CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Classification

The genus *Citrus* is in the family Rutaceae which consists of 150 genera and 1600 species, mainly trees and shrubs, with the largest number of species in the warmer regions of the world. The subfamily Aurantioideae which is commonly known as the Orange subfamily consists of two tribes, namely *Clauseneae* with 3 subtribes and 5 genera very remote to *Citrus* species and the tribe *Citreae* which constitute of 3 subtribes with 28 genera including the *Citrus* species and all of its near relatives (Swingle, 1967) (Table 1). Most of the genera are found in the region from Pakistan to north-central China through the East India Archipelago to New Guinea, North Eastern Australia and New Caledonia. At least 29 of the 33 genera from the sub family Aurantioideae and nearly 65% of the 200 species are native to this region of the world, with 27 genera not found elsewhere. Five genera, belonging to two tribes and three subtribes, are native to tropical Africa and of these, four genera are found only there. The genus, *Clausena*, is native to both regions.

The classification of *Citrus* is difficult because most *Citrus* cultivars have been subjected to natural hybridisation since ancient times, and because of the wide occurrence of mutants and nucellar embryony. Tanaka (1954) recognized 159 species, giving species status to many garden varieties. However, the Swingle and Reece (1967) classification system, which comprises 16 species, is the most widely accepted one. It accords species rank, among others, to mandarin (*C. reticulata* Blanco), shaddock known also as pummelo (*C. grandis* Osbeck), sour orange (*C.*

aurantium L.), sweet orange (*C. sinensis* Osbeck), lime (*C. aurantifolia* Christm.), citron known also as ethrog (*C. medica* L.), lemon (*C. limon* L. Burn. f.), and grapefruit (*C. paradisi* Osbeck).

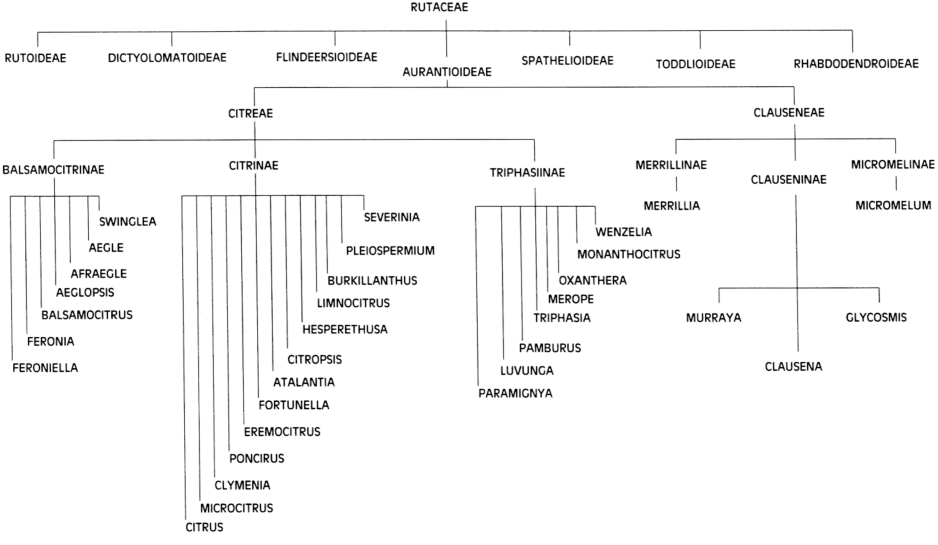
A different classification based on 146 characters of the tree form, leaf, flower, and fruit was suggested by Barrett and Rhodes (1976). They recognized only three valid species within edible citrus viz.; mandarin (*C. reticulata* Blanco), pummelo (*C. grandis* Osbeck), and citron (*C. medica* L.). They also speculated about the interrelationships of the other species of cultivated citrus. They suggested that lime and lemon are trihybrids involving citron, pummelo, and *Microcitrus*, with lemon carrying a greater proportion of the citron genes; and sour orange and sweet orange are hybrids between mandarin and pummelo, having a parallel but separate origin within the polytypic *C. reticulata*.

Citrus classification is still a controversial issue. In this study, Swingle's classification system was adopted.

1.2. History of the crop

Citrus fruits are native to a large Asiatic area extending from the Himalayan foothills of India to north central China and the Philippines in the east, and Burma, Thailand, Malaysia, Indonesia, New Guinea, northeastern Australia, and New Caledonia in the southeast. Centers of origin of the main commercial citrus species

Table 1. Classification of the Aurantioideae (from Swingle 1967)



are difficult to ascertain because they have been subjected to natural hybridisation and probably cultivation since ancient times (Ammirato *et al*, 1984). According to Cooper and Chapot (1977), almost all known citrus fruit cultivars are believed to have originated from China, with the exception of lemon and grapefruit. *Poncirus* is native to central and northern China, whilst *Fortunella* is native to southern China. The mandarin (*C. reticulata*) and pummelo (*C. grandis*), oranges have been mentioned in Chinese writings since 2000 B.C. (Cooper and Chapot, 1977). Citron (*C. medica*) has been suggested to be native to southern China and India. It was the first citrus which interested the Europeans, and was probably cultivated in Babylonia and Egypt before 1500 BC, from which it was introduced to the Near East and Greece (Reuther *et al*, 1967). The sweet orange (*C. sinensis*) probably originated in southern China, while the sour orange (*C. aurantium*) is endemic to Southeast Asia. The sour orange was also known to the Romans and was later carried by the Arabs to many countries, including Spain. There is no written evidence of actual culture of sweet orange in Europe until the fifteenth century. Certainly introductions were made by Genoese trades, from China (around 1400's AD) and later, by the Portuguese. Lemon followed the same route as sweet orange (Barrett and Rhods, 1976). Lime (*C. aurantifolia*) is probably native to the East India Archipelago whilst grapefruit (*C. paradisi*) appeared in the West Indies, during the end of the eighteenth century as a natural hybrid between pummelo and sweet orange (Swingle, 1943; Albaach and Redman, 1969).

Christopher Columbus introduced *Citrus* into what is now North America at the end of fifteenth century. At the beginning of the sixteenth century, the Portuguese and Spanish started to spread it to the rest of of the American continent. Orange, lemon, and pummelo reached South Africa in the mid-seventeenth century and Australia at the end of the eighteenth century (Reuther *et al*, 1967). Citrus world trade was already important during the second half of the nineteenth century and since that time has grown considerably in volume, number of varieties, and countries of production.

1.3. Geographic limitations

Citrus fruits are cultivated from 40° north and south of the Equator. Most of the commercial crop is grown in the subtropical countries between sea level and altitudes of 2000 feet. Most species can stand low winter temperatures not less than -9 °C and a relatively high temperature of up to over 37 °C (Anonymous, 1986). Citrus species are not suited to the very humid tropics as high atmospheric humidity is believed to be associated with susceptibility to pests and diseases. They have capability to grow over a wide range of soil types but a light loamy soil of good fertility and drainage is preferred (Purseglove, 1984)

The three major regions in the subtropical latitudes of the northern hemisphere where citrus are cultivated on a large scale are North America, the Mediterranean, and East Asia including India. Large producers in the southern hemisphere include Brazil, Argentina, South Africa, and Australia. Extreme frost

limits cultivation, and only hardy sour orange types of unshu mandarin grafted on *Poncirus* can be grown successfully in severe climates. In tropical regions, near the Equator, oranges are usually grown for local consumption because of poor fruit colour and high susceptibility to the diseases' infection due to climatic factors such as high temperature and humidity. The colour of fruits is due to the carotenoids located in the chloroplasts which are temperature sensitive as some of these pigments only develop when the temperature remained below 13 ^{o}C for several hours (Samson, 1980).

1.4. Economic importance

Economic importance of the Aurantioideae species have been well documented throughout the tropics and warm regions. These include as a source of food, natural products, medicines and oil and several genera have been used as root stocks (Nobumasa *et al*, 1990).

