoided as this may cause hyperhydricity (Debergh *et al.*, 1992), abnormal shoot morphology (Cambecedes *et al*., 1991), or problems in rooting (Meyer and van Staden, 1988). To overcome these problems, pretreated TDZ explants should be transferred to the basal medium with low concentration of plant growth regulators (PGRs) (Faisal *et al.*, 2005; Ahmad and Anis, 2007; Jahan *et al.*, 2011). In this chapter, the effect of TDZ was investigated as a first report on *in vitro* shoot propagation from nodal segments of the jojoba plant. The overall goal is to develop an efficient and reliable *in vitro* multiplication system

of *S. chinenesis*. There was no previous report to investigate the effects of TDZ on shoot multiplication of *S. chinenesis*. Thus, combinations of BA and TDZ were examined to determine their effects on shoot multiplication of jojoba. Therefore, achieving high number of shoots per explant from efficient sprouting buds within short time can be useful for large-scale mass propagation of elite jojoba genotypes.

2.2 Materials and methods

2.2.1 Plant materials

Seeds of jojoba plants were provided by Dr. Adel Hegazy, University of Sadat City, Egypt, and germinated on soil in pots in a growth room at Osaka University under a 16 h light/8 h dark photoperiod at 26°C and 20% humidity. Explants were collected from 8 months to 1 year-old potted jojoba plants, at this early age sex identification was not determined. Nodal segments, each of which was approximately 2-3 cm long with two axillary buds, were used as explants. After removing leaves, the segments were thoroughly washed in running tap water for 30 min, and then sterilized in 70% ethanol for 15 -20 seconds followed by submerging in 30% sodium hypochlorite solution plus 2-3 drops of Triton X-100 for 30 min. Finally, the explants were rinsed in sterile distilled water three times in a laminar air-flow cabinet.

2.2.2 Shoot initiation

Explants were slightly trimmed at both ends to expose fresh tissue before planting on the medium. Explants (3-5 explants /bottle) were cultured in glass bottles (250 ml) with 50 to 60 ml MS medium containing 3% (w/v) sucrose (KISHIDA, Osaka, Japan), 10 mg L^{-1} thiamine (SIGMA Osaka, Japan), 100 mg L^{-1} *myo*-inositol (Wako Osaka, Japan), 0.8% (w/v) agar (KISHIDA Osaka, Japan) and supplemented with different concentrations (0.0-5.0 mg L^{-1})

of BA and TDZ alone or in combinations. The pH of the media was adjusted to 5.8 (NaOH or HCl) and autoclaved at 121°C for 20 min. Cultures were incubated under a 16 h light (31-35 µmol photon $m^{-2} s^{-1}$) / 8 h dark photoperiod at 25 ± 2°C for 30 days in growth chambers (CLE-303, TOMY). Each treatment was duplicated twice.

2.2.3 Shoot proliferation

Experiments were designed at two-stages of culture procedures; firstly, nodal segments were cultured on MS containing combinations of BA and TDZ to induce shoots. Secondly, explants with induced shoots were subcultured on MS medium containing BA singly for shoot multiplication and elongation. Single-node explants were cultured *in vitro* on solidified MS media supplemented with BA (2.5 mg L^{-1}) in combinations with TDZ at different concentrations (1.0, 2.5 and 5.0 mg L^{-1}) for 30 days to induce sprouting from axillary buds, and rate of sprouting buds, number of shoots per explant and shoot length were measured. The responsive explants were then transferred to MS media containing lower concentrations of BA (0.01, 0.05, 0.5 and 1.0 mg L^{-1}). The shoots were incubated under the same condition for another 45 days. Each experiment was conducted twice.

2.2.4 Root induction and acclimatization

Regenerated shoots were transferred to the glass bottle (100 ml) with root induction medium, which consisted of 30 to 40 ml a half-strength MS medium supplemented with 2% (w/v) sucrose, 10 mg L^{-1} thiamine, 100 mg L^{-1} *myo*-inositol, two concentrations of IBA (0.5 and 2.5 mg L^{-1}) and 0.7% agar (pH 5.8). Cultures were incubated under the same condition as shoot induction and proliferation for 45 days. Each treatment was conducted twice. Plantlets with rooted shoots were transplanted into autoclaved soil in small pots covered with transparent plastic lids and maintained under high humidity for 7 to 15 days, and thereafter

gradually exposed to the growth chamber condition. Established plantlets were then transferred to plastic pots containing soil and cultivated in a growth chamber.

2.2.5 Callus induction:

Leaves were obtained from 8 months to 1 year-old potted jojoba plants, at this early age sex identification was not determined. Leaves were washed with distilled water with shaking for 20 min, and then surface sterilized with 30% sodium hypochlorite solution plus 2-3 drops of Triton X-100 for 20 min. Thereafter, the leaves were washed with sterile distilled water three times in a laminar air-flow cabinet. Finally, the sterilized leaves were incised into small pieces 3 mm x 3 mm and used as explants. Explants were cultured in petri dishes containing 25 ml solidified MS media supplemented with different concentrations of 2,4-D (0.0-2.0 mg L^{-1}), BA (0.1-1.5 mg L^{-1}) and TDZ (0.1 and 0.5 mg L^{-1}) alone or 2,4-D combined with different concentrations of BA and TDZ. The pH of the media was adjusted to 5.8 and autoclaved at 121°C for 20 min. Cultures were incubated under a 16 h light (31-35 µmol photon m^{-2} s^{-1}) / 8 h dark photoperiod at 25 ± 2°C for 30 days in growth chambers (CLE-303, TOMY).

2.2.6 Shoot differentiation

The calli induced from leaf explants were periodically subcultured every 21 days in petri dishes containing 25 ml solidified MS media supplemented with different concentration of TDZ (0 - 1.0 mg L^{-1}) alone or with combinations of BA, TDZ and IBA. Cultures were incubated under the same condition for 90 days.

2.2.7 Statistical analysis

Data on percentage of explants with sprouting buds, number of shoots and shoot length (cm) were recorded after 30 to 45 days from start of the culture. Data were analyzed using one-

way ANOVA and the differences contrasted using Tukey's multiple range test. All statistical analysis was performed at the level of *P* value less than 0.05 using SPSS 22.0 (SPSS Inc., USA). Data on percentage of root induction, average number of days for root induction and root per shoot were analyzed by unpaired t-test at the level of *P* value less than 0.05.

2.3 Results and discussion

2.3.1 Direct organogenesis

2.3.1.1 Shoot initiation

Nodal segments with axillary and apical buds have been used as explants for *in vitro* regeneration of jojoba plants (Mills *et al.*, 1997; Agrawal *et al.,* 1999; Meyghani *et al.,* 2005; Bashir *et al.* 2007b). Preliminary single-node jojoba explants were cultured *in vitro* on solidified MS media supplemented with different concentrations of BA or TDZ alone (Table 2.1) to examine the effect of each on axillary buds sprouting of the plant. Bud sprouting of the explants on the media containing BA took place between 16 to 22 days, while TDZ induced sprouting buds within 17 to 35 days (Table 2.1). On the other hand, buds were not sprouted in some concentrations of BA and TDZ might be due to growth disability of buds. The concentration 2.5 mg L^{-1} BA resulted in the highest number of shoots per explant. However, no sprouted shoot was observed on hormone-free medium. For further analysis, single-node jojoba explants were cultured *in vitro* on solidified MS media supplemented with 2.5 mg L^{-1} BA alone or in combinations with TDZ to investigate the effect of combinations on buds sprouting (Table 2.2). Buds were sprouted in minimum days in respond to the combination of 2.5 mg L^{-1} BA and 2.5 mg L^{-1} TDZ (8.1 ± 0.8 days), which was significantly less than that of 2.5 mg L^{-1} BA and 1.0 mg L^{-1} TDZ (12.6 ± 1.6 days).

These results suggested that the period required for bud sprouting of jojoba explants was significantly affected by combinations of BA and TDZ.

Furthermore, addition of TDZ to the initiation media was drastically enhanced the rates of sprouting buds. The concentrations of TDZ higher than 0.1 mg L^{-1} TDZ showed high rates of sprouting buds in shoot initiation media (90-100%; Table 2.2). Rates of explants with sprouting buds were higher than that in 0.1 mg L^{-1} TDZ in combination with BA, and that in BA alone (70 and 72%, respectively). These results revealed that the rate of sprouting buds was drastically improved in combinations of BA and TDZ compared with BA alone. This is might be due to the regulation of BA metabolism by TDZ (Guo *et al.*, 2011). In contrast, previous reports found that kinetin in combinations with BA delayed buds sprouting of jojoba explants (Bashir *et al*, 2007a; Mohasseb *et al.*, 2009). In comparison to reported results, our results would be better for buds sprouting of jojoba plant within short time. Addition of high concentrations of TDZ to shoot initiation had remarkable positive effects on buds sprouting; therefore these concentrations were selected for further experiments.

Table 2.1. Effect of BA or TDZ alone on shoot initiation after 45 days of culture.

Growth regulators (mg L^{-1})		Period for bud sprouting (days)	Shoots/explant
BA	TDZ		
—	—	—	—
0.5	—	21.5	1.5
1.5	—	—	—
2.5	—	17.3	3.3
3.5	—	22.0	1.5
5.0	—	16.0	2.0
—	0.5	35.0	2.0
—	1.5	—	—
—	2.5	—	—
—	3.5	19.0	1.6
—	5.0	17.7	3.5

Table 2.2. Effect of TDZ in combination with BA on shoot initiation after 30 days of culture.

