CHAPTER 4

DISCUSSION

4. DISCUSSION

In general, plant regeneration is influenced by various factors including the type and physiological age of the explants, exogenous and endogenous levels of phytohormones as well as environmental factors. In essence, the results of current investigations are similar to the findings of other researchers (e. g. George and Sherington, 1984).

Micropropagation for four citrus species, namely citrumelo, *C. suhuiensis, C. limonia*, and *C. reticulata* were achieved with various seedling explants. Complete plants were obtained within 3 to 4 months. Multiple shoots were induced from node explants and adventitious shoots were formed from internodal, leaf, and root explants (3.1.2, 3.1.3; 3.2.2, 3.2.3; 3.3.2, 3.3.3, 3.4.2, 3.4.3). However, the regeneration potential was explant and species dependent.

4. 1. Comparative evaluation of regenerative capacity of explants from various species

4. 1. 1. Nodal explants

The production of plants from nodes or from axillary buds of shoot tip cultures has been proven to be the most reliable and widely used method of *in-vitro* propagation. This method of micropropagation may be useful in seedless clones or clones with few seeds.

From this study it was observed that BAP was not required for the growth of axillary buds, but was necessary to enhance multiple shoot formation (3.1.2.1, 3.2.2.1, 3.3.2.1, 3.4.2.1). This was also observed in sweet orange, citron and lime (Duran-vila *et al*, 1989), and in apple (Lundergan and Janick, 1980). For all the species studied, nodal explants appeared to be suitable explant for clonal propagation when cultured on MT basal medium with 1 to 3 mg/l BAP, because of high regeneration frequency and direct regeneration. Similar reports of successful proliferation from nodal explants were observed in citrus (Barlass and Skene, 1982). Shoot multiplications was achieved in Eureka lemon, cleopatra mandarin, sweet orange, trifoliata orange and grapefruit when cultured on MS basal medium with 1 to 3 mg/l BAP (Marin and Duran-Vila, 1991).

On the other hand, When NAA was added to the media enriched with BAP, the number of shoots per explant of the four citrus species were dependent on the ratio of NAA to BAP used (3.1.3.1, 3.2.3.1, 3.3.3.1, 3.4.3.1). Similar observation was made in citron and lime (Duran-Vila *et al*, 1989).

It was reported that plants regenerated directly from nodal explants were true-to-type (Barlas, *et al*, 1982; Marin and Duran-Vila, 1991). In addition to nodal cultures, shoot micropropagation technique could be useful for species which cannot be rooted readily. Murashige *et al* (1972) has found this to be an alternative method in the propagation of mature tip on a suitable rootstock. This technique also has been

successfully applied in lime, lemon, orange, and grapefruit (Navarro *et al*, 1975; Edriss and Burger, 1984).

4. 1. 2. Internodal explants

Shoot regeneration from internodal explants of the four citrus species was found to be dependent on the species and BAP concentrations of the medium (3.1.2.2, 3.2.2.2, 3.3.2.2, 3.4.2.2). Citrumelo explants exhibited the highest shoot regeneration capacity when 100 % of the explants regenerated shoots at all BAP levels (3.2.2.2), followed by *C. suhuiensis* with optimal concentration was at 3 mg/l BAP and the least responsive was *C. limonia* regenerating 2 shoots per explants only at 2 mg/l BAP while *C. reticulata* did not show any response at the concentrations tried (3.1.2.2, 3.3.2.2).

Similar results was also observed from internodal explants in sweet orange, citron, and lime by culturing the explants on 3 mg/l BAP enriched media (Duran-Vila *et al*, 1989).

4. 1. 3. Leaf explants

There are some reports of direct adventitious shoot regeneration from the leaves of woody plants. Direct regeneration from leaves has been reported in *Morus alba* and *Morus Indica* (Mhatre *et al*, 1985), *Liquidambar styraciflua* (Brand and Lieberger, 1988), and *Garcinia mangostana* (Goh *et al*, 1988). Direct shoot

regeneration has been reported from *C. mitis* (Sim *et al*, 1989). In the present study direct shoot regeneration was found possible from citrumelo (3.2.2.3, 3.2.3.3).