Citrus is grown in over 100 countries extending through Africa, North and South America, Asia, Europe, and Australia making the genus one of the most important fruits commercially (Bajaj, 1985). The important citrus varieties include: (a) the oranges; navel, shamouti, valencia, hamlin, and blood oranges (taroco and moro); (b) grapefruits; marsh seedless, and coloured grapefruit; (c) tangerines and mandarins; clementine, satsuma (unshu), ponkan, avana, and dancy and, (d) lemons; femminello, eureka, Lisbon, verna, fino. Orange is the most important of these citrus

fruits in world trade, both for fresh fruit and fruit by-products followed by grapefruits, mandarins and lemons (Ammirato *et al*, 1984).

Amongst the citrus-producing countries in the world, only 17 of them produce more than 1% of the total world production. The major producing countries include United States (22.3%), Brazil (19.0%), Japan (7.7%), Mexico (5.0%), Spain(5.0%), Italy (5.0%), and South Africa (5.0%), India (3.0%) and Morocco (3.0%), China and Argentina (2.5%), Turkey (2.0%) and Egypt (2.0%). Of these, the major exporters are Brazil, Spain, the United States, Italy, Greece, Morocco, and South Africa. They account for 85.0% of all citrus trade (Ammirato *et al*, 1984).

Large quantities of sweet orange, grapefruit, and mandarin are processed into juice, canned fruits, frozen concentrates, squashes, and beverages. Smaller productions are used for manufacture of marmalade, jam, pectin and essential oils (Cooper and Chapot, 1977). Citrus fruits are also an important source of vitamin C since the juice and peel of orange and lemon fruits contain considerable quantities of ascorbic acid (Reuther *et al*, 1976).

1.5. Breeding and citrus improvement problems

Most citrus and its related genera are diploid in nature (2n = 18). However, natural tetraploids and triploids occur in small frequency. Tetraploids generally arise spontaneously as nucellar seedlings in all major groups of cultivars (Cameron and Frost, 1968). On the other hand, triploids which are of no economic value, regularly

develop as sexually produced seedlings. They can also be systematically produced by controlled crossing of tetraploids with diploids (Esen and Soost, 1972). Genetic studies in *Citrus* are complicated because of nucellar embryony, heterozygosity, self-incompatibility, sterility, and long generation cycle. Nucellar embryony in Citrus is an important obstacle for hybridization, but it is of great value in producing vigorous, uniform, virus-free rootstocks (Frost and Soost, 1968). Self-incompatibility has been demonstrated in *C. grandis* (Soost, 1964), clementine mandarin, tangelos, in some *C. limon* cultivars, and in *C. limettoides* and *C. sinensis* (Soost and Cameron, 1975). Sterility factors in ovule and pollen are widespread in citrus (Iwamasa, 1966). These factors coupled with parthenocarpic tendency of fruit setting have contributed to the selection for seedless *Citrus* fruits. The long juvenile period, high heterozygosity and the paucity of knowledge on mode of inheritance of desirable characters are also serious problems to breeding programs (Vardi and Spiegel-Roy, 1978).

While it is relatively easy to produce new varieties of mandarins or mandarin-types by hybridization, it is very difficult to improve sweet oranges, grapefruit, and lemon with similar methods due to polyembryony, sterility and long juvenile period. In such cases the search for mutants might be a better alternative. Spontaneous mutations occur frequently in Citrus and many of the world's most important cultivars have arisen via this method. Commercially important mutants include the satsuma and clementine mandarins, navel and shamouti oranges, and also marsh grapefruit. Variation between nucellar progeny has also given rise to selections such as the early ripening clones of (*C. unshu*) Miho and Okitsu (Iwasaki *et al*, 1966).

In rootstock breeding, the ultimate goal is the production of rootstocks resistant to diseases (e.g. tristeza, *Phytophthora*, nematodes), and possibly tolerant to environmental stresses such as cold, temperature, drought and salinity. For rootstocks to be successful it should produce high yield, good fruit quality in the scion and have good vigor.

Poncirus trifoliata is often employed in most recent rootstock breeding programs (Bitters *et al*, 1973) because not only does it carry a genetic marker, but it also is resistant to the tristeza virus, *Phytophthora* rot, nematodes, and is cold-tolerant (Hearn *et al*, 1974). Intergeneric hybridization offers the possibility of incorporating multiple desirable traits found in different genera into the germplasm of new improved citrus rootstock (Barrett, 1977).

1.6. Citrus diseases

A citrus crop is threaten by many types of pests and diseases that reduce crop production worldwide. Virus and virus like diseases are the most complex and damaging. Virus diseases are transmitted by grafting or mechanically by contaminated grafting and pruning tools or by insects (e.g. Tristez virus diseases). Tristeza, considered to be the most fatal, killed 20 million trees of sour orange rootstocks in Brazil and Argentina over a 30 year period (Reitz, 1984). Tristeza or one of its several variants is world wide in occurrence today.

Bacterial diseases such as the greening disease is a serious problem in some regions especially in the cooler citrus production parts of the world because most citrus species are affected by the disease regardless of the rootstocks. However, some species like *C. grandis,* which is indigenous to the tropics, are somewhat tolerant to the disease (Samson, 1986). Bacterial canker is another disease that severely affects the young parts of susceptible varieties.

Diseases caused by mycoplasma also affects citrus trees to some degree in California and all the Mediterranean basin. The disease reduce fruit bearing capacity in commercial citrus varieties, particularly in navel oranges and is transmitted by several ways such as grafting and insects (Igwegbe, 1970). Another serious disease, foot rot, is caused by the fungi *Phytophthora* and significantly causes a decline in citrus fruit production (Nito and Akihama, 1990).

Several insects such as the scale insects, white flies, mites and fruit flies cause major problems in citrus farms by, *inter alia,* damaging foliage, and deforming fruits. These types of disorders can be controlled by chemical treatments or cultural practices. However, diseases caused by virus or virus like agents, cannot be adequately controlled by such treatments (Purseglove, 1984).

Vegetative propagation from symptomless carrier trees has been a major source of decline for diseases caused by virus and virus like agents. In this respect, the tissue culture and micropropagation techniques of disease free plant parts, such as

the meristematic tissue have provided great results in controlling such diseases and disorders which are difficult to control by conventional methods.

1.7. Plant Tissue culture

Plant tissue culture is the ability to regenerate and grow plant tissues, callus, isolated organs and embryos aseptically by placing them under controlled environmental conditions such as light, temperature and humidity. It is based on the principle of totipotency, the concept that every living plant cell has the genetic potential to produce an entire plant (George and Sherington, 1984)

It is used, *inter alia,* for propagation, genotype modification, plant breeding, production of biochemical products and plant pathology control. Through micropropagation technique, plant improvement by protoplast technology and gene transformation have become successfully applicable on a considerable diversity of plant species. It has become an invaluable tool in the field of agriculture, horticulture, forestry, pathology and genetic engineering.

Somatic or asexual embryogenesis is the production of embryo like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin. These embryos can develop and germinate into plantlets (Tisserat *et al*, 197). Organogenesis is the formation and outgrowth of shoots from callus or the initiation of axillary buds generated from

culture tips, and their subsequent adventitious rooting (Dixon, 1985). Plant regeneration can be through two different paths, direct and indirect. Indirect plant regenerations are through callus intermediary but not so for direct regeneration.

Tissue culture media play an important role in inducing plant regeneration. Nutrients in tissue culture media usually consist of inorganic salts, one or more carbon source, some vitamins and growth regulators. Other components added for specific purposes include organic nitrogen compounds, and plant extracts such as coconut milk (CM) and orange juice (OJ). Among media which are frequently used include, Murashige and Skoog (MS) (1962), Murashige and Tucker (MT) (1969), Linsmaier and Skoog (LS) (1965), and Gamborg *et al* (B5) (1968).

The choice of media used is dependent on the purpose of the tissue culture and the species or type of plants. A relatively small number of mineral salts are used as components of media for plant tissue culture compared to that used to sustain normal plant. The media usually contain inorganic nitrogen and potassium. Lesser amounts of ammonium salts are used since the effect of ammonium salts can vary from inhibitory to essential depending upon the tissue and purpose of the culture. Small amounts (1 - 3 mM) of calcium, sulfate and phosphorous are usually adequate (Kochba and Spiegel-Roy, 1973; Button and Kochba, 1977).