Growth regulators (mg L^{-1})		Period for bud sprouting* (days)	Explants with spouting buds (%)
BA	TDZ		
—	—	—**	0
2.5	—	10.6 ± 0.5^{ab}	72
2.5	0.1	9.9 ± 1.6^{ab}	70
2.5	0.5	10.9 ± 0.8^{ab}	90
2.5	1.0	12.6 ± 1.6^{b}	100
2.5	2.5	8.1 ± 0.8^{a}	90
2.5	5.0	10.6 ± 1.3^{ab}	90

* Values represent means ± standard error of 10-15 replicates per treatment. Same letters (a or b) are not significantly different (*P*: 0.05) by Tukey-kramer test.

** Sprouting buds were not observed after 30 days in control without hormones.

2.3.1.2 Shoot multiplication

TDZ has been proven to be potentially effective regulator of shoot morphogenesis (Huettenman and Preece 1993; Murthy *et al*., 1998; Wang *et al.*, 2008). It has been reported that prolonged exposure to TDZ may cause hyperhydricity (Debergh *et al.*, 1992), abnormal shoot morphology (Cambecedes *et al*., 1991), or problems in rooting (Meyer and van Staden, 1988). To succeed in dealing with these problems, it has been recommended to transfer

TDZ-pretreated explants to the basal medium with low concentration of plant growth regulators (PGRs) (Faisal *et al.*, 2005; Ahmad and Anis, 2007; Jahan *et al.*, 2011). Among these PGRs, BA was found to be superior over others in promoting shoot multiplication and elongation in TDZ-exposed explants of *Cassia sophera* (Parveen and Shahzad, 2010).

In the present study, the effectiveness of BA in promoting shoot multiplication and elongation of TDZ-exposed jojoba explants was investigated in two stages, Stage I with TDZ and Stage II without TDZ. The percentages of bud sprouting were very high in Stage I (80-100%; Table 2.3), which were consistent with previous results in Table 2.2. At the same time, rates of sprouting buds per explant were dramatically increased compared to BA or TDZ alone (Table 2.1). The number of shoots per explant in MS media supplemented with combinations of BA and TDZ was remarkably increased (Stage I of Table 2.3) compared with whose containing BA or TDZ individually (Table 2.1). The combination of 2.5 mgL^{-1} BA and 5.0 mg L^{-1} attained the highest number of shoots per explant (7.5 ± 1.7), followed by the combination of 2.5 mg L^{-1} BA and 2.5 mg L^{-1} TDZ (7.0 ± 1.6). While the lowest one (5.2 ± 1.6) was produced by the combination of 2.5 mg L^{-1} BA and 1.0 mg L^{-1} TDZ (Stage I of Table 2.3, Fig 2.1a-c). The results indicated that the number of sprouted shoots per explant was induced by higher concentration of TDZ treatments; however, the difference was not significant. The results presented in Stage I of Table 2.3 revealed that the length of shoot was not significant differences among the three conditions. It was noticed that the average of shoot length in range 0.77 to 1.39 cm after 30 days as shown in Fig. 2.1.

To avoid the negative effects of prolonged exposure to TDZ, explants with sprouted shoots obtained from shoot induction media were subsequently transferred to solidified MS media supplemented with different concentrations of BA (Stage II). Cultures were incubated

for 45 days and sprouted shoots per explant and shoot length were measured. Generally, the results found that the number of sprouted shoots per explant was increased in most of BA concentrations (Stage II of Table 2.3, Fig. 2.1d-f). Explants obtained from combination of BA with the highest concentration of TDZ exhibited higher number of sprouted shoots per explants in most of BA concentrations. Among BA concentrations, explants from media with the combination of 2.5 mg L^{-1} BA and 5.0 mg L^{-1} TDZ significantly attained the highest sprouted shoot number (29.5 ± 0.9) in the media supplemented with 1.0 mg L^{-1} of BA. Otherwise, the number of proliferated shoots per explant in Stage II was increased 3.9-fold higher than that in Stage I. However, the average length of shoot (0.92 cm, Stage II of Table 2.3) was shorter than before the transfer (1.10 cm, Stage I of Table 2.3). The reduction in shoot length might be due to the newly-regenerated shoots and the competition among them. On the other hand, explants from media with the combination of 2.5 mgL^{-1} BA and 2.5 mgL^{-1} TDZ significantly exhibited the highest sprouted shoot number (21.8 ± 1.5) in the medium supplemented with 0.5 mgL^{-1} of BA. In the meantime, the number of proliferated shoots per explant was increased 3.3–fold in Stage II (Table 2.3). The average of shoot length in Stage II (1.81 cm) was remarkably increased more than 2-fold higher than that in Stage I (0.77 cm). While regenerated shoots of explants obtained from media with the combination of 2.5 mg L^{-1} BA and 1.0 mg L^{-1} TDZ showed lower number of shoots per explant (5.8–9.2) than other combinations. However, the maximum length of shoots (2.81 cm in average) was obtained from these explants in the medium supplemented with 0.5 mg L^{-1} of BA. These results indicated that the high multiple shoots were produced from the combination containing high concentrations of TDZ in shoot initiation medium followed by transferring to the shoot multiplication medium containing suitable concentrations of BA.

TDZ may be involved in the regulation of metabolism of endogenous plant growth regulators (Guo *et al.,* 2011).

In this study, TDZ was effective hormone for shoots proliferation of jojoba plant. We have achieved the highest number of sprouted shoots per explant (more than 20 shoots explant) on MS media supplemented with the combination of BA and TDZ. The number of shoots per explant obtained in this study was higher than the maximum numbers in previous reports using media with BA alone (19.0 shoots/explant in average; Bashir *et al.*, 2007a) or with the combination of BA and kinetin (8–15 shoots/explant; Heba Allah *et al.,* 2009). TDZ has been reported to increase the biosynthesis and accumulation of endogenous adenine-type cytokinins (Huettman and Preece 1993), effective to stimulate the shoot formation. Lata *et al.* (2009) found that TDZ resulted in shoot regeneration of *Stevia rebaudiana* better than other cytokinins. Ahmed *et al.* (2013) reported that the number of shoots per explant was enhanced two-fold when cotyledonary node explants of *Cassia alata* were pretreated with TDZ and transferred into multiplication media containing BA. Yorgancilar and Erisen (2011) obtained the highest sprouted shoot number of *Astragalus schizopterus* (23.6 shoots/explant) when TDZ-exposed explants (1 mg L^{-1}) were subcultured on the media containing BA. Sujatha *et al*. (2005) achieved maximum shoot proliferation rate (12.3 shoots/explant) when nodal explants of jatropha were initially cultured on medium containing TDZ (4.5 µM) and transferred into medium containing BA. They recorded a linear correlation between the concentration of TDZ and number of shoots per explant. Our results indicate that TDZ raise the rate of explants with sprouting buds, as well as the number of sprouted shoots per explant in jojoba, which are consistent with the previous results in other plant species described above. From these results, we conclude that

transferring nodal explants to MS medium with BA after exposure to high concentrations of TDZ and BA is effective for proliferation of jojoba shoots.

Table 2.3. Effect of TDZ on multiple shoot regeneration from nodal segments after transferring to MS medium with BA.

Stage I					**Stage II**		
Growth regulators (mg L^{-1})		Explants with sprouting buds (%)	Shoots /explant*	Shoot length* (cm)	BA (mg L^{-1})	Sprout shoots /explant*	Shoot length* (cm)
BA	TDZ						
2.5	1.0	100	5.2 ±1.6[a]	1.39 ± 0.39[a]	0.01	9.2 ± 0.8[b]	2.3 ± 0.6[a]
					0.05	9.1 ± 0.5[b]	1.5 ± 0.8[a]
					0.50	5.1 ± 0.6[a]	2.8 ± 0.7[a]
					1.00	9.1 ± 0.7[b]	1.4 ± 0.6[a]
2.5	2.5	100	7.0 ±1.6[a]	0.77 ± 0.39[a]	0.01	9.7 ± 1.1[ab]	1.7 ± 0.7[a]
					0.05	5.8 ± 0.7[a]	1.1 ± 0.6[a]
					0.50	21.8 ± 1.5[c]	1.8 ± 0.4[a]
					1.00	11.0 ± 0.9[b]	1.5 ± 0.6[a]
2.5	5.0	80	7.5 ±1.7[a]	1.10 ± 0.41[a]	0.01	9.0 ± 0.6[a]	1.6 ± 0.4[a]
					0.05	22.0 ± 1.1[b]	1.0 ± 0.3[a]
					0.50	20.8 ± 1.1[b]	0.9 ± 0.3[a]
					1.00	29.5 ± 0.9[c]	0.9 ± 0.3[a]

*Values represent means ± standard error of 10 replicates per treatment. Same letters are not significantly different ($P < 0.05$) by Tukey-kramer test.

