CHAPTER I

Introduction

Historical Reviews of *Carthamus tinctorius* Linn.

Safflower (*Carthamus tinctorius* Linn.) is one of the world's oldest food and edible oil crops grown primarily in Asia, Mediteranean, Africa and Europe, as a source of dye (Vaughan 1970). It is also use as a source of edible and industrial oil distribute in the region of arid and semi-arid tropics of India, Mexico, U.S.A., Ethiopia and Australia (Litzenberger 1974). Although it constitutes only about 0.5% of the total oil seeds production of the world (FAO 1985), safflower oil is considered as a premium cooking oil due to its high content of linoleic acid (C18:2), high iodine value, light yellow color and a characteristic pleasant flavour (Salunkhe *et al.* 1992). Safflower seeds oil is used for culinary purpose in most countries where it is grown. Recently, the demand for safflower oil in developed countries is increased and it is now commonly utilized for many food products, including cooking oils, margarine, mayonnaise, salad dressing, frozen desserts and speciality breads. Many of these uses are direct results of the considerable publicity given to the possibility of replacing saturated fatty acids by unsaturated fatty acids in the diet to reduce the high cholesterol content of the blood, which is allegedly associated with artherosclerotic disease (Salunkhe *et al.* 1992).

For oil quality improvement program, there are many strains and varieties of safflower grown in many parts of the world. The breeding of varieties has been based on conventional methods which are now available with higher yielding ability and oil content, varying plant height, of growing periods and disease resistance

(Litzenberger 1974). Another interesting aspect related to sources of oil is the desired fatty acids composition of safflower oil. The fatty acid composition of safflower has been studied for breeding and selection programs (e.g. Fuller *et al.* 1966, Knowles 1969 and Fedeli *et al.* 1972).

In the present times, plant tissue culture biotechnology can lead to improve in the nutritional characteristics of oils and can improve oil yield and other agronomic characteristics in a shorter period of time than conventional breeding method (Sharp 1985). The biotechnology techniques such as somaclonal and gametoclonal variation, protoplast fusion and clonal propagation could be selected against particular oil crop species to develop improved genetic traits (James 1985). Furthermore, the application of genetic engineering to oil seed crops appears to be feasible and has to use a broad knowledge base on lipid biosynthetic enzymes (Knauf 1987). The application of these techniques for improvement of genetic traits required continued development of tissue culture procedures for isolated single cell clones from a mixed cell population, as well as for the selection of clones containing increased quantities of desired fatty acids. For these techniques, regeneration of plants from cell clones using tissue culture can result in the recovery of genetically modified plants called somaclonal variants (Mangold 1986).

Botanical aspect of *Carthamus tinctorius* Linn.

Safflower is a member of the compositae which is a very large, widespread, successful group of plants that is found throughout the world. The family includes other important crops such as sunflower (*Helianthus annuus*), niger seed (*Guizotia abyssinica*), pyrethrum (*Chrysanthemum cinerariifolium*) and lettuce (*Lactuca sativa*) (Cobley 1976). Safflower is an annual, erect herbaceous and highly branches plant which grows to a height of 35 to 150 cm., depending upon the planting time,

environmental conditions and variety (Purseglove 1974). The stem is stiff, cylindrical, fairly thick at the base and tapering towards the growing end. The color of stem is gray or green to white and the main stem grows to primary, secondary and tertiary branches, each terminating into flowering head called capitulum (Litzenberger 1974). The leaves are simple, dorsiventral, sessile, oblong to lanceolate and alternate. The leaves and the bracts below the flower of most commercial cultivars have short spines, although spineless cultivars have been developed to facilitate harvesting. The root system strongly developes tap root with branches that penetrate into the soil to a depth of 2 to 3 m. giving drought tolerant character to the plant. The fruits or seeds of the plant are achenes. The achenes consist of 40-60% by weight of pericarp or hull, 22-42% of oil and 20-55% of protein. The percentage of oil and protein is very variable depending on the thickness and weight of the pericarp (Cobley 1976).

Chemical compositions of safflower (*Carthamus tinctorius* Linn.) seeds.