Most of the shoot buds were formed from the mid-rib and petiolar regions for citrumelo. Similar observations were reported in petiole and main veins of *C. mitis* and *P. trifoliata* and from sweet gum leaves (Brand and Lineberger, 1988). Sim *et al* (1988), assumed that the vascular cells in the veins contain some factors which favor adventitious shoot formation and these factors are less available in the lamina regions. It seems likely that the tissues which respond to shoot inducing substances are associated with vascular cells which are absent in the lamina. In other words, this may be due to the meristematic activity and the presence of endogenous growth substances in the petiole and mid-rib.

Amongst the four species attempted, leaf explants from Citrumelo were the most regenerative while those from *C. suhuiensis* and *C. limonia* were the least and leaf explants of *C. reticulata* failed to show any response on BAP enriched media. In citrumelo, shoots were formed either directly from the explants or after a short intervening callus stage. The difference in response could be due to genotype, especially for citrumelo, which is a different genus.

Adventitious organs differentiation was regulated by a balance between auxin and cytokinin in the culture media (Skoog and Miller, 1975). However, leaf, nodal,

internodal and root of citrumelo could regenerate shoot buds with cytokinin (BAP) alone. Stimulatory effects of cytokinin on adventitious bud formation were also observed in mangosteen (Goh *et al.*, 1988), apple (Natalie *et al*, 1994), perilla (Tanimoto and Harada, 1980) and Rudbeckia (Tanimoto and Harada 1982).

4. 1. 4. Root explants

Direct bud formation from cultured roots has been reported in other species such as *Brassica alboglabra* (Wong and Loh, 1988), Troyer citrange(Edriss and Burger, 1984) and *C. sinensis* (Burger and Hackett, 1986). In *C. sinensis*, the greatest number of buds from root segments was obtained in medium containing both NAA and BAP (Burger and Hackett, 1986). Sim *et al* (1989), suggested that bud formation from root explants of *C. mitis* not only depend on BAP in the medium but also on a factor derived from the shoot, presumably auxin.

In the present study, no shoot was produced from root explants for all species when cultured on MT basal medium (3.1.2.4, 3.2.2.4, 3.4.2.4). This showed that normal levels of endogenous growth substances were not conducive to bud formation. However, for media supplemented with BAP, a striking increase in shoot regeneration was observed (3.4.2.4). Production of shoot buds from root segments and root tips on BAP enriched media was achieved in citron and lime while sweet orange root explants failed to produce shoots (Duran- vila *et al*, 1989).

Root explants of *C. reticulata* were highly responsive to BAP producing high number of shoots through callus phase followed by citrumelo and the least response was *C. suhuiensis* while no shoots were produced from root explant of *C. limonia* (3.4.2.4). The number of shoots regenerated per explant was highest for *C. reticulata* where about 15.6 shoots per explant were obtained after 2 months on BAP enriched medium, 5.3 shoots for *C. suhuiensis* and 7.3 for Citrumelo (Tables 30, 7, 16). In *C. reticulata* shoots were regenerated from green compact callus up to 10 mg/l BAP, while in citrumelo and *C. suhuiensis* high levels of BAP was inhibitory for shoot formation where only callus was produced. Therefore, root explants are a good source for micropropagation in *C. reticulata,* citrumelo and *C. suhuiensis* since high number of shoots were obtained within a relatively short period of time. In contrast to this finding, however, Sim *et al*, (1989) reported that high number of shoots produced per root explant for *C. mitis* nine weeks in culture on MT with 0.5 mg/l BAP while 4 mg/l BAP was inhibitory

It is conceivable then that root explants are a good source for micropropagation of *C. reticulata* and citrumelo when high number of shoots were produced over a period of 18 months (3.2.2.4, 3.4.2.4). Root segments of *C. aurantifolia* regenerated shoots on BAP enriched medium after a short callus phase in a continuos culture for 3 years and the regenerated plants contained normal diploid chromosome number (2n = 18) (Bhat, *et al*, 1992).