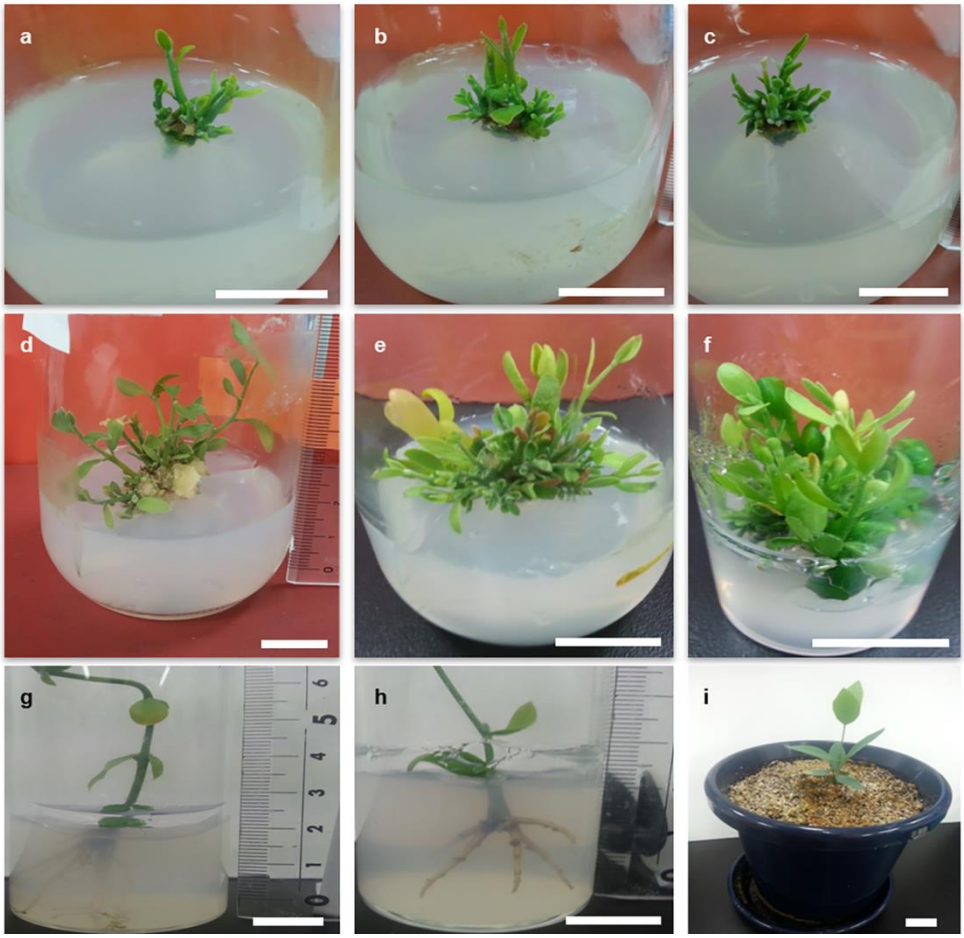

Fig. 2.1 *In vitro* propagation of *Simmondsia chinensis.*

a-c Shoot induction on MS media supplemented with different combinations of BA and TDZ after 45 days of culture. Explants on media containing 2.5 mg L^{-1} BA + 1.0 mg L^{-1} TDZ **(a),** 2.5 mg L^{-1} BA + 2.5 mg L^{-1} TDZ (**b)**, and 2.5 mg L^{-1} BA + 5.0 mg L^{-1} TDZ (**c)** are shown. **d-f** Shoot multiplication on MS media supplemented with BA after 6 weeks of culture. Explants on media containing 1.0 mg L^{-1} BA after exposed to 2.5 mg L^{-1} BA + 1.0 mg L^{-1} TDZ (**d),** 0.5 mg L^{-1} BA after exposing to 2.5 mg L^{-1} BA + 2.5 mg L^{-1} TDZ (**e)**, and 1.0 mg L^{-1} BA after exposing to 2.5 mg L^{-1} BA + 5.0 mg L^{-1} TDZ (**f**) are shown. **g** and **h** Rooted plantlets in ½ MS medium supplemented with IBA (0.5 and 2.5 mg L^{-1}, respectively). **i** A plantlet after acclimatization. Bars =2 cm.

2.3.1.3 Rooting

Previous studies of jojoba showed that the indole butyric acid (IBA) was the most effective auxin for root induction of micropropagated shoots (Elhag *et al*., 1998; Khanam *et al*., 1999; Bashir *et al.,* 2007c). For root induction elongated shoots carefully dissected from explants grown on the medium containing BA and cultured on ½-MS media supplemented with two concentrations of IBA (0.5 and 2.5 mgL^{-1}). The results showed that concentrations of IBA did not significantly affect duration for root induction and also the rooting efficiency (Table 2.4). In number of roots per shoot, however, 2.5 mg L^{-1} IBA showed significantly higher number of roots per shoot (7.4 ± 0.9) than 0.5 mg L^{-1} IBA (3.6 ± 0.7) (Table 2.4, Fig. 2.1g-h). The effectiveness of IBA on *in vitro* rooting of shoots has been shown in the jojoba plants (Agrawal *et al.,* 2002; Tyagi and Prakash 2004; Bashir *et al.*, 2007c; Kumar *et al.,* 2009). Our results proved that *in vitro* regenerated jojoba shoots were rooted on media containing IBA that were in consistent with previous results in jojoba. Twenty-one rooted plantlets out of twenty-eight were successfully acclimatized inside growth chamber for 21 days (75% survival rate). The acclimatized plants exhibited normal morphological appearance (Fig. 2.1-i).

Table 2.4. Effect of IBA on root induction after 45 days of culture.

Growth regulator (mg L^{-1}) IBA	Average number of days for root induction[a]	Roots/shoot[a]	Rooted shoot (%)
0.5	32.3 ± 0.9	3.6 ± 0.7	46.7
2.5	30.8 ± 0.6	7.4 ± 0.9*	40.0

[a] Values represent means ± standard error of 20 replicates per treatment.
* Significantly different ($P < 0.05$) from 0.5 mg L^{-1} IBA by unpaired *t*-test.

2.3.2 Indirect organogenesis

There were a few reports that succeeded in regenerating jojoba shoots via indirect organogenesis (Kumar *et al*., 2013). In this study, we found that 2,4-D alone or in combinations with BA or TDZ were sufficient for callus induction (Fig. 2.2). White roots with root hairs were observed in media containing 2,4-D alone or in combinations with BA or TDZ, they were not observed in media containing BA or TDZ alone (Fig. 2.3). Calli derived from the callus induction media failed to differentiate into shoots in all conditions used for shoot differentiation. Formation of roots seems to be due to 2,4-D activity as auxin, since cytokinins (BA and TDZ) did not induce roots formation. Our results were in accordance with previous reports in other plant species (Kartha *et al*., 1974; Aragao, 1976).

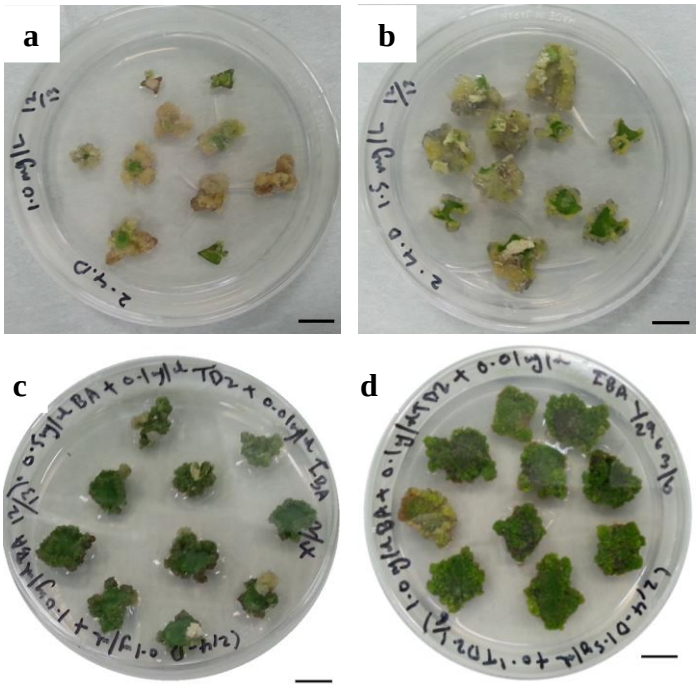

Fig. 2.2 Callus induction of *S. chinensis.*

Explants on media containing 1.0 mg L^{-1} 2,4-D **(a),** 1.5 mg L^{-1} 2,4-D **(b),** 0.1 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA **(c),** and 1.5 mg L^{-1} 2,4-D + 0.1 mg L^{-1} TDZ **(d)** are shown. Bars =1 cm

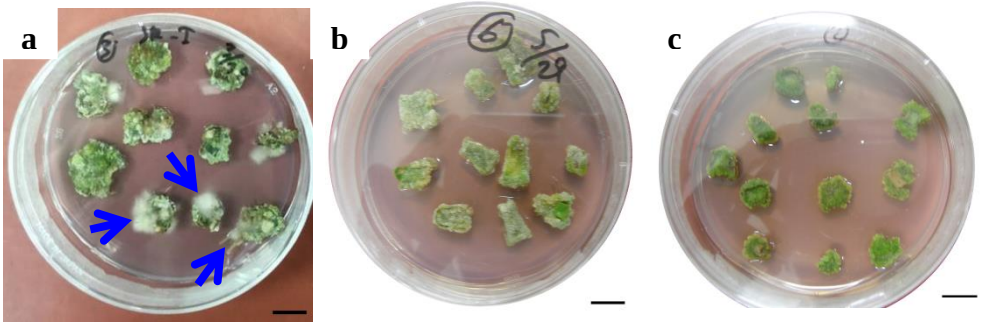

Fig. 2.3 Root induction of *S. chinensis* during callus formatin

Explants on media containing 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA **(a),** 0.5 mg L^{-1} BA **(b),** 0.1 mg L^{-1} TDZ **(c)** are shown. Blue arrows are regenerated roots. Bars =1 cm

2.4 Conclusion

Fluctuation of jojoba seeds production might be due to the lack of elite jojoba genotypes or an efficient mass propagation method. In this study, we investigate the potential effects of TDZ on shoots multiplication of jojoba using nodal segments. We established an efficient shoot regeneration method using TDZ in combination with BA from jojoba segments. TDZ in combination with BA enhanced shoot initiation and multiplication from jojoba nodal explants. The highest shoot number per explant was observed in the combination of 2.5 mg L^{-1} BA and 5.0 mg L^{-1} TDZ followed by 1.0 mg L^{-1} BA. Our results showed the highest shoots multiplication rate of jojoba nodal segments compared previous reports on jojoba plant. The regenerated shoots were rooted on media containing IBA. The plantlets were acclimatized and transferred to a growth chamber with 75% survival rate. The developed protocol in this study concluded that the best conditions were 2.5 mg L^{-1} BA and 5.0 mg L^{-1} TDZ for shoot initiation, followed by 1.0 mg L^{-1} BA for shoot multiplication and 2.5 mg L^{-1} IBA for rooting. This protocol would be useful for large-scale microporpagtion of elite jojoba genotypes as well as for genetic transformation.