Sucrose and glucose are the standard sources of carbon. Other sugars such as sorbitol are used less often. Vitamins usually added to the culture medium include thiamine, nicotinic acid, pyridoxine and calcium pantothenate. Thiamine is required

for plant growth, while the rest enhance growth in some systems. Other vitamins such as nicotinamide, folic acid, and riboflavin have been used in tissue culture media. Practically all media contain myo-inositol, which is beneficial as a component of cell wall metabolism (George and Sherington, 1984).

Growth regulators used in tissue culture consist of four classes of compounds, namely auxins, cytokinins, gibberellins and abscisic acid. The auxins are required for the induction of cell division and are often used in combinations with cytokinins (Lindsey, 1991). The cytokinins play an essential role in differentiation and plant regeneration of most species whilst the gibberellins are used in plant regeneration from meristems. Abscisic acid is believed to be important in somatic embryogensis (Kochba and Spige-Roy, 1977 ; Murashige and Tucker, 1969).

Tricarboxylic acid cycle intermediates such as malate and citrate are commonly used in the media as organic acids for protoplast culture. These compounds appear to alleviate the inhibitory effects of ammonium salts (Gamborg and Shyluk, 1970).

As mentioned above, a variety of organic supplements have been used in tissue culture. The one most commonly used is orange juice (OJ) for the stimulation of callus growth. Coconut milk (CM) has also been found to be helpful for callus

initiation, while malt extract (ME) has been reported to be beneficial for embryogenesis in nucellus cultures of citrus (Kochba *et al*, 1972).

1. 7. 1. Tissue culture of citrus

Since citrus is one of the important commercial crop that enjoys a worldwide distribution, numerous studies have been conducted on selected species and cultivars. Morphogenesis of citrus tissue cultures have been described from different plant organs such as nucelli, seeds, (Kochba *et al* , 1972), stem segments (Kitto and Young, 1981) and somatic callus derived from leaf, stem and root segments (Chaturvedi and Mitra, 1974). A summary of some of these studies is shown in table 2.

The production of *in-vitro* plants directly from proliferating shoot tips, node and stem segments, epicotyl segments, root tips and root segments have also been reported (. Alskief and Riedel, 1978; Tesserat *et al*, 1979; Burger and Hackett, 1981; Barlass and Skene, 1882; Edriss and Burger, 1984).

Schroeder and Spector (1957) used explants from the fruit mesocarp of citron and reported that callus formation occurred best on a nutrient containing indol-3-acetic acid (IAA) and gibberellic acid (GA_3). Unlimited growth can be obtained *in-vitro* from callus regenerated from juice vesicles of *C. limon* (Kordan, 1959) and satsuma (*C. unshiu* Marc.) (Nito and Akihama, 1990).

Citrus plants originated *in-vitro* indicate that large genotypic differences were extende (Barlass and Skene, 1982). Nodal and internodal explants have been the preferred source of multiple shoot culture. Nodal explants with their pre-existing axillary meristems give the best chance for trueness-to-type of the resultant plants. Conversely plants regenerated from internodal explants provided a potentially useful system for genetic changes through mutagenesis (~~Broerties *et al*, 1978~~; Barlass, and Skene, 1982; Bajaj, 1986).

The media used for the micropropagation of citrus have largely involved the basal salts of Murashige and Skoog (MS) (1962) for shoot regeneration. Since an increase in the sucrose concentrations in the medium from 3 to 5% has been favoured by some researchers (Murashige and Tucker, 1969; Chaturvedi and Mitra, 1974). Addition of cytokinins such as 6-Benzyl-aminopurine (BAP) and kinetin (K) have been found to be essential for shoot regeneration of citrus *in-vitro* (Bouzid, 1975; Primo Millo and Harada, 1976; Raj Bhansali and Arya, 1978*a*). Sim *et al* (1989) using different vegetative parts of *C. mitis* Blanco seedlings and mature plants as explants to initiate cultures, reported that shoots were obtained from intact roots cultured in MS medium with 0.5 mg/l BAP. Shoot tips and axillary buds regenerated multiple shoots after 3 weeks in culture medium supplemented with 0.1 - 2 mg/l BAP (Table2). Reports have shown that 2,4-dichlorophenoxyacetic acid (2,4-D) was superior to 3-indolacetic acid (IAA), and 1-naphthaleneacetic acid (NAA) as the supplied auxin (Murashige and Tucker, 1969). Bahnsali and Arya (1979) reported that exogenous auxins were not a critical requirement since growth occurred

Table 2. A summary of tissue culture work on some *Citrus* species and its relatives.

Species	Source of explants	Culture media	Responses	References
(*C. aurantifolia*)	nucellus	MWM + YE (500 mg/l) + CH (400 mg/l)	callus ---> shoots, roots	Singh, 1963.
	unpolinated ovaries	MMS + ADS ± K ± GA3	callus --->	Mitra & Chaturvedi, 1972.
	ovules	MMS + K(0.5 mg/l) + IAA (0.25 mg/l)	embryos	Mitra & Chaturvedi, 1972.
	unpolinated buds	or K (0.5 mg/l) + IAA (0.25 mg/l) + GA3 (5mg/l) or ADS (10 mg/l) + GA3 (10 mg/l)	callus ---> embryos	
	stem	(1) MMS + NAA (0.5 mg/l) 2,4-D (0.25 mg/l) + K (0.25 mg/l) (2) MS + BAP (0.5 mg/l) ± NAA (0.01 - 0.1 mg/l)	(1) callus (2) adventitious shoots	Raj Bhansali & Arya, 1978 b.
	anther	MMS + BAP (0.5 mg/l) + IAA (1mg/l)	plantlets (2n = 18)	Chaturvedi & Sharma, 1985.
	undeveloped ovules	MT + ME (500 mg/l)	embryoids	Gmitter & Moor, 1986.
(*C. aurantium*)	nodes, internodes stem segments	MS + NAA + K	multiple shoots adventitious shoots	Bouzid, 1975.
	ovules	MT + galactose or glucose	hab. callus --->	Kochba *et al*, 1982 .
	anther	(1) MS + K (0.02 mg/l) + IAA (2mg/l) (2) MS + sucrose (2%)	embryoids embryoids plantlet (n = 9)	Hidaka *et al*, 1982,
(*C. deliciosa*)	ovules	MT	callus ---> embryoids	Kobayashi *et al*, 1984.
(*C. junosa*)	ovules	MT	callus ---> embryoids	Kobayashi *et al*, 1984.
(*C. kharna*)	nucellus	MWM + YE (500 mg/l) + CH (400 mg/l)	callus ---> embryoids	Singh, 1963.
(*C. limettioides*)	stem segments	MMS + BAP (1 mg/l)	callus ---> embryoids	Raj Bhansali & Arya, 1979.
(*C. limon*)	nucellus	MS + ME (500 mg/l) or MMS + ME (500 mg/l) or + ADS (25 mg/l) NAA (0.5 mg/l) + OJ (5%)	embryoids	Rangan *et al*, 1968,1969.
	juice vesicles	MT + K (0.2 mg/l) + 2,4-D (0.66 mg/l) or NAA (1 mg/l)	callus	Murashige & Tucker, 1969.