The chemical composition of safflower seeds has been reported by several researchers (e.g. Applewhite 1966, Knowles 1969, Betchart 1979 and Guggolz *et al.* 1968). The factors that influence the concentration of various chemical constituents in seed include the genetic background of the variety, geographic region exerting the effect of latitude and altitude, climate with respect to day and night temperatures, the amount of rainfall during periods of flowering and seed setting, agronomical practices such as application of fertilizers, sowing dates, plant population and irrigation, soil fertility and use of chemicals like growth regulators. Among these variations, plant types and geographic effects cause significant differences in the chemical composition as compared to other factors (Salunkhe 1992).

1. Lipid content and fatty acid composition.

Safflower oil is pale or golden yellow. The oil constitutes mainly triglycerides or neutral lipids. Linoleic acid (C18:2) is a major fatty acid found in phosphatides of safflower oil. Many researchs have been reported clearly on the genetic variability of the oil content in different available safflower genotypes. The common white, normal hull, high linoleic acid (C18:2) types usually contain 25-40% oil. Many researchs about fatty acid composition of safflower oil has been studied (e.g. Bratcher 1969, Knowles 1969). The fatty acid composition of commercial safflower cultivars contains 55-81% linoleic acid (C18:2), 7-42% oleic acid (C18:1) as major fatty acids, followed by 1-10% stearic acid (C18:0) and 2-10% palmitic acid (C16:0) as minor fatty acids. The unsaturated fatty acids make up about 90% while the remaining 10% of total lipids are saturated fatty acids. The factors that influence the fatty acid composition of safflower oil include variety, location, temperature and plant types (Stumpf 1975). Stumpf (1975) reported that the types of fatty acids synthesized in safflower seed may be quite variable as defined by the genetics of the specific plant but the fatty acid composition in safflower leaf lipids is highly conserved in the part where synthesis is taken place and these fatty acids are not transported to other parts of the plant.

Plant tissue culture for regeneration (micropropagation).

The methods of micropropagation have more advantages than traditional methods in several aspects (Bajaj 1988):

- cultures are initiated with very small pieces of plants (explants), and only a small amount of space is required to maintain large number of plants.
- propagation is carried out in aseptic conditions, free from pathogen.

- plant production can be continued throughout the year and independent of seasonal changes.
- vegetatively reproduced material can often be stored over a long period.
- no need for laborious attention between subculturing.

Micropropagation techniques have been developed and widely used in many areas as shown in Figure 1.

Five main stages for *in vitro* multiplication of plants has been defined by Murashige (1974). These stages have been used widely in many commercial and institutional tissue culture laboratories. For the main five steps are explained below:

Stage 1: Selection and preparation of explants

Plant which is a representative typical of each variety and disease free would be selected as a mother plant for *in vitro* culture. Growth, morphogenesis and rates of *in vitro* propagation can be improved by appropriate environmental and chemical pretreatment of mother plant.

Stage 2: Development of aseptic culture

The second step in the micropropagation process is to obtain an aseptic culture of the selected plant material. The explants should be aseptically transferred to the culture environment and completed a number of survived explants without contamination.

Stage 3: The production of suitable propagates

The object of this step is to bring about the multiplication of organs and structures that are able to give rise to new intact plants. This step includes the prior induction of meristematic centres from which adventitious organs may develop.

Stage 4: Preparation for growth in the natural environment

Shoots or plantlets derived from stage 3 are very small and not yet capable of self-supporting for growth in soil or compost. This step is taken to grow