Chapter three

Agrobacterium-mediated transformation of jojoba

3.1 Introduction

Jojoba [*Simmondsia chinensis* (Link.) Schneider] is a unique industrial oil crop that received important commercial applications because its seeds accumulate valuable liquid wax esters (50% of dry weight). The liquid wax (jojoba oil) is different from ordinary vegetables oil, which is triglyceride (Benzioni *et al.,* 1999). It has now diverse uses in cosmetics due to high similarity to human skin sebum (Ash *et al.,* 2005) and lubricants due to its stability under the high temperature and pressure. Several environmental conditions are known to limit the extension of jojoba cultivation and production, including frost sensitivity of flower buds in freezing areas (Dunstone, 1988), requirement of low temperature (5 to 20°C) to break the dormancy of jojoba flower buds (Dunstone, 1980), and sensitivity to waterlogging in rainy areas. Variability in chilling requirements has been found among jojoba genotypes (Benzioni, 1992). To extend the production of jojoba, these problems need to be solved. Jojoba does not have any close relatives, and genotypes have similar general characteristics. These prevent to solve the problems by conventional breeding. Genetic transformation, however, would be the alternative way to solve the problems. Because genetic transformation of jojoba has not been reported yet, establishment of the protocol in jojoba should be desirable.

Agrobacterium-mediated transformation has been commonly used to transform various important plant species because it offers several advantages such as the defined integration of transgenes, potentially low copy number, and preferential integration into transcriptionally active regions of the chromosome (Koncz *et al.,* 1989; Hiei *et al.,* 2000). Sonication has been widely used to enhance the efficiency of *Agrobacterium*-mediated transformation by producing small and uniform fissures and channels throughout the plant

tissue, allowing *Agrobacterium* to access into especially recalcitrant target plant tissues (Trick and Finer, 1997; Tang *et al.,* 2001; Liu *et al.,* 2006; Subramanyam *et al.,* 2011). Therefore, sonication-assisted *Agrobacterium* transformation would be useful for transformation of jojoba with desired genes.

Establishment of an efficient regeneration method is a prerequisite for genetic transformation efficiency. An efficient regeneration protocol can be achieved through direct organogenesis from axillary buds or indirect organogenesis from callus cells. Jojoba plant lacks an efficient regeneration method from callus cells. Results of the chapter two showed the difficulty of establishment of efficient regeneration from calli, because shoots were not differentiated (also refer Aragao, 1976). Although many reports have shown the successful regeneration of transgenic plants from callus in various species (Cheng *et al.*, 1997; Li *et al.*, 2008; Maheshwari and Kovalchuk, 2016), there are only a few reports of successful plant regeneration from callus of jojoba, and their results are not reproducible (Kumar *et al*., 2013). On the other hand, several studies succeeded to establish an efficient regeneration shoots from axillary buds of nodal explants of jojoba (Gao and Cao, 2001; Prakash *et al.*, 2003; Tyagi and Prakash, 2004; Bashir *et al.*, 2007a). In the chapter two we have developed a more effective shoot multiplication protocol from axillary buds of jojoba nodal segments than previous reports, which would be suitable for regeneration of jojoba transformants. Indeed, nodal segments have been successfully used for transformation in some plant species. Suzana and Zlata (2005) reported a transformation protocol of *Humulus lupulus* using nodal explants, and Ramesh *et al.* (2011) reported high transformation efficiency in *Bacopa monnieri* using nodal segments as target explants. Sontikun *et al.* (2013) found that nodal segments were more suitable as explants than the leaf-base tissue for *Agrobacterium*-

mediated transformation of teak. Recently, Sivanandhan *et al.* (2015) showed an efficient regeneration shoots and *Agrobacterium*-mediated transformation of *Withania somnifera* using nodal segments.

The aim of this chapter is to establish an efficient *Agrobacterium*-mediated genetic transformation system for stable transformation of foreign genes into jojoba. We report here *Agrobacterium*-mediated genetic transformation of jojoba, by using nodal segments as explants and regeneration of multiple shoots with transformed cells from them.

3.2 Materials and Methods

3.2.1 Plant materials

Seeds of jojoba plants were provided by Dr. Adel Hegazy, University of Sadat City, Egypt, and germinated on soil in pots in a growth room at Osaka University under a 16 h light / 8 h dark photoperiod at 26°C and 20% humidity. Nodal segments approximately 2-3 cm in length, each including a node with two axillary buds, were collected from 1 year-old jojoba plants grown in the lab of Osaka University, and were used as explants to proliferate shoots. After removing leaves, the cuttings were thoroughly washed in running tap water for 30 min then sterilized in 70% ethanol for 15-20 seconds followed by submerging in 30% sodium hypochlorite solution plus 2-3 drops of Triton X-100 for 30 min. Finally explants were rinsed in sterile distilled water three times under laminar air-flow cabinet.

3.2.2 Shoot proliferation

The explants were cultured in glass bottles (250 ml) with 50 to 60 ml Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 10 mg L^{-1} thiamine, 100 mg L^{-1} *myo*-inositol, 0.8% (w/v) agar, and a combination of 2.5 mg L^{-1} 6-benzyladenine (BA) and 5.0 mg L^{-1} Thidiazuron (TDZ) (pH 5.8) autoclaved at 1.06 kg cm^{-3}

and 121°C for 20 min (shoot initiation medium). They were incubated under a 16 h light (31-35 µmol photon m^{-2} s^{-1})/8 h dark photoperiod at 25 ± 2°C for 15 days in growth chambers (CLE-303, TOMY), were transferred to the MS medium supplemented with 0.5 mg L^{-1} BA, and were incubated for 45 days to multiply regenerated shoots. The regenerated shoots of jojoba, consisting of 4-6 young leaves, were cut into uninodal segments, which were then used for the *Agrobacterium tumefaciens*-mediated transformation. To examine survival of the shoots in the presence of hygromycin, the explants were cultured on the shoot initiation medium without hygromycin, and then were subcultured on the media with 0.5 mg L^{-1} BA, as well as 0, 10, 20, and 30 mg L^{-1} hygromycin, at the same environmental condition as above for 45 days.

3.2.3 Plasmid vector and A*grobacterium* strain

Plasmid pCAMBIA1305.1 (Cambia, GenBank accession number AF354045) with the *GusPlus* gene and *A. tumefaciens* strain (LBA4404) were used for transformation. The binary vector contains the neomycin phosphotransferase II (*nptII*) gene conferring for bacterial kanamycin resistance and hygromycin phosphotransferase II (*hptII*) gene for plant hygromycin selection. The reporter *GusPlus* gene encodes beta-glucuronidase, and has an intron sequence of the caster bean catalase gene within the coding sequence to avoid expression in *Agrobacterium*. The *GusPlus* gene is fused with the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator sequences.

3.2.4 Transformation of *Agrobacterium* by binary vectors

Agrobacterium tumefaciens was transformed by pCAMBIA 1305.1 using freeze/thaw method (Hofgen and Willmitzer, 1988). Stored cells were thawed on ice prior to transformation. Competent agrobacteria were mixed with 0.5-1.0 µg plasmid DNA. The

cells were incubated successively 5 min on ice, 5 min in liquid nitrogen and 5 min at 37°C. After dilution in 1 ml YEB-medium the cells were shaken for 2-4 h at 28°C. Aliquots of 200 µL were plated on YEB-plates containing appropriate antibiotics and incubated for 2 days at 28°C. Single colonies were picked and inoculated for plasmid analysis. Presence of the binary vector in the transformed *Agrobacterium* cells was confirmed by polymerase chain reaction (PCR) and electrophoresis. The forward and reverse primers were 5'-AACTGGTGAACGACGGACTG-3' and 5'-CGTGAAATTCCTGGCGGAGA-3', respectively, both of which were designed to hybridize with the *GusPlus* gene sequence. A 20 µL mixture containing 10 µL 2 x GoTaq GreenMaster Mix (Promega Corporation, Madison WI, USA), 9.2 µL MilliQ water, 0.4 µL each of forward and reverse primers (1 ng each) and 2 µL plasmid DNA was used. PCR was conducted as follows: an initial denaturation at 94°C for 2 min, followed by 40 cycles of 30 second at 94°C, 1 min at 55°C, and 2 min at 72°C, and final extension at 72°C for 10 min. PCR products were then separated by electrophoresis on 1.5 % agarose gels, stained with ethidium bromide and visualized by the UV transilluminator. *Agrobacterium* cells were grown overnight at 30°C with shaking at 180 rpm to an optical density 0.5 (OD_{600} = 0.5) in 20 mL liquid YEB medium (5 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1} peptone, 5 g L^{-1} sucrose and 0.5 g L^{-1} $MgSO_4.7H_2O$) containing 50 mg L^{-1} kanamycin and 20 mg L^{-1} acetosyringone. The cultures were centrifuged at 4000 x *g* for 10 min and the pellets were re-suspended in 25 ml liquid MS basal medium containing 20 mg L^{-1} acetosyringone.