		MS + ascorbic acid 100 mg/l or citric acid 100 mg/l	normal devloped vesicle	Tissart and Galleta, 1987.
	ovules	MT + galactose/lactose	callus embryoids	Kochba *et al*, 1982.
	root meristem	MS + BAP 1 mg/l + 2,4-D 0.01 mg/l	shoots and callus	Sauton *et al*, 1982.
	undeveloped ovules	MT + ME 500 mg/l	embryoids	Moore, 1985; Gmitter & Moore, 1986.
(*C. limon*) va. jambhiri	nucellus	MWM + CH 400 mg/l + YE 500 mg/l	callus, embryoids	Singh, 1963.
	undeveloped ovules (ripe fruit)	MT + ME 500 mg/l	callus, embryoids	Starrantino & Russo, 1980.
(*C. maxima*) (*C. grandis*)	nucellus	MS + ME 500 mg/l	embryoids	Rangan *et al*, 1968, 1969.
	juice vesicles	MT + K 0.2 mg/l + 2,4-D 0.66 mg/l + NAA 1.8 mg/l	callus	Murashige & Tucker, 1976.
	stem and leaves	(1) MMS + K 0.25 mg/l + 2,4 -D 0.25 mg/l + NAA 0.5 mg/l (2) MMS + BAP 0.25 mg/l (3) MMS + NAA 0.1 or 0.5 mg/l	callus shoots plantlets	Chaturvedi & Mitra, 1974, 1975.
	cotyledon	R (Barba's medium) + BAP 1 mg/l	callus, adventitious shoots	Patena *et al*, 1978.
	endosperm	(1) MT + 2,4-D 2 mg/l + BAP 1 mg/l + CH 1000 mg/l (2) 2 MT + GA3 (2 - 5 mg/l)	(1) callus (2) embryoids	Wang & Chang, 1978. Rao *et al*, 1981.
	cotyledon embryo axis	MS + K 2.5 mg/l + NAA 5 mg/l	callus, roots	
~~(*C. medica*)~~	~~juice vesicles~~	~~MT + K 0.2 mg/l + 2,4-D 0.66 mg/l or NAA~~	~~callus~~	~~Murashige *et al*, 1981~~
(*C. mitis*) (*C. microcarpa*)	globular embryos	MWM + CH 400 mg/l	embryoids	Masheswari & Rangaswamy, 1958.
	nucellus	MWM + CH 400 mg/l	embryoids	Rangaswamy, 1961.
(*C. mitis*)	shoot tips	WM + adenine 40 mg/l or CM 40%	shoots, roots	Rangaswamy, 1975.
	seedling internodes	WM	Adventitious shoots & roots	Rangaswamy, 1975.
	seedling stems	MS + BAP 10 mg/l + ME 500 mg/l + sucrose 5% (w/v)	callus & shoots	

	decapitated seedling	Turke's medium	callus, adventitious shoots & r00ts	Rangaswamy, 1975.
	cotyledon	R (Barba's medium) + BAP 1 mg/l or 5 mg/l + 2,4-D 0.05 mg/l	callus & shoots	Rangaswamy, 1975.
	anther	(1) N6 + KT or BAP 2 mg/l + 2,4-D 0.5 - 2 mg/l + sucrose 8% (2)MS + BAP (2mg/l) + IAA 0.1 mg/l + LH 500 mg/l + sucrose 2% (3) MS + IAA 0.1 mg/l + GA3 1 - 4 mg/l +LH 500 mg/l + sucrose 2%	(1) small embryoids (2)mature embryoids	Chen *et al*, 1980.
	cotyledon, embryoids	MS + K 2.5 mg/l + NAA 5 mg/l	callus	Rao *et al*, 1981.
C. paradisi	juice vesicles	MT + K 0.2 mg/l + 2,4-D (0.66 mg/l) or NAA (0.18 mg/l)	callus	Murashige & Tucker, 1969.
	nucellus ovules	MT + ME (500 mg/l)	embryoids embryoids	Kochba *et al*, 1972. Kochba *et al*, 1972.
	undevloped ovules	MT + 2,4-D (0.01 mg/l) + BAP (0.01 mg/l)	embryoids	Moore, 1985.
	stem & leaf	MT + ME (1000 mg/l) + NAA (0.15 mg/l) + BAP (0.5 mg/l)	Adven. shoots & embryoids	Raj Bhansali & Arya 1978
C. paradiisi × *C. reticulata* cv. Orlando	undevloped ovules	MT + ME (500 mg/l)	embryoids	Gmitter & Moore, 1986.
C. reshni (Hort ex Tanaka)	seedling internodes adventitious shoots	MS + BAP (2.25 mg/l)	callus --->	Barlass & Skene, 1982.
	nodal sections of seedlings & mature explants	MS + BAP (2.25 mg/l)	axil shoots	Barlass & Skene, 1982.
C. reticulata (*C. limonia*, *C. nobilis*)	nucellus (micropylar halves)	MWM + CH (400 mg/l) + CM (10%)	Callus ---> embryoids	Sabharwal , 1963.
	seedling internodes	MS + BAP (2.25 mg/l)	callus ---> adventit. shoots	Barlass & Skene, 1982.
	nodal sections (mature seedl.)	MS + BAP (2.25 mg/l)	axillary shoots	Barlass & Skene, 1982.
	undeveloped ovules (mature fruits)	MT + ME (500 mg/l)	embryoids	Moore, 1985.
	ovules	MT + galactose/lactose	hablo. callus --->	Kochba *et al*, 1982.

	decapitated seedling	Turke's medium	callus, adventitious shoots & r00ts	Rangaswamy, 1975.
	cotyledon	R (Barba's medium) + BAP 1 mg/l or 5 mg/l + 2,4-D 0.05 mg/l	callus & shoots	Rangaswamy, 1975.
	anther	(1) N6 + KT or BAP 2 mg/l + 2,4-D 0.5 - 2 mg/l + sucrose 8% (2)MS + BAP (2mg/l) + IAA 0.1 mg/l + LH 500 mg/l + sucrose 2% (3) MS + IAA 0.1 mg/l + GA3 1 - 4 mg/l +LH 500 mg/l + sucrose 2%	(1) small embryoids (2)mature embryoids	Chen *et al*, 1980.
	cotyledon, embryoids	MS + K 2.5 mg/l + NAA 5 mg/l	callus	Rao *et al*, 1981.
C. paradisi	juice vesicles	MT + K 0.2 mg/l + 2,4-D (0.66 mg/l) or NAA (0.18 mg/l)	callus	Murashige & Tucker, 1969.
	nucellus	MT + ME (500 mg/l)	embryoids	Kochba *et al*, 1972.
	ovules		embryoids	Kochba *et al*, 1972.
	undevloped ovules	MT + 2,4-D (0.01 mg/l) + BAP (0.01 mg/l)	embryoids	Moore, 1985.
	stem & leaf	MT + ME (1000 mg/l) + NAA (0.15 mg/l) + BAP (0.5 mg/l)	Adven. shoots & embryoids	Raj Bhansali & Arya 1978
C. paradiisi × *C. reticulata* cv. Orlando	undevloped ovules	MT + ME (500 mg/l)	embryoids	Gmitter & Moore, 1986.
C. reshni (Hort ex Tanaka)	seedling internodes adventitious shoots	MS + BAP (2.25 mg/l)	callus --->	Barlass & Skene, 1982.
	nodal sections of seedlings & mature explants	MS + BAP (2.25 mg/l)	axil shoots	Barlass & Skene, 1982.
C. reticulata (*C. limonia*, *C. nobilis*)	nucellus (micropylar halves)	MWM + CH (400 mg/l) + CM (10%)	Callus ---> embryoids	Sabharwal , 1963.
	seedling internodes	MS + BAP (2.25 mg/l)	callus ---> adventit. shoots	Barlass & Skene, 1982.
	nodal sections (mature seedl.)	MS + BAP (2.25 mg/l)	axillary shoots	Barlass & Skene, 1982.
	undeveloped ovules (mature fruits)	MT + ME (500 mg/l)	embryoids	Moore, 1985.
	ovules	MT + galactose/lactose	hablo. callus --->	Kochba *et al*, 1982.