Since roots have high regenerative capacity, and it is difficult to obtain clean roots from mature trees, it may be feasible to induce roots on shoots regenerated from meristem tips or nodal explants of a mature tree, and use the induced roots as a source of adventitious shoots. Theoretically, the plantlets obtained should be true-to-type (Bhat *et al*, 1992).

4. 2. Rooting

Rooting was done in MT basal medium or MT supplemented with 1 to 3 mg/l NAA, and solidified with 0.8 % (w/v) agar. Rooting was relatively easy and root initiation was ~~induced with different percentage~~ dependent on the species and concentration of NAA in the medium Section (3. 5), with no lateral roots were produced .

In citrumelo and *C. limonia*, shoots rooted in auxin free MT basal medium after 28 and 36 days with success rates of 33.5 and 60% respectively (3. 5). Similar results was reported for shoots of *C. aurantium*, *Poncirus trifoliata* and carrizo citrang (Naima, 1991). Addition of NAA to the medium induced root formation on shoots of the four citrus species studied (3. 5) .

For citrumelo, 100% of shoots produced roots within 10 and 8 days at concentrations of 2 and 3 mg/l NAA with number of shoots 5.4 and 6 respectively. One mg/l NAA gave the highest percentage for root initiation in *C. suhuiensis* and

C. limonia whereas 3 mg/l NAA was optimal for shoot initiation in *C. reticulata* (3.5). Root initiation usually occurred near the cut surface of the shoot explants basal end.

The results obtained are similar to those of Matsumoto and Yamaguchi (1983) when shoots of *P. trifoliata* initiated roots on MT enriched with 2 mg/l NAA. Nel (1987) reported root development from shoot meristems of *in-vitro* grown seedlings of citrus species after 2 months in half-strength MT medium supplemented with NAA only. Starrantino and Carious (1988) reported rooting from excised shoots of citrange and trifoliata orange (*P. trifoliata*) on MS medium with 1 mg/l NAA. In general, higher shoots (4 cm) seemed to root better although the rooting potential of shoot height was not tested. Such influence was observed in Douglas fir (Boulay, 1979) and radiate pine (Smith, 1983), where 4 cm and 2.4 to 2.7 cm shoots respectively were best for rooting. The number of roots per shoot is also reportedly important as 'Kinnow' mandarin plantlets with greater number of roots gave higher survival percentages compared to plantlets with less number of roots (Gill, 1994).

4. 3. Acclimatisation of tissue culture plantlets

Exposure of cultured plantlets to desiccant for 7 days before transplanting significantly increased the survival percentage (3. 6). It seems to be that loosening culture jars introduced the regenerated plantlets to an environment with a lower humidity, without exposing them to the stress of transplanting to non-aseptic

conditions. If the plantlets are transferred direct from jars to pots, they will suffer from shock of sudden reduction in relative humidity even though the pots were covered with plastic bags.

4. 3. 1. Establishment of plantlets

The acclimatisation period between transplanting *in-vitro* regenerated plantlets and full establishment under greenhouse conditions is complex and not fully understood and often results in low rates of plantlet survival (Wardle *et al*, 1983). During this period, any physiological abnormalities arising from *in-vitro* culture need to be corrected. These include the transition from heterotrophic mode of nutrition to growth by photolithotrophy and the establishment of normal water relations, both uptake and loss (Grout and Aston, 1977; ~~Wardle and Simpkins, 1979~~). A major cause of plantlet loss on transplanting is desiccation apparently caused by uncontrolled water loss. Increased survival is generally obtained if the rate of foliar water loss is restricted (Bush and Langhans, 1976; Clarke and Collin, 1974; Walkey and Matthews, 1979; ~~Wardle and Simpkins, 1979~~).