3.2.5 *Agrobacterium*–mediated transformation of jojoba nodes

The uninodal segments were aseptically excised (1-2 cm) from sprouted shoots of axenic cultures of jojoba. The segments were then transferred to the 50 ml falcon tubes, and

immersed in 25 ml hormone-free MS liquid medium with 20 mg L^{-1} acetosyringone (AS) containing *A. tumefaciens* LBA4404 cells (OD_{600} = 0.5) harboring pCAMBIA1305.1. The tubes were placed at the center of a bath sonicator (ULTRA SONIC CLEANER), with the lower part of the tube touching at the bottom of the bath. The explants were subjected to sonication at 60 kHz for 1, 2 or 5 minutes. After sonication, the explants were subjected to shaking on an orbital shaker (EYELA, Tokyo Rikakikai Co., Japan) at 120 rpm, 30°C in the same *Agrobacterium* suspension for 10, 20 and 30 min. Followed by these treatments, explants were blotted on the sterile paper towel to remove excess of *A. tumefaciens* cells. The explants were transferred to a solid co-cultivation medium consisting of the MS medium supplemented with 20 mg L^{-1} acetosyringone (AS), 2.5 mg L^{-1} benzyladenine (BA), 5.0 mg L^{-1} TDZ and 0.8% (w/v) agar, were incubated for 3 days at 25 ± 2°C in the dark, and were washed five times with distilled water containing 500 mg L^{-1} cefotaxime to eliminate *Agrobacteria*. Shoots were regenerated from the explants by using the direct regeneration protocol described above. The regenerated shoots were then subcultured on the medium containing 0.5 mg L^{-1} BA and 30 mg L^{-1} hygromycin at the same condition as above for 45 days, and the frequency of survived hygromycin-resistant shoots was calculated.

3.2.6 Histochemical GUS assay

The GUS (beta-glucuronidase) assay was performed on nodal explants according to the method described by Jefferson *et al*. (1987) with some modifications. The explants were immersed in X-Gluc staining solution (100 mM $NaHPO_4$ buffer (pH 7.0), 10 mM EDTA (pH 7.0), 2 mM $K_3[Fe(CN)_6]$, 2 mM $K_4[Fe(CN)_6]3H_2O$, 0.1% (v/v) Triton X-100, 1 mg mL^{-1} X-Glu), were subjected to vacuum for 20 min, and were further incubated in staining solution overnight at 37°C. The stained explants were washed with 0.1 M sodium phosphate

buffer (pH 7.0) followed by dehydration with series of ethanol (30, 50 and 70%) for 5 min in each. They were then placed in 100% ethanol to remove chlorophyll, followed by rehydration with series of ethanol (70, 50 and 30%) for 5 min in each. Finally, the explants were kept in 0.1 M sodium phosphate buffer (pH 7.0) and were observed using a stereomicroscope (Leica MZ FLIII). Percentages of explants with GUS positive expression were calculated by the number of explants that showed GUS positive to the total number of evaluated explants. The frequency of transient transformation is expressed as the number of blue areas of each explant.

3.2.7 RT-PCR analysis

Total RNA was extracted from hygromycin resistant shoots (100 mg) using Plant RNA Isolation Reagent (Invitrogen, CA, USA) following manufacturer's instructions. The synthesis of cDNA from 100 ng RNA was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit. (TOYOBO CO., OSAKA, Japan). PCR was performed in the 20 µL reaction mixture containing 10 µL of 2 x GoTaq GreenMaster Mix (Promega), 2 µL of reverse transcription (RT) products, and the following primers. A Primer set used to amplify the *GusPlus* sequence was 5'-AACTGGTGAACGACGGACTG-3' and 5'-CGTGAAATTCCTGGCGGAGA-3', and the primers 5'-GGCGAGAAAGGCATTGGAAA-3' and 5'-TAGATTGACTCGCCTCCGGT-3' were used to detect an endogenous jojoba homolog of the potato cyclophilin gene, which was used as internal control for RT-PCR by Nicot *et al*. (2005). PCR was conducted as follows: an initial denaturation at 94°C for 2 min, followed by 40 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C, and final extension at 72°C for 10 min. PCR products were then

separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized with the UV transilluminator.

3.3.8 Statistical analysis

From 15 to 20 explants were used for each treatment and experiments were repeated two times. Data were analyzed using one or two-way ANOVA and the differences contrasted using Tukey-Kramer test. All statistical analysis was performed at the level of *P* value less than 0.05 using SPSS 22.0 (SPSS Inc. Chicago, USA). Results are shown in supplementary materials.

3.3 Results and Discussion

3.3.1 Genetic transformation of nodal segments

Meristem cells in axillary buds of nodal segments have been used as target for *Agrobacterium*-mediated transformation in several plant species (Suzana and Zlata 2005; Ramesh *et al.* 2011; Sontikun *et al.* 2013; Sivanandhan *et al.* 2015). To perform *Agrobacterium*-mediated transformation of jojoba, nodal segments were used as explants. Nodal explants were exposed to combinations of sonication and shaking treatments in the presence of the *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1305.1 in the MS liquid medium, to introduce the reporter *GUS* (beta-glucuronidase) gene to jojoba cells. The explants were then co-cultivated on the shoot initiation medium containing acetosyringone in the dark for 3 days, and were washed five times with water containing 500 mg L^{-1} cefotaxime to eliminate *Agrobacteria*. To examine introduction and expression of the *GUS* gene in jojoba cells, histochemical GUS assay was performed. Note that the *GUS* gene used in this study (*GusPlus*) had an intron to avoid detection of the GUS activity in *Agrobacterium* cells. From the results of histochemical

assay, we could detect the GUS staining in explants infected with *Agrobacterium*, but not in non-infected explants (Fig. 3.1a-f; Table 3.1). Observation of the GUS activity indicated the successful of introduction and expression of *GUS* gene in jojoba cells of nodal segments by inoculation with *Agrobacterium*. We also observed the GUS staining in apical cells and axillary buds (Fig. 3.1e,f), which have ability to multiply and generate new shoots. The detection of *GUS* expression in these cells confirmed the transformation of target cells and eventually ensured the generation of transgenic plants.

3.3.2 Transformed cells in regenerated shoots

To examine regeneration ability of putative transformed cells, putative transgenic and non-transgenic explants were cultured on the shoot initiation medium for 15 days, and then subcultured them on MS medium containing 0.5 mg L^{-1} BA for 45 days. Multiple shoots were regenerated from axillary buds, and histochemical GUS assay was performed using regenerated shoots. We observed allocation of the GUS staining on the regenerated shoots of *Agrobacterium*-treated explants but not in those of untreated ones (Fig. 3.1g-l). Transformation stability and expression of transgene in jojoba cells were proved by the observation of the GUS staining in regenerated shoots. Presence of transgenic cells in regenerated shoots suggests that regeneration of multiple shoots from nodal explants would be an effective way to generate transgenic jojoba plants, this is in agreement with the results of previous reports in other plant species (Suzan and Zlata, 2005; Ramesh *et al.*, 2011; Sontikun *et al.*, 2013; Sivanandhan *et al.*, 2015).

Table 3.1. Transformation efficiency of jojoba nodal explants with different sonication and shaking conditions after 3 days of co-cultivation with *Agrobacterium tumefaciens* strain LBA 4404 harboring pCAMBIA1305.1

Sonication (min)	Shaking treatment (min)	Total No. of explants	No. of GUS positive explants	Percentage of GUS positive explants (%)	GUS spots/positive explant**	Estimate for variance of average value in each factor	Estimate for standard error based on ANOVA
1	10	35	24	69	6.1 ± 1.1	14.7	3.8
1	20	35	24	69	8.5 ± 2.2	14.7	3.8
1	30	39	17	44	12.0 ± 6.6	20.7	4.6
2	10	28	19	68	6.6 ± 1.3	18.5	4.3
2	20	32	17	53	15.5 ± 6.0	20.7	4.6
2	30	32	21	66	13.8 ± 5.4	16.8	4.1
5	10	34	15	44	16.4 ± 8.1	23.5	4.8
5	20	35	10	29	5.0 ± 3.3	35.2	5.9
5	30	38	13	34	5.4 ± 1.6	27.1	5.2
—*	—	25	0	0	0.0 ± 0.0	0.0	0.0

*The bottom row shows results of explants without *Agrobacterium* co-cultivation, sonication, and shaking as the reference.

**Values represent means ± standard error. Two-way ANOVA showed that effects of sonication, shaking and their interaction on average of GUS spots were not significant (Supplementary table 1).