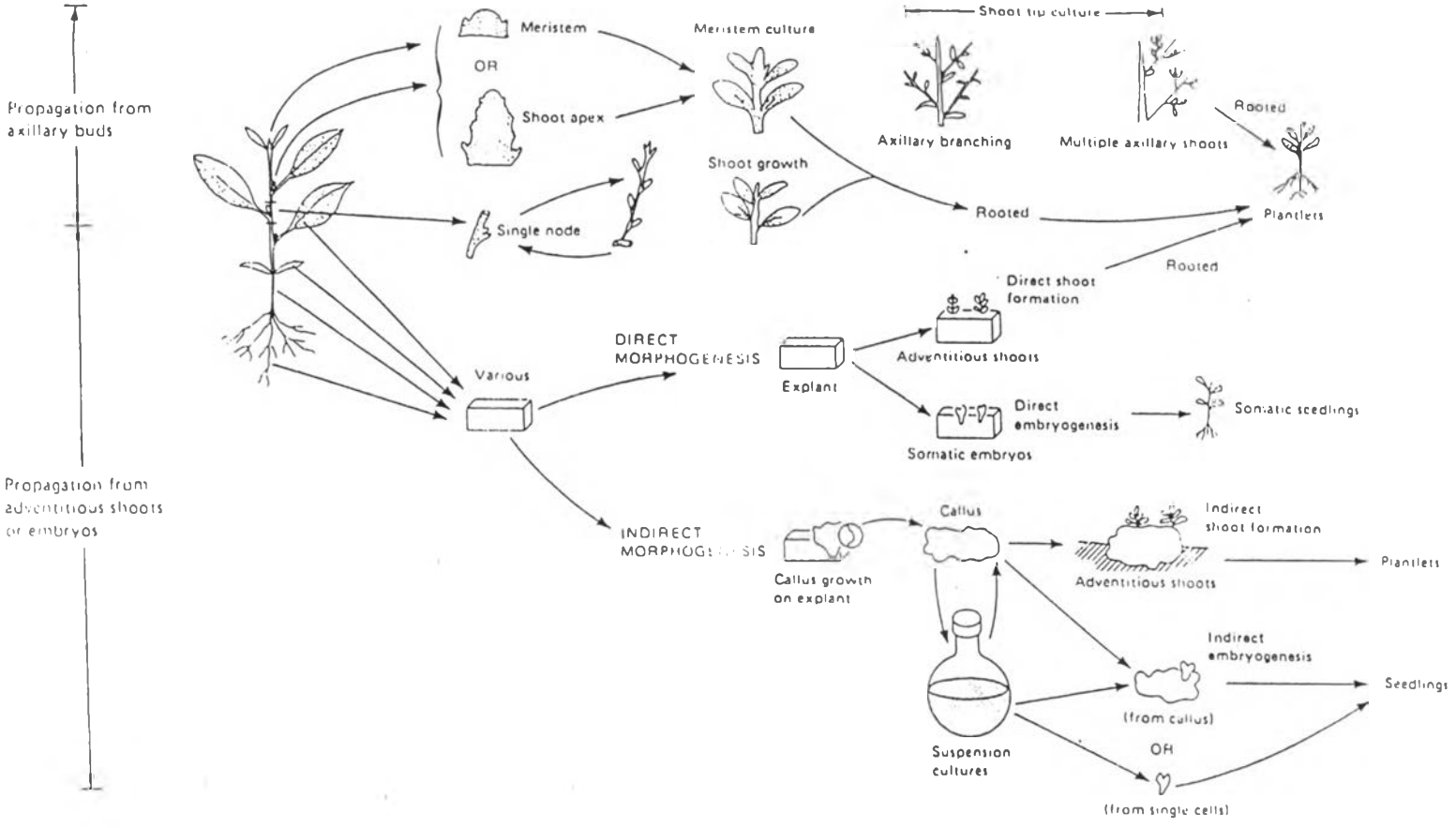

Figure. 1 The principle methods of micropropagation (George and Sherrington 1984)

individual plantlets that can carry out photosynthesis and survive without an artificial supply of carbohydrate. This step includes the *in vitro* rooting of shoots prior to their transfer to soil. In some species, this step is necessary to have elongated shoots ready for rooting.

Stage 5: Transfer to the natural environment

This step is to transfer plantlets from the *in vitro* growth to the extra external environment carefully. This step is very important because improper transfering methods can result in a significant loss of new plants.

Some plants in which tissue culture has been used successfully for regeneration are listed in Table 1.

Plant tissue culture of safflower (*Carthamus tinctorius* Linn.)