Some species of plants grown in-vitro can be acclimatised to reduced levels of relative humidity within 5 days (Prainerd and Fuchigami 1981). The reduction of stomatal aperture during culture by the use of low humidity reduce water loss and may therefore increase survival rates at transplanting. In addition, several workers have demonstrated that reduced surface wax of tissue cultured plantlets is related to the

and Fuchigami
high humidity (Prainerd, 1981; Fuchigami *et al*, 1981; Sutter *et al*, 1979; ~~Wardle *et al*, 1979~~).

In the present study, gradually reducing the relative humidity over a period of 4 weeks enabled the plantlets to form well developed cuticle layer, palisade and spongy parenchyma and increased the density of chloroplasts in the leaf cells (3. 6). These features changed the plantlets from heterotrophic to autotrophic plants and eventually increased their survival rate.

Exposure of cultured plantlets to desiccant by keeping the cap loosened for 7 days prior to transferring them to pots increased the survival percentage (3.6). This method was also suggested by Wardle *et al*, (1983). It seems to be that loosening the caps of culture vessels introduced the plantlets to an environment with a lower humidity, without exposing them suddenly to the stress of transplanting to *in-vivo* conditions. The high percentage of survival of tissue culture derived plants upon transfer to soil in citrumelo and *C. reticulata* (3. 6) points to the feasibility of this technique.

4. 4. Leaf Anatomy

The anatomical characteristics observed in tissue cultured leaves (3.7) reflected the malfunctioned leaves which could not perform normal physiological function to support independent plant growth. The undifferentiated structure of mesophyll would result in an extended cell wall surface. This, associated with non

functional stomata and extensive cuticular transpiration, ~~could~~ resulting in significant water losses. After 2 to 4 weeks of acclimatisation, leaves had a higher cell density, functional stomata and developed cuticle, thus approaching seedling leaves in anatomical characteristics. Leaf anatomy is influenced by environmental factors, such as light intensity, humidity, temperature, and nutrition (Hazel *et al*, 1982). Further investigations are important to determine how each of these factors effect leaf anatomy under culture conditions.

It appears that the cuticle in *in-vitro* cultured plantlets, is less developed than in acclimated plantlets (3. 7). However, environmental parameters affect not only wax quantity but also wax composition (Baker, 1974). The chemical composition of epicuticular waxes is also as important as their density in reducing leaf transpiration (Hadley, 1980). Apart from development of cuticle, another possible cause of water stress in cultured plantlets may be the poor development of vascular tissues and roots. For instance, Grout and Aston (1977) reported incomplete vascular development between roots and shoots in cultured cauliflower plantlets.

Tissue culture plantlets are grown under heterotrophic nutritional conditions. Thus photosynthetic processes are not required. The palisade parenchyma is commonly thought to be a specialized type of photosynthetic tissue, bringing chloroplast into a more suitable position with reference to light (Esau, 1965). The lack of internal membrane development and lack of differentiated palisade

parenchyma cells would suggest that the photosynthetic capacity of plantlets in culture is lower than those in the field or acclimated plantlets (3. 7).

Considering the observed leaf anatomical changes, it thus appears that cultured plants during acclimation undergo changes as associated with water relations including a decrease in intercellular spaces and cuticular development. This agrees with the previous reports of Grout and Aston (1977, 1978); Sutter and Langhans (1979) and Brainerd *et al,* (1981).

In the present work, tissue cultured leaves were found much thicker than seedling leaves (3. 7). This is probably because plantlet leaves contain high amount of water and more extensive intercellular space compared to greenhouse plants. Contrary to the present finding, Hazel *et al,* (1982) reported that field grown leaves were thicker than those of cultured leaves.