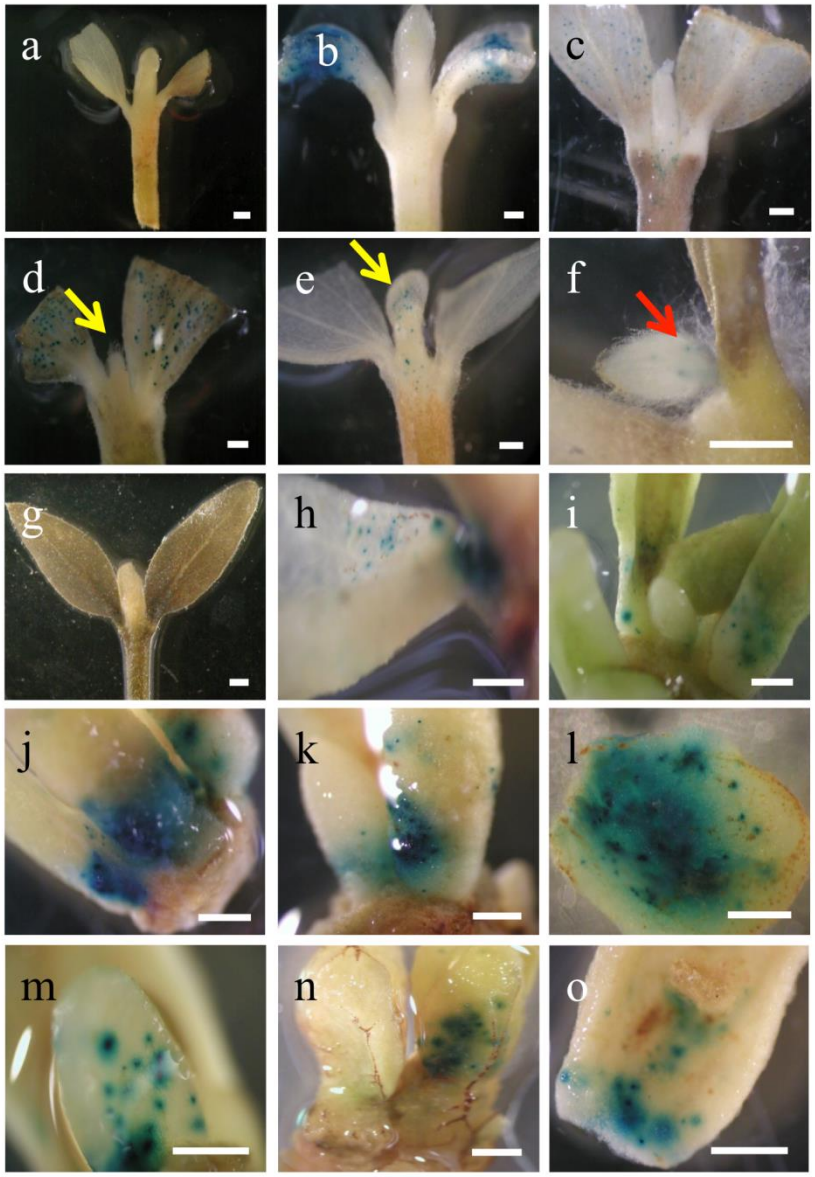

Fig 3.1. Histochemical assay of the β -glucuronidase (GUS) expression in nodal explants of *Simmondsia chinensis*. a-f. Explants 3 days after co-cultivation with the *Agrobacterium* strain. The explant without *Agrobacterium* co-cultivation, sonication and shaking treatments (a), and those co-cultivated with *Agrobacterium* after 1 min sonication and 30 min shaking (b), 2 min sonication and 20 and 30 min shaking (c and d), and 5 min sonication and 10 and 30 min shaking (e and f). g-l. Explants 45 days after shoots regeneration. The explant without *Agrobacterium* co-cultivation, sonication and shaking treatments (g), and those co-cultivated with *Agrobacterium* after 1 min sonication and 20 min shaking (h), 2 min sonication and

20 and 30 min shaking (i and j), and 5 min sonication and 10 and 30 min shaking (k and l). m-o Hygromycin-resistant shoots. Yellow arrows indicate apical cells and the red arrow indicates the axillary bud. Bars = 1 mm.

3.3.3 Effects of physical treatments on transformation efficiency

To examine the effects of physical treatments on transformation efficiency, explants were exposed to three different periods of sonication (1, 2 and 5 min) in the presence of *Agrobacterium*, followed by shaking for 10, 20 and 30 min, and the percentage of GUS-positive explants and the number of GUS-stained spots on them were counted after 3 days of co-cultivation. Data were analyzed, and the results of two-way analysis of variance of the effects of sonication, shaking, and their interaction on the number of stained spots per explant were not significant (Fig. 3.1; Table 3.1). Although there were reports of other plant species showed that wounded tissue caused by physical treatment produced phenolic substances mediating the binding accessibility of *Agrobacterium* to the cell surface (Stachel *et al.,* 1985; Pathak and Hamzah, 2008; Bakshi *et al*., 2011; Subramanyam *et al*., 2011), but our results showed that sonication, shaking and their interaction did not significantly affect the transformation efficiency of jojoba nodal explants. The reason might be due to the variation in sensitivity of plant tissue toward these treatments.

After that we investigated the effects of different sonication and shaking on the shoot regeneration from transformed explants by comparing the average of sprouted shoots per explant. The results showed that the minimum sonication and shaking times (1 and 10 min, respectively) resulted in the highest number of shoots per explant (Table 3.2). The results of two-way analysis of variance found that sonication, shaking, and interactions had statistically significant effects on shoot numbers per explant ($P < 0.05$), which showed that they had negative effects on shoot regeneration. While the average of sprouted shoots from explants without *Agrobacterium* co-cultivation, sonication and shaking was higher than

explants with the treatments (Table 3.2). Our results concur with those of Meurer *et al.* (1998) who reported that the sonication treatment in *Agrobacterium*-mediated transformation significantly decreased number of regenerating shoots in soybean.

Table 3.2. Shoot formation from *Agrobacterium*-infected explants

Sonication (min)	Shaking treatment (min)	Total No. of explants	Shoots per explant**	Estimate for variance of average value in each factor	Estimate for standard error based on ANOVA
1	10	31	4.2 ± 0.3	0.06	0.25
1	20	30	3.4 ± 0.3	0.07	0.26
1	30	31	2.7 ± 0.2	0.06	0.25
2	10	29	2.8 ± 0.2	0.06	0.25
2	20	31	2.5 ± 0.2	0.07	0.26
2	30	31	2.9 ± 0.2	0.06	0.25
5	10	29	3.2 ± 0.3	0.07	0.26
5	20	29	2.8 ± 0.2	0.07	0.26
5	30	31	2.9 ± 0.2	0.06	0.25
—*	—	12	8.5 ± 1.1	0.17	0.41

*The bottom row shows results of explants without *Agrobacterium* infection, sonication, and shaking as the reference.

**Values represent means ± standard error. Two-way ANOVA showed that effects of sonication, shaking and their interaction on the shoot number per explant were significant ($P < 0.05$; Supplementary table 2).

3.3.4 Selection by hygromycin

To test the sensitivity of nodal explants to hygromycin, nodal explants from wild type jojoba plant were cultured on the medium containing different concentrations of hygromycin. The effective concentration of hygromycin was determined. It was observed that with increasing hygromycin, growth and survival response of nodal explants were decreased. Green shoots were observed with 10 mg L^{-1} of hygromycin in 19% of the explants. When the concentration of hygromycin was increased to 20 mg L^{-1}, the rate of explants with green shoots drastically decreased into 4%. The complete death of cells was observed at the concentration 30 mg L^{-1} of hygromycin. The nodal explants cultured on medium without hygromycin (control) grew healthy and showed the 98.1% survival rate (Table 3.3). The results of one-way analysis of variance showed the significant effects of hygromycin on nodal growth. Based on these observations, 30 mg L^{-1} hygromycin was selected to be used in selection of transgenic jojoba shoots from non-transgenic ones.

After the effective hygromycin concentration was determined, *Agrobacterium*-treated and untreated explants with regenerated shoots were cultured on the MS medium containing 30 mg L^{-1} hygromycin for 45 days. The results found a considerable number of shoots from *Agrobacterium*-treated explants could survive in the presence of 30 mg L^{-1} hygromycin (Fig. 3.2). The average frequencies of hygromycin-resistant shoots from *Agrobacterium*-treated explants were in range from 3.3 to 16.4 % (Table 3.4). In contrast, there were no shoots regenerated from *Agrobacterium*-untreated explants could survive (Fig. 3.2; Table 3.4). These results strongly suggest that survived shoots had hygromycin-resistant transgenic cells. A two-way analysis of variance found that sonication, shaking and their interaction had no significant effects on the frequency of hygromycin-resistant shoots. The

results indicated again that sonication and shaking did not affect the transformation efficiency.

To confirm the existence of transgenic cells in survived shoots, we analyzed the expression of the *GUS* transgene in these shoots. RNA was extracted from the survived shoots on the medium with 30 mg L^{-1} hygromycin, as well as from shoots of non-transgenic explants on the medium without hygromycin, and RT-PCR analysis was performed. RT-PCR results showed that the transgene was expressed in regenerated shoots selected by hygromycin, but not in those from *Agrobacterium*-untreated explants (Fig. 3.3). These results strongly support the idea that the survived shoots had transgenic cells; therefore, transgenic jojoba shoots were produced.