Plant tissue culture of *Carthamus tinctorius* Linn. was first observed by Nag and Pandey (1978) (cited by George and Sherrington 1984). They reported that roots regeneration (rhizogenesis) could be induced from hypocotyl callus which was cultured on MS medium supplemented with 0.2 mg/l NAA, 0.1 mg/l BA and 3.0% (w/v) sucrose under dark incubation period at $25\pm2^{\circ}C$.

George and Rao (1982) obtained shoot buds and plantlets regeneration from cotyledons and hypocotyl callus cultured on MS medium with various growth regulators. They found that cotyledons were more regenerative than hypocotyls. BA was proved to be effective for shoot bud regeneration than other cytokinins such as kinetin, 2-iP and zeatin. Rooting of shoot buds was obtained on hormone free MS medium containing with high concentrations of sucrose (6-8%w/v).

Tejovathi and Anwar (1984) obtained *in vitro* capitula (head-inflorescence) induction and shoot initiation from the cut end of inner surface of cotyledons whereas callus initiation was also observed at the outer surface of tissues cultured

Table. 1 *In vitro* regeneration studies on some plant species.

Plant species	Source of explants	Morphogenesis/propagation	References
Allium cepa	meristem segments	plantlets regenerated	Hussey 1978
Arabidopsis thaliana	immature cotyledons	shoots formation	Patton and Meinke 1988
Camllia sasanqua	shoot tips	shoots, roots organogenesis	Torres and Carlisi 1986
Capsicum annuum	mature embryos	plantlets regenerated	Agrawal and Chandra 1983
Centaurea junoniana	cotyledons, leaves	shoot organogenesis	Hammatt and Evans 1985
Cichorium intybus L.	mature tap roots	plantlets regenerated	Heirwegh et al 1985
Coffea arabica	shoot tips seedling	plantlets regenerated	Kartha et al 1981
Cucumis melo	cotyledons	shoots,roots organogenesis	Chee et al 1991
Digitalis thapsi L.	shoot tips	shoot multiplication, roots	Herrera et al 1990
Gossypium arboreum	immature embryos	plantlets regenerated	Gill and Bajaj 1984
Helianthus annuus L.	immature embryos	shoots,roots embryogenesis	McCann et al 1988
Lotononis bainesii	cotyledons and leaf	shoots organogenesis	Bovo et al 1986
Medicago spp.	roots, hypocotyls	embryogenesis	Nagarajan et al 1986
Mentha arvensis	nodal segments	plantlets regenerated	Rech and Pires 1986
Papaver bracteatum	seeds	plantlets regenerated	Day et al 1986
Paspalum notatum	immature embryos	plantlets regenerated	Bovo and Mroginski 1989
Phytolacca dodecandra	shoot tip and nodal	plantlets regenerated	Demeke and Hughes 1990
Pinus ponderosa	mature embryos	buds regenerated	Ellis and Bilderbach 1984
Trifolium medium L.	petiole segments	plantlets regenerated	Choo 1988
Trifolium pratense L.	hypocotyl and petiole	plantlets regenerated	Maclean and Nowak 1989
Zea may	immature embryos	embryogenesis	Songstad et al 1988
Zingiber officinale	rhizome	embryoids and plantlets	Hosoki and Sakawa 1977

on MS medium supplemented with 0.5 mg/l BA and 0.1 mg/l NAA. Continuous subcultured onto the same medium, capitula and callus subsequently turned brown.

Prasad *et al.* (1991) induced *in vitro* haploid plants from anther culture of safflower (*Carthamus tinctorius* Linn.) and recommended that MS medium was the most suitable for inducing androgenic callus. Differences in induction of androgenic calli among ten genotypes revealed that the most response genotype was a local cultivar; Manjira, with 48.6% anther initiating callus formation. They also observed that pre-treatment of immature capitula resulting in increasing callus induction.

A good callus proliferation rate and greening in shoot tip cultures were achieved by adding 5-10 mg/l adenine sulphate into medium (Singh 1991). The result found that BA was approved to be a critical cytokinin supplement during regeneration phase. The plantlets and bud initiation were proliferated extensively from shoot tips of wild safflower (*Carthamus oxycantha* MB.) in MS medium containing proper concentration of NAA, ascorbic acid and low level of GA_3.