Hazel *et al*, (1982) reported that in addition to the water relations adaptations seen in new growth during the acclimation period, other factors apparently are involved. Changes in chloroplasts ultra structure, cytoplasmic content, ergastic vascular components, and organelle frequency were seen. Perhaps changes in cell metabolism occurred associated with cytoplasmic changes and chloroplast development during acclimation. The mortality rates and sensitivity of cultured plantlets to the normal environmental conditions may thus be twofolds. Firstly due to

difficulties incurred as a result of water stress during the transition from high to low humidity, and secondly due to physiological changes which presumably took place during transition from heterotrophic to autotrophic conditions.

From the results, we conclude that exposure of cultured plantlets to a desiccant for 7 days increased their survival rate (3. 6). Loosening caps of the culture vessels for 7 to 10 days introduced the regenerated plants to an environment with a lower humidity, without exposing them to the stress of *in-vivo* condition. This procedure could be efficient method for hardening of *in-vitro* propagated plants.

The structural differences in leaf reported in the present study give an indication of the important role of leaf anatomy during acclimatisation. Additional studies are needed to verify physiological and developmental aspects. Thus further research is suggested to understand the acclimatisation of culture plantlets from *in-vitro* to the field environment.

4. 5. Isoenzyme

Isoenzyme studies have been used widely in citrus to determine the phylogenic relationship and the degree of affinity between species (Kozak and Kajiura, 1986; Masashi and Ichiro, 1987; Masashi *et al*, 1990), in taxonomic studies (Germana *et al*, 1994; Tusaet *et al*, 1990), in genetic mapping of citrus (Jarrel *et al*, 1992), for identification of zygotic and nucellar seedlings (Lima and Simon, 1974).

Isoenzyme study was carried out to detect the presence of somaclonal and nucleic variation in tissue culture produced plants. The same banding patterns were observed in the isoenzyme peroxidases, and glutamate oxaloacetate transminase (GOT) in the plants citrumelo, *C. limonia* and *C. reticulata* regenerated from internodal, leaf and root explants for citrumelo; internodal explants for *C. limonia* and from root explants for *C. reticulata* the seedlings regenerated from seeds for the parents plants *C. suhuiensis* was not tested because the ~~deficiency~~ of plant material. For the plants tested there were no changes in isoenzyme band patterns indicating that there was no somaclonal variation in the regenerated plants (3. 8).

4. 6. The isolation, culture and division of protoplast

Osmotic pressure, enzyme combination as well as the duration of callus exposure to the enzymes, strongly affect the protoplast yield, viability and division capability. It was found that 0.7 M mannitol or combination of mannitol 0.3 M and sucrose 0.3 M provides an appropriate osmolarity during callus maceration (Vardi and Galun, 1988).

The success of protoplast culture to induce plant regeneration depends on the ability to isolate large numbers of viable protoplasts. For citrumelo embryo callus protoplast, it was found that their number and viability depend on the enzyme combination. Cellulase at 0.30%, macerozyme at 0.35 % and driselase at 0.10 % (E_3) was the best combination for high yield whereas cellulase R-10 at 3.0 % and

macerozyme R-10 at 0.30 % (E_1) gave highest protoplast viability (3.9.1.1) (Appendix D).

The presence of driselase at 0.1 % in the enzyme mixture (E_3) (Appendix D) which increased the yield but reduced the viability may be because driselase is somewhat toxic to the protoplasts or the incubation time was too long. Therefore, enzyme mixture (E_1) was chosen for protoplast isolation. Vardi and Galun (1988), reported that plating efficiency of grapefruit protoplast isolated from embryo callus was increased when driselase was omitted from the enzyme mixture containing cellulase and macerozyme. Ochatt *et al,* (1989) reduced incubation time for wild pear rootstock leaves to 8 hours in enzyme mixture containing a combination of cellulase R-10, macerozyme R-10 and driselase to obtain appropriate viability.