Histochemical GUS assay of the survived shoots was performed. GUS activity was observed on tissues of survived shoots (Fig. 3.1 m-o). This confirmed presence of transgenic cells in the selected shoots. The results indicated that the selected shoots were chimera of transgenic and non-transgenic cells. Previously, many reports showed chimeric transgenic plants by *Agrobacterium*-mediated transformation other plant species (McHughen and Jordan, 1989; James *et al.*, 1990; Sangwan *et al.*, 1991; Dong and McHughen, 1993). The possibility of that is due to instability of T-DNA by rearrangement or deletion in some of the cells early in the shoot differentiation (McHughen and Jordan, 1989). Non-chimeric transgenic plants can be recovered either by repeating cycles of selection with increasing concentration of selectable reagent on shoot regeneration medium (Sivanandhan *et al.*, 2015), or by examining a large number of progenies (Dong and McHughen, 1993).

Table 3.3. Effect of hygromycin concentration on shoot regeneration from non-transgenic nodal explants

Hygromycin conc. (mg L^{-1})	Total no. of explants	No. of response explants	Percentage response* (%)
0	52	51	98.1 ± 1.8
10	63	12	19.2 ± 6.6
20	51	2	4.0 ± 2.4
30	50	0	0.0 ± 0.0

*Values represent means ± standard error. One-way ANOVA showed that the effect of hygromycin concentration on shoot regeneration was significant ($P < 0.01$; Supplementary tables 3 and 4).

Table 3.4. Hygromycin resistant shoots from nodal explants

Sonication (min)	Shaking treatment (min)	Total No. of explants	Hygromycin-resistant shoots** (%)	Estimate for variance of average value in each factor	Estimate for standard error based on ANOVA
1	10	39	5.3 ± 2.7	0.0023	0.05
1	20	23	13.2 ± 8.3	0.0023	0.05
1	30	25	16.4 ± 4.5	0.0023	0.05
2	10	32	3.3 ± 3.3	0.0023	0.05
2	20	21	7.9 ± 4.0	0.0023	0.05
2	30	27	8.3 ± 4.8	0.0023	0.05
5	10	25	7.0 ± 3.5	0.0023	0.05
5	20	27	4.2 ± 4.2	0.0023	0.05
5	30	29	5.6 ± 5.6	0.0023	0.05
—*	—	28	0.0 ± 0.0	0.0	0.0

*The bottom row shows results of explants without *Agrobacterium* infection, sonication, and shaking as the reference.
**Values represent means ± standard error. Two-way ANOVA showed that effects of sonication, shaking and their interaction on the rate of hygromycin-resistant shoots were not significant (Supplementary table 5).

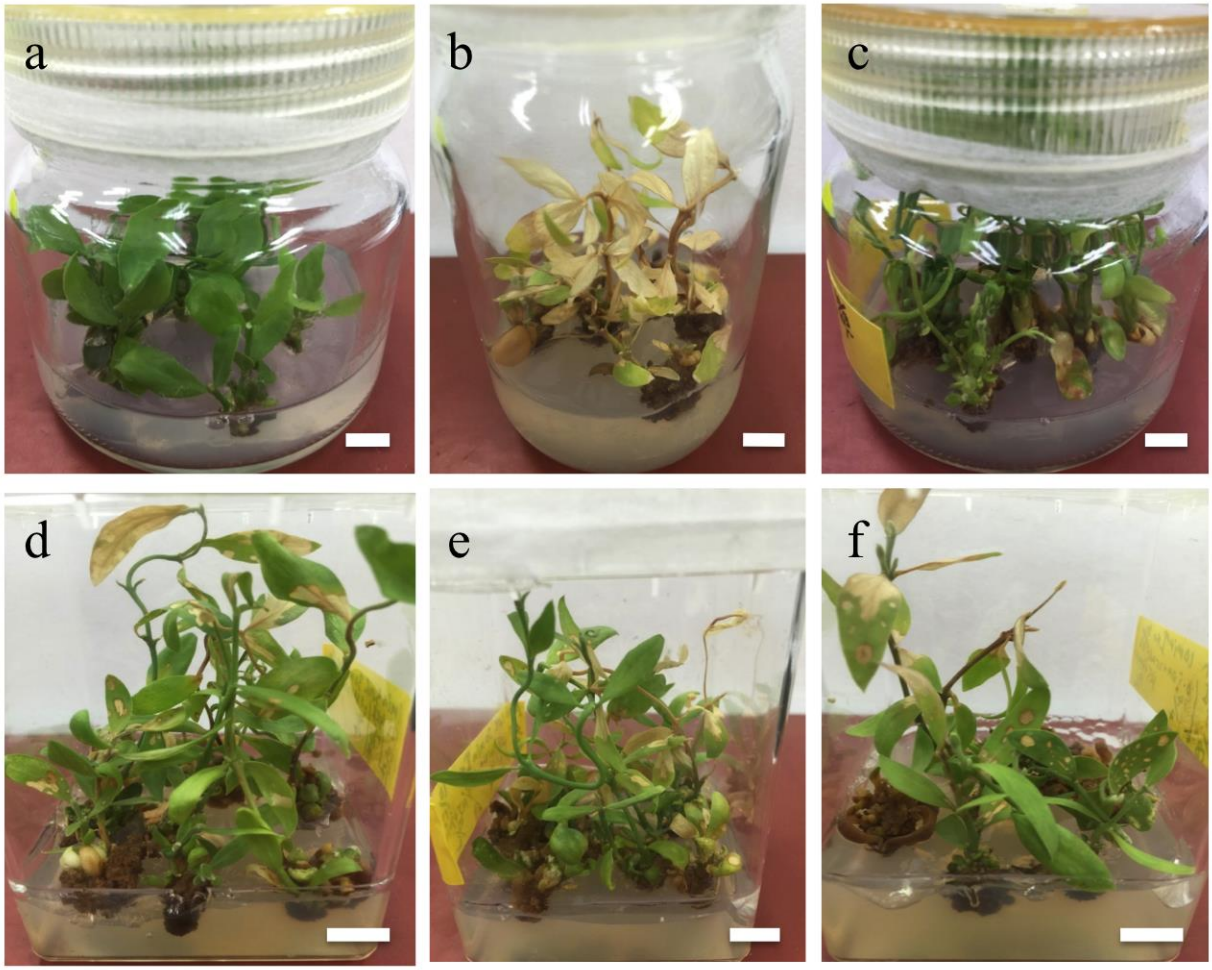

Fig 3.2. Selection of hygromycin-resistant jojoba shoots regenerated from nodal segments. a,b. Regenerated shoots of *Agrobacterium*-untreated explants on MS medium without (a) and with 30 mg L^{-1} of hygromycin (b). c-f. Regenerated shoots of *Agrobacterium*-treated explants on MS medium with 30 mg L^{-1} of hygromycin. Bars = 1 cm.

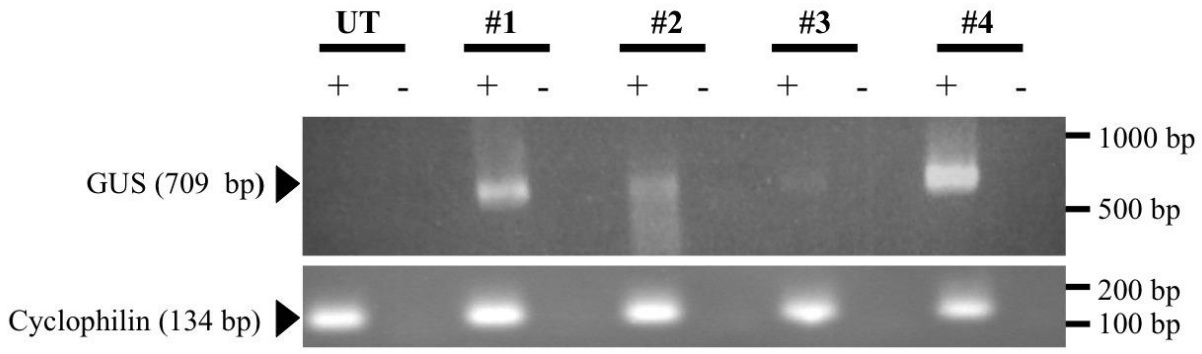

Fig 3.3. RT-PCR analysis. RT-PCR products of the *GUS* transgene (the upper panel) and those of an internal control gene (a jojoba homolog of the potato cyclophilin gene; the lower panel) in regenerated shoots were shown. UT, shoots from the *Agrobacterium*-untreated explant; #1-#4, shoots from four independent *Agrobacterium*-treated explants; (+): with reverse transcriptase; (-) without reverse transcriptase.

3.4 Conclusion

Jojoba cultivation limited to arid and semiarid subtropical regions. In order to improve and expand jojoba cultivation, we have established *Agrobacterium*-mediated transformation system of jojoba. In this study, nodal segments were found to be sufficient for shoots regeneration and genetic transformation of jojoba. Jojoba cells were stably transformed by a foreign gene by using *Agrobacterium*-mediated genetic transformation. Regenerated jojoba shoots from nodal segments contained stably transformed cells. Thirty mg L^{-1} of hygromycin can be used to select the transgenic jojoba shoots. The results of this study represented the successful introduction and expression of a foreign gene into jojoba cells. Obtained results should contribute to improvement of jojoba cultivation and production via generating transgenic plants with desired genes.

3.5 Future prospects

3.5.1 Production of non-chimeric transgenic jojoba plants

Rooting of chimeric transgenic jojoba shoots can be achieved by culturing on MS media containing 2.5 mg L^{-1} IBA. To recover non-chimeric transgenic plants, new shoots will be regenerated from chimeric shoots by repeating cycles with increasing concentrations of hygromycin.