			embryoids	
C. reticulata × *C. sinensis*	nucellus	MS + ME(500mg/1) MMS + ME (500mg/1) + ADS (25mg/1) + NAA (0.5mg/1) + OJ (5%)	embryoids embryoids	Rangan *et al*, 1968. Rangan *et al*, 1969.
C. sinensis (sweet orange)	nucellus nucellus	MWM + YE (500mg/1) (1) MT+ ME(500mg/1) (2) MT+ GA_3 (1mg/1) MT + IAA (0.1mg/1)	embryoids (1) embryoids ---> (2) plantlets callus ---> embryoids	Singh, 1963. Kochba *et al*, 1972. Kobayashi *et al*, 1984.
	juice vesicles	MT K(0.2mg/1) 2.4 - D (0.66mg/1) or NAA (1.8mg/1)	callus	Murashige & Tucker, 1969.
	ovules	(1) MT+ ME(500mg/1) (2) MT+ GA_3 (1mg/1) +IAA (0.1mg/1)	(1) callus --->(2) embryoids	Kochba *et al*, 1972.
		MT	callus ---> embryoids	Kobayashi *et al*, 1984.
	ovules	MT	hab. callus ---> embryoids	Kochba & Button , 1974.
		MT+ ME (100mg/1)	,,	Kochba & Spiegel-Roy, 1973.
		MT	,,	Kochba & Spiegel-Roy, 1977a.
		MT	,,	Button, 1978.
		MT+ galactose/ lactose	,,	Kochba *et al*, 1982.
			,,	Epstein *et al*, 1977.
	unpollinated ovaries	MS +K (0.5mg/1) + IAA (0.25mg/1) ± GA_3 (5mg/1) or MS +ADS (10mg/1) + GA_3 (10mg/1)	callus ---> embryoids	Mitra & Chaturvedi, 1972.
	nodes (mature tree) stem & leaf	MT + BAP (0.22 - 2.25 mg/l)	multiple shoots	Altman & Goren, 1976.
		(1) MMS+K(0.25 mg/l)+NAA 2.5 mg/l + 2,4-D 0.25 mg/l	(1) callus (2) shoots	Chaturvedi & Mitra, 1974.
		(2) MMS + BAP (0.25 mg/l) + NAA (0.1mg/l)	(3) planlets	
		(3) MMS + NAA (0.1 - 0.5 mg/l)		
	nodes or internodes stem segments	MS + IBA (1mg/1) + CM10%	axillary buds	Bouzid, 1975.
			callus ---> shoots	Bouzid, 1975.

	macerated callus	MS + ME (500 mg/l) +CM (10%)	single cell ---> embryoids	Button & Botha, 1975.
	endosperm	(1) MS + 2,4-D (2mg/l) + BAP 5mg/l + CH 1000mg/l (2) 2MT + GA (2 - 5 mg/l)	(1) callus (2) embryoids	Wong & Chang, 1978.
	fragmented apices	MS + BAP (2.25 mg/l)	adven. shoots	Barlass & Skene, 1982.
	seedling internodes	MS + BAP (2.25 mg/l)	callus ---> shoots	Barlass & Skene, 1982.
	nodal sections	MS + BAP (2.25 mg/l)	callus ---> shoots	Barlass & Skene, 1982
	seedling root meristems	MS + BAP (1mg/l) + 2,4-D (0.1 mg/l) or Farve medium	shoots or callus ---> shoots	Sauton *et al*, 1982.
	anther	(1) MS ± IAA + K (2) MS + sucrose (2%)	(1) embryos (2) plantlets	Hidaka, 1984.
(Navel orange)	undeveloped ovules mature fruits dormant buds	MT + ME (500 mg/l)	embryoids, pseudobulbils adven. buds	Starrantino & Russo, 1980. Altman & Goren, 1978.
(Washington navel)	nucellur (from unfert. ovules)	MT MMT + Adenine (400 mg/l) + ME (400 mg/l) + sucrose 4%	embryoids	Bitters *et al,* 1970. Button & Bornman, 1971.
(Valeencia)	ovules & nucelli	(1) MT + ME (500 mg/l) (2) MT + GA_3 (1 mg/l)	(1) embryoid ---> (2) plantlets	Kochba *et al*, 1972.
	root & epicotyl	MS + BA (2 mg/l) + NAA (0.2 mg/l)	advent. shoots	Burger & Hackett, 1986.
(Hamlin, Peel Navel Pineapple)	undeveloped ovules	MT + 2,4-D (0.01 mg/l) + DZ (0.1 mg/l) or MT + ME (500 mg/l)	embryoids	Moore, 1985. Gmitter & Moore, 1986.
C. sinensis × *P. trifoliata* (Carrizo citrange rootstock)	seedlings shoot tips	(1) MS (micro) + Knop' medium (organic) + BAP (5 mg/l) + sucrose (3 - 4 %) (2) MT + NAA (1mg/l)	(1) shoots --->(2) plantlets	Kitto & Young, 1981.
	fragmented apics	MS + BAP (2.25 mg/l)	advent. shoots	Barlass & Skene, 1982.
	seedling internodes	MS + BAP (2.25 mg/l)	callus ---> shoots	Barlass & Skene, 1982
	nodes	MS + BAP (2.25 mg/l)	axillary shoots	Barlass & Skene, 1982
	mature apex		shoot proliferation	Barlass & Skene, 1982

	matture nodes	$\frac{1}{2}$ MS + BAP (0.56 mg/l)	shoot proliferation	Barlass & Skene, 1982
		$\frac{1}{2}$ MS + BAP (0.56 mg/l)		
(Troyer citrange)	seedling root meristem	MS + BAP (1 mg/l) + 2,4-D (0.1 mg/l) or Favre medium	adventitious shoots	Sauton *et al*, 1982.
	epicotyl	(1) MS + BAP (0.25 mg/l) + NAA (0.1 mg/l) + sucrose 5 % (2) MS + BAP (0.5 mg/l) + NAA (0.1 mg/l) or (3) MS + NAA (2mg/l)	(1) callus (2) shoots (3) plantlets shoots planlets	Edriss & Burger, 1984
	Root segments	(1) MS + BAP (10 mg/l) + NAA (1mg/l) (2) MS + NAA (2mg/l)	(1) shoots ---> (2) plantlets	Edriss & Burger, 1984.
P. trifoliata	nucellus	MWM + CH ($00 mg/l) + YE (400 mg/l)	callus ---> embryoids	Singh, 1963.
	endosperm	(1) MT + 2,4-D (2 mg/l) + BAP (5 mg/l) + CH (1000 mg/l) (2) 2MT + GA3 (2 mg/l)	(1) callus ---> (2) embryoids (3n = 27)	Wang & Chang, 1978.
	seedling internodes	MS + BAP (2.25 mg/l)	adventi. shoots	Barlass & Skene, 1982.
	nodes	MS + BAP (2.25 mg/l)	axillary shoots plantlets	Barlass & Skene, 1982.
	seedling root meristem	MS + BAP (1mg/l) + 2,4-D (0.1mg/l) or Favre medium	callus ---> shoots	Sauton *et al*, 1982.
	pollen	(1) MS + K(0.2 - 2 mg/l) + IAA (0.2 mg/l) + sucrose (5%) (2) MS + sucrose (2%)	(1) embryoids --->(2) planlets (n = 9)	Hidaka *et al*, 1979.
Satsuma (*C. unshiu* Marc.)	juice vesicles	MS + NAA 1.0 mg/l + K 1.0 mg/l + GA_3 1.0 mg/l + sucrose 3 % (w/v)	callus, embryos, roots	Nobumasa and Mesoo 1990a.
(*P.trifoliata*) carizo citrang (*C. sinensis* × *P. trifoliata*) *C. aurantium*	embryos	MT + NAA 1.0 mg/l + BAP 5 mg/l + ME 1000 mg/l + sucrose 3 % (w/v)+ agar 0.8%	embryoids adventi. shoots	Naima, 1991.
sweet orange (*C. sinensis* L. Osb.)	stem segments	MS + NAA 3 - 10 mg/l + BAP 1 - 3 mg/l + sucrose 3 % (w/v) + agar 1 %	shoots, buds roots & callus	Duranvila, *et al*, 1989.
citron (*C. medica*) lime (*C. aurantifolia* Christm. swing)				
C. mitis Blanco)	cotyledon	full or $\frac{1}{2}$ strenght MS + sucrose	callus, shoots	Sim *et al*, 1989.