Leaves of citrumelo, *C. suhuiensis* and *C. limonia* grown *in-vitro* plants were used for mesophyll protoplast isolation. It was reported aseptic cultured plants are an ideal source for mesophyll protoplasts (Ahuja *et al*, 1982). Leaves from *in-vitro* cultured plants are thin, fragile and contain loose mesophyll cells. Therefore, separation of cells and digestion of cell wall are easier compared to greenhouse plants, ~~which~~ thus producing higher protoplast yields. The yield and viability of isolated protoplasts from *in-vitro* plants were found to be higher compared to *in-vivo* plants and was reportedly due to the effect of chemicals during sterilisation process (Okumura *et al*, 1984). Plant material obtained from aseptic culture usually are free of microbial contamination, and

furthermore the controlled growth conditions ensure a relatively uniform physiological state of the plants (Cassells and Barlass, 1978). It was also reported that higher yields from leaves were usually obtained from young tissues with thin cuticle, while relatively old leaves with heavily lignin tissue may be difficult to digest. Mesophyll cells are usually used in protoplast fusion and somatic hybridisation. Plant regeneration from mesophyll protoplast in citrus and woody plants are reportedly very rare (Oka and Ohyama, 1985; Tusa *et al*, 1991).

The effects of enzyme combination on the yield and viability of mesophyll protoplasts was found to be species dependent. Furthermore, for the same species the highest yield was obtained from one enzyme mixture while the highest viability was obtained from another (3.9.1.1). Therefore the appropriate enzyme mixture should be determined empirically for each species. Similar observations was reported for mesophyll protoplasts of some citrus species (Vardi and Galun, 1988). Attempts were made to culture mesophyll protoplasts of citrumelo, *C. suhuiensis*, and *C. limonia* on MS basal media without growth substances. No division was observed possibly due to the lack of protoplast capability for division.

4. 6. 1. Embryo callus of citrumelo

Plant regeneration has been reported in embryogenic callus derived protoplasts from plant material other than nucellar callus such as embryo (Hidaka and Kajiura, 1988; Hidaka and Omura, 1989) leaf (Tusa *et al*, 1991) hypocotyl region (Hidaka

and Kajiura, 1988; Ling *et al*, 1989, 1990) epicotyl derived callus (Grosser *et al*, 1988) and root (Sim, 1989).

Embryo callus are preferred material source of protoplasts because of their uniformity and ease of production as experimental material. Furthermore, the use of embryo callus for protoplast isolation overcomes the difficulties associated with the production of leaf material and enables adequate and rapid supply of experimental material (Xu *et al*, 1981), because the embryo is a good source of dividing cells with high mitotic index.

Embryo callus protoplasts also provide a useful system for genetic manipulation, as they are capable of withstanding treatment with agents such as polyethylene glycol (Uchimiya, 1982) and calcium ion (Ca^{+2}) at high pH (Keller and Melchers, 1973) which are used as fusion agents. Embryo callus protoplast fused readily (Power *et al*, 1970), and being a colourless partner, morphological identification of heterokaryons following fusion of embryo callus protoplast with leaf mesophyll protoplast is easy (Kobayashi *et al*, 1988). Moreover, it was reported that embryo callus retained its embryogenic potential after 5 years of culture without exhibiting any somaclonal variation (Kobayashi *et al,* 1988; Hidaka and Omura, 1989).

In view of the advantages mentioned, embryo callus protoplasts of citrumelo were cultured (3. 9. 1. 2.). Plant growth substances enhanced cell division in embryo callus protoplasts of citrumelo. The highest rate of cell division obtained after 7 days was approximately 3.3 % on MT liquid medium with 2,4-D and BAP at 0.5 and 1 mg/l respectively (3.9.1.2). Generally, protoplasts cultured on liquid media responded better than those on solid media.

It is unfortunate that the contamination problems that are inherent in the culture room used (Laboratory F, Botany Department; University of Malaya) during protoplast culture did not allow visual observation beyond the first division. Thus, more experiments should be carried out on embryo callus protoplasts to determine their regenerative potentials.