Ying *et al.* (1992) obtained a reproducible transformation of safflower tissue and regeneration of transgenic shoot buds expressing the GUS reporter gene by inoculated with *Agrobacterium tumefaciens* containing NPT II and GUS genes.Their results suggested that callus formation from cotyledon, leaf and stems explants were successful on MS medium containing 1.0 mg/l NAA and 1.0 mg/l BA. Substitution of 2,4-D for NAA did not improve callus forming efficiency. The best tissue source for bud regeneration was observed on leaf calli.

Lipid biosynthesis in plant cell culture.

In recent years the study of lipids in plant tissue cultures had attracted much attention for academic as well as applied interest. The lipid biosynthesis and metabolism of lipid in plant cell cultures such as callus as well as cell suspension

cultures was quite low. Some of the studies clearly showed that fatty acid composition of total lipids was influenced by chemical and physical properties of the media in which cells were cultured (Mangold 1986).

In Rape (*Brassica napus* cv. *Target*) and Turnip rape (*Brassica campestris* cv. *Echo*) callus cultures, the constituent fatty acids of the triglycerides from the two tissue cultures contained more than 50% of linolenic acid (C18:3), but they were free of erucic acid (C22:1) which is a major fatty acid in the triglycerides from seed oils of both species (Staba *et al.* 1971).

In most tissue cultures of plants such as *Yucca glauca* Nutt., *Corchorus olitorius* L., and *Corchorus capsularis* L. the predominant fatty acids were palmitic acid (C16:0) and oleic acid (C18:1). In each case the seeds contained linoleic acid (C18:2) as the predominant fatty acid (Stoller *et al.* 1974).

Pence *et al.* (1981) demonstrated that there was a shift in the fatty acid composition from predominantly polyunsaturated to saturated and monounsaturated fatty acids. It was characterized by an increase in the mole percents of stearic acid (C18:0) and oleic acid (C18:1) and a decrease in the mole percents of linoleic acid (C18:2) and linolenic acid (C18:3) when the sucrose concentration in culture was increased.

Lipid composition in callus cultures from *Ricinus communis* endosperm upon transfer from darkness into light. In light grown cultures the major lipid classes were phospholipids and glycolipids while dark grown cultures were rich in triacylglycerol. The major fatty acids were linolenic acid (C18:3) and palmitic acid (C16:0) in both cultures (Gemmrich 1982).

Comparative study of fatty acid composition of the total lipids extracted from different developmental stages and callus formation of *Cucumis melo* cotyledons yielded a large proportion of unsaturated fatty acids with linoleic acid (C18:2) as the

major component, whereas those of cotyledon callus show a marked reduction in linoleic acid (C18:2), together with the increment in linolenic acid (C18:3) and palmitic acid (C16:0) as a predominant components (Halder and Gadgil 1984).

Manoharan *et al.* (1988) studied on the lipid composition of callus cultures of *Datura innoxia* grown on media supplemented with phospholipid precursors. They found that choline or ethanolamine supplementation inhibited greening and shoot differentiation and led to an increase in the content of the major phospholipids, phosphatidylcholine and phosphatidylethanolamine and to decrease in the content of galactolipids. The relative content of phosphatidylinositol having an increase relationship with the degree of differentiation, was increased to the content comparable to that of the non-green undifferentiated callus.

In leaf callus culture of safflower, the oil content in callus cultured on MS medium under dark incubation period with high sucrose content (5.0% w/v) enhanced the oil level upto two-fold while inclusion of precursor bases of lipid synthesis reduced level of oil content (Singh and Chatterjee 1991).

In order to understand more about the regeneration nature and the regulation mechanism of lipid biosynthesis in *Carthamus tinctorius* Linn. tissue culture in this present study, cultures of *Carthamus tinctorius* Linn. were established from various parts of explants especially cotyledons and hypocotyls from steriled natural seed cultured. Several nutritive including hormonal conditions were optimized to obtain the reproducible protocol for safflower regeneration system with the local variety of Thailand. The biosynthesis and regulation mechanism of fatty acids components were also studied with the ultimate goal of using the developed model to the crop improvement.