		2% + agar 0.8% (W/V)+ BAP 0.1 - 2 mg/1 +NAA 0.2-2.0mg/1+ 2,4-D 0.1- 0.5mg/1		
(*C. sinensis* ovules Osb.)	ovules	MT+ BAP 10mg/1+ galactose 5% + agar 0.8% (W/V)	callus, shoots	Sim *et al.* 1989.
Kinnow (*C. nobilis*)	leaf, epicotyl cotyledon & root segm.	MS or MT + sucrose 5 %(W/V)+ ME 500mg/1+ agar 0.7% + NAA2-10 mg/1 +2,4-D2-5mg/1+ BAP 0.5-3mg/1+ K 0.5mg/1	embryogeniccallus somatic, embryos	Gill, *et al.* 1994.
sweet orange (*C.sinensis* L. Osb.)	stem seg.	MS+ sucrose 3% (W/V) +agar1.0% +NAA 10mg/1+ BAP 1-3 mg/1	callus, shoots	Duran-Vila, 1992.
lime (*C. aurantifolia*)	root segments	MS + sucrose 3% (w/v) + BAP 0.89 - 5 mg/1	shoots	Bhat, *et al*, 1992.

in its absence. Callus and root induction on the other hand, was promoted by the presence of auxins in the medium (Murashige and Tucker, 1969; Kochba *et al*, 1972).

The complex organic substance, malt extract (ME) have been used extensively for the culture of citrus material, from both nucellar and vegetative organs (Rangan *et al*, 1968). Shoots regenerated from stem segments of sweet lime (*C. limettioides*) on MS medium were greatly enhanced when (500 mg/l) malt extract was added to the medium (Bahnsali *et al*, 1979).The progression from embryo to plant is stimulated by gibberellic acid (GA_3), mainly by encouraging root and shoot growth (Button and Bornman, 1971; Kochba *et al*, 1972, 1974).

Adventitious embryogenesis have been induced in nucelli excised from fertilized (Bitters *et al*, 1970) as well as from unfertilized ovules of seedless oranges (Button and Bornman, 1971; Kochba *et al*, 1972; Mitra and Chaturvedi, 1972) and callus proliferation has been obtained from ovary walls of unfertilized polyembryonic citrus cultivars (Mitra and Chturvedi, 1972). Long term callus cultures obtained from stem explants of a monoembryonic cultivar (Chaturvedi and Mitra, 1974) which had lost their capacity to produce shoots were able to initiate embryos after appropriate manipulations to the medium, including a change of cytokinin type (Chaturvedi and Mitra, 1975). Multiplication of embryos in sufficient number for propagation requires an extended callus phase, which would

increase the occurrence of somatic variation, including changes in ploidy levels. However, Vardi *et al*, (1982) reported that only one out of eight citrus cultivars showed a high proportion of tetraploid cells in the nucellar callus whereas all others were diploid.

The main practical benefits of adventitious embryos to citrus improvement are the propagation of virus-free plants and asexual breeding. Preservation of fruit trees germplasm by tissue culture has great potential to avoid problems related with field preservation such as damages caused by diseases, insects and adverse weather. On the other hand protoplast culture systems provide a unique opportunity for somatic cell hybridization, transformation and genetic manipulation of important citrus species. Somatic hybridisation has great potential for cultivar improvement and for overcoming problems such as heterozygosity, sterility, nucellar embryony and long juvenile period .

1.7.2. Development of protoplast culture in citrus

Citrus ranks among the first fruit trees to have been regenerated from protoplast culture. Kochba *et al*, (1972) described a system by which citrus embryo and plants could be regenerated from ovule callus. These systems were utilized to isolate protoplasts from shamouti orange nucellar callus. The protoplasts could then be cultured *in-vitro* and regenerated embryos from which plants were derived (Vardi *et al*, 1975). As in other protoplast systems, the division of citrus protoplasts commenced only at a certain plating density (10^5 protoplasts per ml or more).

However, the deficiency in plating density could be compensated by plating over a layer of division arrested by X-irradiated protoplasts from either citrus callus or *Nicotiana* leaf mesophyll protoplast (Vardi and Raveh, 1976). The protoplast to tree system was extended, in addition to orange (*C. sinensis*), to some other species such as grapefruit (*C. paradisis*), lemon (*C. limon*), sour orange (*C. aurantium*) and mandarin (*C. reticulata*) (Vardi *et al*, 1982). A summary of protoplast culture of some citrus species are shown in table 3.

Citrus trees derived from protoplast culture have a remarkable similarity to the plants from which the protoplasts were isolated (Bajaj, 1986). Vardi *et al*, (1986) extended the protoplast system to *Microcitrus* trees, a relative of the citrus trees, with high efficiency. In addition to this, trovita orange protoplasts derived from nucellar callus have been cultured *in-vitro* and ultimately gave rise to plants, following methods identical to those used by Vardi and collaborators (Kobayashi *etal*, 1983).

Nucellar calluses of some citrus species such as orange, mandarin, grapefruit, lemon and sour orange, showed high chromosomal stability and maintained their diploid number. They have also shown regeneration ability through somatic embryogensis over a long period of time (5-10 years). These merits made it possible to regenerate whole plants from protoplasts (Kobayashi *et al*, 1983; Kobayashi 1987; Vardi *et al*, 1982, 1986).

However, the deficiency in plating density could be compensated by plating over a layer of division arrested by X-irradiated protoplasts from either citrus callus or *Nicotiana* leaf mesophyll protoplast (Vardi and Raveh, 1976). The protoplast to tree system was extended, in addition to orange (*C. sinensis*), to some other species such as grapefruit (*C. paradisis*), lemon (*C. limon*), sour orange (*C. aurantium*) and mandarin (*C. reticulata*) (Vardi *et al*, 1982). A summary of protoplast culture of some citrus species are shown in table 3.

Citrus trees derived from protoplast culture have a remarkable similarity to the plants from which the protoplasts were isolated (Bajaj, 1986). Vardi *et al*, (1986) extended the protoplast system to *Microcitrus* trees, a relative of the citrus trees, with high efficiency. In addition to this, trovita orange protoplasts derived from nucellar callus have been cultured *in-vitro* and ultimately gave rise to plants, following methods identical to those used by Vardi and collaborators (Kobayashi *etal*, 1983).

Nucellar calluses of some citrus species such as orange, mandarin, grapefruit, lemon and sour orange, showed high chromosomal stability and maintained their diploid number. They have also shown regeneration ability through somatic embryogensis over a long period of time (5-10 years). These merits made it possible to regenerate whole plants from protoplasts (Kobayashi *et al*, 1983; Kobayashi 1987; Vardi *et al*, 1982, 1986).

In the last decade protoplast fusion have been accomplished with citrus species. Among these are the protoplast fusion of trovita orange (*C. sininses*) with *Poncirus trifoliata* and true somatic hybrids were obtained (Ohgawara *et al*, 1985). Vardi *et al*, (1987) reported that when the donor-recipient protoplast fusion procedure was employed with citrus protoplasts, it can lead to the production of cybrid trees (Table 3).

The protoplast fusion technique has also been applied to produce Rutaceae hybrids which can not be obtained by sexual crossing because of sexual incompatibility (Grosser *et al*, 1988, 1990 a) or male and female sterility (Kobayashi *et al*, 1988; Ohgawara *et al*, 1989). The somatic hybridisation obtained between Rutaceae species are amphidiploid and have morphological characteristics intermediate between those of their parents. Citrus tetraploids plants have no economic value, but they are of great interest as breeding material, because when crossed with diploids, they are able to produce triploids that are potentially useful since they have thin rinds and lack seeds (Ohgawra *et al*, 1991).

Thus, fertility of amphidiploid somatic hybrid plants is desirable for the integration of the somatic hybridisation technique into citrus breeding programs (Grosser *et al*, 1990 b). Somatic hybridisation of citrus with related genera has potential for citrus rootstock improvement by providing access of potentially useful genetic variation that were inaccessible previously (Swingle , 1967; Grosser *et al,* 1988 a, b; Grosser and Gmitter, 1990). Sweet orange (*C. sinensis*) has been a common partner in most of the reported interspecific or intergeneric somatic

Table 3. A summary of protoplast culture of some *Citrus* species and its relatives.