4. 7. Photosynthetic capability

It has been well documented that transplanting of plantlets *in-vitro* requires prolonged regulation of both temperature and relative humidity to enable acclimatization of plantlets to glasshouse conditions (Grout, 1984). One of the major problems commonly encountered at transplanting are the inability of plantlets to regulate the transpiration stream in addition to an inefficient photosynthetic capability (Grout and Aston, 1978). The ability of plantlets formed *in-vitro* to exhibit full photosynthetic competence after transplanting depended largely on the species studied. Grout (1978)

working on cauliflower and Hazel *et al*, (1982) on sweet gum reported that plantlets regenerated *in-vitro* attained full photosynthetic capacity after they were transplanted from culture to soil.

In the studies carried out here on citrus species it was observed that the various plantlets generated from tissue culture exhibited similar photosynthetic competence when compared to those grown from seeds. Photosynthetic rates between 6 and 8 μmol CO_2 m^{-2} s^{-1} were observed from the various species studied (3.10.1). Rates between 1 to 70 μmol CO_2 m^{-2} s^{-1} have been reported in the literature from various studies (Coombs *et al*, 1985; Norrizah., 1993; Norlaila, 1994). Photosynthetic rates in C_4 grasses are among the highest recorded. Norlaila (1994) working on 6 species of legumes reported similar assimilation rates whilst Norrizah (1993) studied 5 species of local Malaysian vegetables and salads "Ulam" observed slightly higher rates.

Light saturation for all the plantlets and seedlings studied was achieved between 350 to 500 μmol m^{-2} s^{-1} of light intensity (3.10.1.1). Leaves of many C_3, plants of which citrus is one, are unable to utilize additional light above 500 μmol m^{-2} s^{-1} (Coombs *et al*, 1985). Generally shade plants exhibit lower light saturation (50 - 400 μmol m^{-2} s^{-1}) compared to sun plants (400 - 1200 μmol m^{-2} s^{-1}). However,

some C_4 plants fail to saturate even at full sun light (Coombs *et al*, 1985). The apparent lower light saturation observed in this study could probably be due to the plants, being grown under laboratory conditions, has acclimatised under low light intensity regime. Nevertheless the plantlets exhibited similar light saturation patterns compared to those shown by seedlings (3.10.1.1).

As shown in Table 40 the light compensation points in citrumelo and *C. limonia* plantlets and seedlings were similar. A 2 to 3 fold difference was observed in *C. reticulata*. Norrizah, (1993) reported higher values ranging between 40 to 70 $\mu mol\ m^{-2}\ s^{-1}$ for the five species of "Ulams" she studied. The light compensation points for the species studied were within the range typical for C_3 plants.

All the plantlets and seedlings studied showed similar and relatively high quantum yield (3.10.1.3) with values ranging between 0.02 to 0.05, indicating efficient use of light for CO_2 assimilation. Lower values, between 0.01 to 0.02 was observed for 6 species of legumes (Norlaila, 1994). Water use efficiency, has been defined as the amount of carbon fixed relative to water loss as a result of the fixation (Nobel *et al*, 1978), reflects the spontaneous ability of the plant to conserve water without causing a reduction in CO_2 assimilation. It involves the regulation of the stomatal opening such that water loss is minimal but sufficient for the CO_2 intake.

The values obtained from the studies here showed that the plantlets were slightly more competent than the seedlings registering higher values (3.10.1.4).

These observations are in agreement with the light microscopy studies on the leaf anatomies of the plantlets and seedlings during the hardening period. It was shown that after 2 weeks of transplanting the plantlets developed distinct palisade and spongy tissues and a well developed cuticle layer (3.7). This confirmed the observations that the plantlets regenerated *in-vitro* were photosynthetically competent.

From these results we may conclude that the citrus plantlets regenerated *in-vitro* were able to develop the full complement of the photosynthetic machinery after transplanting and exhibited similar photosynthetic characteristics to the plants grown from seedlings.