3.5.2 Production of frost tolerance jojoba plants

Spinacia oleracea Betaine Aldehyde Dehydrogenase (*SoBADH*) gene has been reported to enhance frost tolerance in cotton (Luo *et al.*, 2008). For jojoba improvement *SoBADH* gene will be introduced into jojoba plant using established genetic transformation protocol in this study. The *SoBADH* plasmid for ectopic expression in plants was constructed and introduced in *Arabidopsis* to confirm the function of the gene in a foreign plant species.

3.5.3 Candidate genes for jojoba improvement in other traits

- Vernalization: *FLOWERING LOCUS C (FLC*) (Sheldon *et al.*, 2000)
- Waterlogging tolerance: *Vitreoscilla Hemoglobin* (*VHb*) (Du *et al.*, 2016)

Chapter four

General discussion

Jojoba is a unique oil crop that has received much attention in recent years (Arya and Khan, 2016). Jojoba is the only plant that accumulates seed oil consists of liquid wax esters, makes it a valuable industrial crop (Committee on Jojoba Utilisation, 1975). The chemical structure of jojoba is ester and totally different from triglycerides, which is the main component of ordinary vegetative oil, but ester of long-chain fatty acids and fatty alcohols, both of which are unsaturated. It is similar to sperm whale oil, and therefore jojoba oil was firstly used as a replacement for whale oil in many applications (National Academy of Science, 1985; Wisniak, 1987). It is also similar to the wax of human skin, so jojoba oil is now widely used in cosmetic and pharmaceutical industries as one of the best moisturizers. A potential wide range of uses as lubricants, anti-foaming agents, electrical insulators, and plastic industries are also suggested (Reddy and Chikara, 2010). Although jojoba has these advantages, but still there are agronomic and environmental obstacles that affect the productivity and need to be solved. The most important among them are the lack of elite genotypes and efficient mass propagation method for them as well as the severe damage caused by frost. Therefore, this study aims the establishment of an efficient mass vegetative micropropagation method and genetic transformation of jojoba will contribute in improvement of jojoba cultivation and productivity.

Jojoba now is mainly cultivated in several subtropical countries, such as Peru, Israel or Egypt, as an adopted new crop (Benzioni, 1995). In these areas seeds propagation and cutting were used for cultivation. However, the production is not enough for commercial large-scale, due to the shortage of high-yield varieties and lack of effective mass production method. Jojoba is a dioecious plant, is propagated by seeds as well as cuttings, and gives flowers after 3 to 5 years from germination. Seed propagation of jojoba negatively affected

plant growth and predictable yield due to genetic heterogeneity. Thus, vegetative propagation methods should be used for mass production of elite jojoba genotypes. Many studies have been focused on the way to overcome these problems. Bashir *et al.* (2013) found that planting time of jojoba cuttings significantly affected percentage of rooted cuttings and number of propagules. Inote *et al.* (2016) reported successful rooting of jojoba cuttings using different auxins, however, percentage of rooting was still low. Although conventional vegetative propagation methods have been used to propagate jojoba, but they are not effective for mass production due to the limitation of propagules number and time dependent.

Micropropagation is a better alternative to conventional vegetative propagation methods, produces huge numbers of homogenous superior, with conservation of space and time (Nehra and Kartha 1994; Rao *et al.* 1996). Several research groups developed an efficient micropropagation protocols for elite jojoba genotypes (Kacker *et al.*, 1993; Gabr, 1993; Agrawal *et al.*, 2002; Tyagi and Prakash, 2004; Bashir *et al.*, 2008; Singh *et al.*, 2008; Mohasseb *et al.*, 2009; Llorente and Apóstolo, 2013; Hegazi *et al.*, 2014; Bakheet *et al.* 2015). Successful *in vitro* regeneration of jojoba is influenced by various factors such as explants and plant growth regulators (Rai *et al.*, 2010). In most of the previous studies in jojoba, nodal segments were used as explants for shoot initiation and multiplication. Nodal segments have shown to be better choice for explants than the callus or other explants, because of the absence of apical dominance and the presence of axillary buds at an advanced stage of the development (Amin and Jaiswal 1987). In this study, we also have used nodal segments as explants, and they exhibited high efficiency of shoot initiation and multiplication, as expected.

Plant growth regulators played a crucial role in plant *in vitro* regeneration. Success of an efficient regeneration protocol depends on the type and concentration of PGRs in the culture medium. Cytokinins have been found to be the most important for multiplication in many plant species. BA has been commonly used for propagation of jojoba in all previous reports. The advantages of BA for shoot induction and multiplication might be due its ability of to induce production of natural hormones such as zeatin within the tissue (Malik *et al*., 2005; Rai *et al*., 2010). Although BA has been used for jojoba shoot multiplication, but the rate of multiplication is low.

On the other hand, TDZ has been used for shoot multiplication in several plant species and exhibited high efficiency of multiple shoots per explant (Lata *et al.*, 2013; Alatar, 2015; Gambhir and Srivastava, 2015). The effects of TDZ on jojoba shoot initiation and multiplication have not been reported yet. In this study, we have achieved the highest shoot number per explant in jojoba by using TDZ, compared with previous reports. In conclusion, our results would provide the best protocol for a large-scale micropropagation of elite jojoba genotypes and also for the genetic transformation.

Jojoba does not have any breeding program for its improvement. It is only species in the family Simmondsiaceae and does not have any closely-related species. Because it is difficult to introduce useful traits from other species by crossing, the conventional breeding program cannot be useful for the drastic improvement of jojoba. Genetic transformation would be the only effective way to improve jojoba traits to extend its cultivation and production. Because genetic transformation of jojoba has not been established yet, this study aimed to establish it. Here, we have succeeded to stably introduce a foreign gene into regenerated jojoba shoots. Based on results in this study, we will establish the method to

generate non-chimeric transgenic jojoba plants, which would contribute to the jojoba improvement.

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List of publications

Ismail A. MOHAMMED, Suguru TSUCHIMOTO, Hiroe SAKAI, Naoki WADA, Kiichi FUKUI. High-frequency Shoots Regeneration of an Oil Crop, *Simmondsia chinensis* (Link) Schneider Using Axillary Buds. Tropical Agriculture and Development in press.

Ismail A. MOHAMMED, Suguru TSUCHIMOTO, Hiroe SAKAI, Naoki WADA, Kiichi FUKUI. *Agrobacterium*-Mediated Transformation of Jojoba [*Simmondsia chinensis* (Link.) Schneider]. Tropical Agriculture and Development in press.

Supplementary materials

Supplementary table 1. Two-way ANOVA for number stained spots of GUS/ positive explants

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	2671.595[a]	8	333.949	.948	.479	.048
Intercept	14649.517	1	14649.517	41.602	.000	.216
Sonication	348.647	2	174.323	.495	.611	.007
Shaking	16.463	2	8.231	.023	.977	.000
sonication * shaking	2213.689	4	553.422	1.572	.185	.040
Error	53172.405	151	352.135			
Total	71844.000	160				
Corrected Total	55844.000	159				

a. R Squared = .048 (Adjusted R Squared = -.003)

Supplementary table 2. Two-way ANOVA for shoot formation from *Agrobacterium*-treated explant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	58.554[a]	8	7.319	4.919	.000	.131
Intercept	2512.752	1	2512.752	1688.823	.000	.866
Sonication	22.074	2	11.037	7.418	.001	.054
Shaking	15.668	2	7.834	5.265	.006	.039
sonication * shaking	19.806	4	4.951	3.328	.011	.048
Error	389.822	262	1.488			
Total	2966.000	271				
Corrected Total	448.376	270				

a. R Squared = .131 (Adjusted R Squared = .104)

Supplementary table 3. ANOVA for shoot regeneration from non-transgenic nodal explants

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.183	3	1.061	119.682	.000
Within Groups	.151	17	.009		
Total	3.333	20			

Supplementary table 4. Multiple comparisons shoot regeneration from non-transgenic nodal explants by Tukey-Kramer test

(I) Hygromycin	(J) Hygromycin	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
without hygromycin	10 mg L hygromycin	.79015*	.05701	.000	.6281	.9522
	20 mg L hygromycin	.94182*	.05955	.000	.7726	1.1111
	30 mg L hygromycin	.98182*	.05955	.000	.8126	1.1511
10 mg L- hygromycin	without hygromycin	-.79015*	.05701	.000	-.9522	-.6281
	20 mg L hygromycin	.15167	.05701	.071	-.0104	.3137
	30 mg L hygromycin	.19167*	.05701	.018	.0296	.3537
20 mg L- hygromycin	without hygromycin	-.94182*	.05955	.000	-1.1111	-.7726
	10 mg L hygromycin	-.15167	.05701	.071	-.3137	.0104
	30 mg L hygromycin	.04000	.05955	.906	-.1293	.2093
30 mg L- hygromycin	without hygromycin	-.98182*	.05955	.000	-1.1511	-.8126
	10 mg L hygromycin	-.19167*	.05701	.018	-.3537	-.0296
	20 mg L hygromycin	-.04000	.05955	.906	-.2093	.1293

*. The mean difference is significant at the 0.05 level.

Supplementary table 5. Two-way ANOVA for hygromycin resistant shoots from nodal explants

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.045[a]	8	.006	.811	.602	.265
Intercept	.169	1	.169	24.488	.000	.576
sonication	.019	2	.010	1.385	.276	.133
shaking	.011	2	.006	.808	.461	.082
sonication * shaking	.015	4	.004	.527	.717	.105
Error	.124	18	.007			
Total	.338	27				
Corrected Total	.169	26				

a. R Squared = .265 (Adjusted R Squared = -.062)

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