Species	Source material	Enzyme mixture		Culture media	Responses	References
(C. aurantium)	nucellus callus	Pectinase Cellulase R10 Driselase sucrose mannitol	0.3% 0.2% 0.1% 0.14M 0.56M	MT + mannitol (0.3M) + sucrose (0.3M) + agar (0.6%)	colonies embryoids	Vardi *et al*, 1982.
C. limon (L) Burm. cv. Valencia	nucellus callus	Pectinase Cellulase R10 Driselase sucrose mannitol	0.3% 0.2% 0.1% 0.14M 0.56M	MT + mannitol (0.3M) + sucrose (0.3M) + agar (0.6%)	colonies embryoids	Vardi *et al*, 1982.
C. paradisi cv. Dumcan	nucellus callus	Pectinase Cellulase R10 Driselase sucrose mannitol	0.3% 0.2% 0.1% 0.14M 0.56M	MT + mannitol (0.3M) + sucrose (0.3M) + agar (0.6%)	colonies embryoids	Vardi *et al*, 1982.
C. reticulata (a) Murcott (b) Dancy (c) Ponkan	nucellar callus	Pectinase Cellulase R10 Driselase sucrose mannitol	0.3% 0.2% 0.1% 0.14M 0.56M	MT + mannitol (0.3M) + sucrose (0.3M) + agar (0.6%)	colonies embryoids	Vardi *et al*, 1982.
C. reticulata Ponkan	callus from embryos	Pectolyase 23 Cellulase RS sucrose glucose mannitol MES	0.01% 0.33% 0.09M 0.18M 0.33M 5mM	MS + sucrose (0.09M) + glucose(0.08M) + mannitol(0.23M) + MES (5mM) ± growth substances [liquid or agar (0.6%)]	callus embryoids	Hidaka & Kajiura, 1988.
C. sinensis (Shamouti)	ovular callus	Cellulase R10 Pectinase (Koch-Light) PDS sucrose mannitol sorbitol	1.0% 1.0% 0.3% 0.14M 0.28M 0.28M	MT+ mannitol(0.23M) + sorbitol(0.23M)	callus, embryoids	Vardi *et al*, 1975.
		Pectinase Cellulase Driselase PDS sucrose sorbitol	0.2% 0.2% 0.15% 0.3% 0.28% 0.28%	MT+ mannitol (0.23M) + sorbitol (0.23M)	callus, embryoids	Vardi, 1977, 19
C, sinensis (Shamouti)	nucellus callus	Pectinase Cellulase R10 Driselase sucrose mannitol	0.3% 0.2% 0.1% 0.14M 0.56M	MT + mannitol (0.3M) + sucrose (0.3M) + agar (0.6%)	colonies, embryoids	Vardi *et al*, 1982.

C.sinensis (trovita orange)	nucellus callus	Maserozyme 0.3% Cellulase R10 0.2% Driselase 0.1% mannitol 0.7% $\frac{1}{2}$ MT (macro only)	MT + sucrose (0.15M) + glucose (0.45M) + agar (0.6%)	colonies, embryoids	Kobayashi *et al*, 1983.
		Macerozyme R10 0.3% Cellulase R10 0.2% Driselase 0.1% mannitol 0.7% $\frac{1}{2}$ MT (macro only)	MT + sucrose (0.15M) + glucose (0.45M) + agar (0.6%)	colonies, embryoids	Kobayashi *et al* 1985.
C. sinensis (Washington navel orange)	callus from embryos	Pectolyase Y23 0.01% Cellulase RS 0.33% sucrose 0.09M glucose 0.18M mannitol 0.33M MES 5mM	MS + sucrose (0.09M) + glucose (0.08M) + mannitol (0.23M) + MES (5mM) ± growth substances [liquid or agar (0.6%)]	callus embryoids	Hidaka & Kajiura, 1988.
(*C. yuko* Hort ex. Tanaka)	callus from embryos	Pectolyase Y23 0.01% Cellulase RS 0.33% sucrose 0.09M glucose 0.18M mannitol 0.33M MES 5mM	MS + sucrose (0.09M) + glucose (0.08M) + mannitol (0.23M) + MES (5mM) ± growth substances [liquid or agar (0.6%)]	callus embryoids	Hidaka& Kajiur 1988.
Microcitrus natural hybrid (*M. australis* × *M. ustralasica*)	ovular callus	Macerozyme 0.2% Cellulase 0.3% mannitol 0.35% sucrose 0.35M $\frac{1}{2}$ MT (macro only)	MT + mannitol (0.3M) + sucrose (0.3M)	callus embryoids	Vardi *et al*, 1986.
Valencia (*C. sinensis* L. Osb.)	cotyledon	Celulysin 3.0% Macerase 0.3% mannitol 0.6M	MT + mannitol 0.6% NAA 15µm Kinetin 4.6µm ME 1000mg/l	colonies	David & Weste 1982.
Sour orange *C. aurantifolia* + Rugh lemon (*C. jambhiri* L)	nucellar callus	Macerozyme 0.2% Cellulase R10 0.3% Driselase 0.1% mannitol 0.07%	MT + sucrose (0.3M) + mannitol (0.3M) + agar (0.6%)	colonies microcalli embryos	Vardi *et al*, 1989.
Sour orange (C. aurantium)	nucellar callus	Macerozyme 0.2% Cellulase R10 0.3% Driselase 0.1% sucrose 0.35M mannitol 0.35M	MT + sucrose (0.3M) + mannitol (0.3M) + agar (0.6%)	colonies embryos	Vardi *et al*, 1987.
Satsuma mandarin (*C. unshiu*)	*embryogenic* callus	*Cellulase R10* 0.3% Macerozyme 0.3% Driselase 0.1% sorbitol 0.7M	*MT + sucrose(0.15M)* + mannitol (0.45M) + gellan gum 0.1%	*embryoids*	Hisato *et al*, 1991.

Maxima lime (*C.aurantifolia* Swingle + Grapefruit (*C.paradisi* Macf)	embryo calli	Pectolyase Y23 0.33% CellulaseRS 0.33% mannitol 0.35% sorbitol 0.35M	MS + glucose (0.2%) + sorbitol (0.2%)	colonies callus	Hidaka *et al*, 1982.
Washington navel orange (*C.sinensis* Osb.) + Satsuma mandarin (*C. unshiu*)	nucellar	Macerozyme 0.3% Cellulase R10 0.2% Driselase 0.1% mannitol 0.7% $\frac{1}{2}$ MT (macro only)	MT + sucrose 0.6M + agar 0.6%	embryoids	Kobayashi *et al*, 1988.
(*C. sinensis* L. Osb.) Yuko (*C.yuko* Hort. Tanaka) + Poncu (*C. reticulata* Blanko)	embryo calli	Pectolyase Y23 0.01% Cellulase RS 0.33% + sucrose 0.09M + glucose 0.08M mannitol 0.33M + N-morpholinoethane-sulfonic acid (5mM)	MS + sucrose 0.09 M + glucose 0.08M + N-morpholinoethan-esulfonic acid (5mM) + agar 0.6%	embryos	Hidaka *et al*, 1988.
(*C. mitis*)	embryonic suspension		MT + sucrose 0.15M + glucose 0.45M	somatic embryos	Sim *et al*, 1989.
Calamndin *C.madurensis*	hypocotyl callus	Macerozyme10 0.4% Cellulase R10 0.2% Driselase 0.1% $\frac{1}{2}$ M T (in organic salt) sorbitol 0.7 M	MT+ sucrose 0.15M sorbito 0.25 - 0.45M agarose 0.60%	embryos	Ling *etal*, 1989.
Satsuma *C. unshiu* Marc.	embryo callus	Macerozyme R10 0.4% CellulaseR-10 0.2% Driselase 0.1% Sorbitol 0.7M $\frac{1}{2}$ M T medium	MT+ sucrose 0.3%M sorbitol 0.30M agar 0.6%	colonies embryos	Ling, *et al.* 1990
(*C.reticulata*) + *Citropsis gilletiana*	ovule suspension	AS in Grosser *et al.*, 1987	MT + sucrose 200g/l ME 0.5g/l	embryos	Grosser *et al*, 1990.
(*C. sinensis*) + Murcot tungor	nucellur suspension culture leaf	Macerozyme R10 0.3% Cellulase 0.2% Driselase 0.1% mannitol 0.7% $\frac{1}{2}$ (MT macroelement)	MT + sucrose 0.60M agarose 0.60%	somatic embryos	Kobayashi *et al*, 1991.

hybrids of the Rutaceae family because of its tendency for cell-to-plant regeneration via somatic embryogenesis *in-vitro* (Grosser *et al*, 1990 b). Hybridisation between commercially important Rutaceae germplasm has great benefits to creat new potential of citrus rootstocks by transfer of the genetic merits such as disease resistance and stress resistance (e.g. salinity, drought) since sources for disease and stress resistance are known to be latent in citrus relatives, and sexual hybridisation between rootstocks or cultivars of citrus and its relatives are difficult (Barrett, 1977). Thus, cell-manipulation methodologies, and especially protoplast manipulation, should provide great benefits for citrus breeding and improvement.

Troyer citrange, which is the sexual hybrid between the navel orange and trifoliate orange, has been used as useful rootstock because it produces a large number of seeds and has inherited virus resistance characteristics from the trifoliate orange. Grosser *et al*, (1988 a) have produced several somatic hybrids, including the hamlin orange plus the trifoliate orange which possess dwarf characteristics. In addition they have produced the hamlin orange plus its sexually incompatible wild relatives, *Severinia disticha* (Grosser *et al*, 1988 b), and the cleopatra mandarin plus *Citropsis gilletiana* (Grosser *et al*, 1990 a). These somatic hybrids are useful as rootstocks possessing genes for resistance against diseases and pests even though they are sterile. The somatic hybrid between the trovita and trifoliate orange, can be induced to produce flowers, and bear fruits containing normal seeds (Kobayashi *et al*, 1991).

Thus the protoplast culture and somatic hybridisation established in the Rutaceae family has great potential value for the improvement of both scion and rootstock.

1. 8. The objectives of the present study include the following goals :-

1- To achieve clonal propagation of citrus species through shoot proliferation and adventitious shoot production from seedling explants.

2- To isolate and culture protoplasts from embryo callus and leaf.

3- To establish a regime for acclimatization of micropropagated citrus plants.

4- To study changes in the leaf anatomy of the micropropagated plants during the acclimatisation period.

5- To compare the CO_2 assimilation efficiency of citrus plants propagated by tissue culture with those grown from seeds in *in-vivo* conditions.

1. 9. Description of citrus species used in study

Limau langkat (*Citrus suhuiensis* Hort. ex Tan.)

Limau langkat is a local loose-peel citrus species believed to have originated from China. The species is widely cultured in Malaysia especially in the states of Terengganu, and Perak. It was introduced to the Cameron Highlands when the plantings in the lowlands were severely affected by *Phytophthora* collar rot disease. It proved to be more successful when planted on steep land compared to the lowlands (Ko, 1992).

The tree is medium in size, canopy oval in shape and leaves broadly lanceolate. The fruits are medium to large, with smooth surface and orange in colour with a greenish tint. It is juicy, low in acid content and contain plenty of seeds, rind medium in thickness, adherence, aroma weak, mesocarp cream in colour (Plate 1 a). Seeds vary 12-15 per fruit, size medium, shape fusiform to ovoid, seed coat cream in colour, cotyledon light green (Ko, 1992).

Citrumelo (*Poncirus trifoliata* × *Citrus paradisi* Swingle)

It is one of the *Poncirus trifoliata* hybrids. The fruit contains large number of seeds with only nucellar embryos and has been considered a promising rootstock, because trees on this rootstock are cold tolerant, produce high quality fruits, and are resistant to *Phytophthra* foot rot and citrus nematode. In Italy for instance, *Citrus* trees grafted on citrumelo Swingle suffered only negligible damage and sustained high yield the following seasons after episodes of frosts (Recupero and Russo, 1989). As a result the demand is great for citrumelo rootstock and for establishing new plantations. Citrumelo is normally propagated by seeds as in other citrus rootstocks. However, the amount of seeds available is inadequate to supply the current demand for these trees. Thus tissue culture technique could be exploited in rootstock mass production.

The leaves are darkgreen, medium in size and mainly trifoliate. The fruit is medium to large, globose and has an acidic bitter flavour. The seeds are highly polyembryonic and rarely developed zygotic embryos.

~~globose~~, The flower buds are small, single, lateral, and protected by small fleshy scales. The rind is relatively thick and has abundant oil glands (Plate 1 b) (Reuther *et al*, 1967; Ko, 1992).

Swingle citrumelo has become a significant commercial rootstock in Florida because it has many attributes such as tolerance to cold, citrus tristeza virus, citrus nematode and *Phetophthora* foot rot. Trees loss from blight in commercial planting has been generally less than 2 % after about 10 years of culture in Florida (Castle *et al,* 1988).

Rangpur lime (*Citrus limonia* Osbeck)

Many common names have been used for this fruit and these include rangpur in India, lemon in Japan, canton lemon in China, japanch citrus in Java, and rangpur lime or mandarin lime in the United States (Reuther *et al*, 1967).The branches are very thorny, with leaves medium in size, dull green, broadly lanceolate. The fruits are small to medium, rind thin and moderately loose, surface coarsely pitted and rough, reddish orange in colour (Plate 1 c). Pulp juicy, flavour strongly acid. Seeds moderate in number, small, cotyledons light green, and highly polyembryonic (Ko, 1992).

The plants are hardy to cold weather and are used mainly as rootstock and ornamentals as potted plants. It is especially well adapted for such uses because it is easily dwarfed when the roots are cut (Reuther, *et al*., 1967). The trees of these

rootstocks are vigorous, high yielding and tristeza tolerant (Reuther *et al*, 1967; Eliezer *et al*, 1992).

Cleopatra (*Citrus reticulata* Blanco Hort. ex Tan)

Its common name is sour mandarin and it originated from Kuangtung, China where it is commonly used as rootstock and it has been considered as one of the original species of the subgenus *Citrus* (Zhong, 1993). Cleopatra mandarin has become increasingly important because it is less susceptible to citrus blight. The trees grafted on cleopatra mandarine are moderately vigorous, resistant to *Phytophthora citrophthora* and the citrus tristesa virus (Broadbent, 1993; Eliezer, 1992). It has a moderately vigorous habit with thornless branches, and the leaves are dark green. Fruits are small, surface coarsely pitted, rough, reddish orange in colour. Pulp intensely juicy with an acidic flavour (Plate 1 d). The seeds are small, with green cotyledons and are polyembryonic (Ko, 1992).

Many interspecific hybrids have been made between cleopatra and other Citrus species such as Tangelo (*C. reticulata* × *C. paradisi*) with fruits resembling oranges and a flavour intermediate between parents. Tangors (*C. reticulata* × *C. sinensis*) is a natural hybrid (Purseglove, 1984).

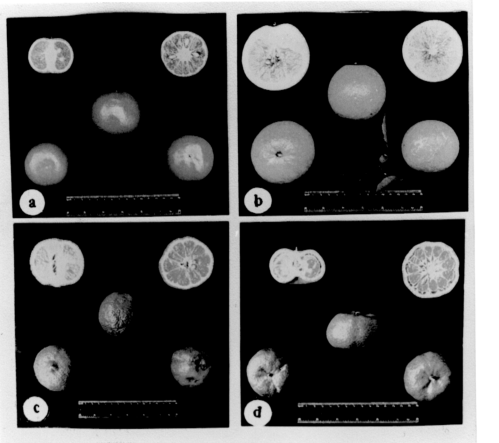
Plate 1. Fruits of the citrus species.

a *C. suhuiensis* fruit.

b Citrumelo fruit and leaves.

c *C. limonia* fruit.

d *C. reticulata* fruit.


a
b
c
d