CHAPTER 3

RESULTS

3. RESULTS

3. 1. *C. suhuiensis*

3. 1. 1. Effect of NAA

3. 1. 1. 1. Nodal explants

Nodal explants of *C. suhuiensis* responded to all concentrations of NAA tested forming shoots and/or roots. The optimal concentration of NAA for root and shoot regeneration was found to be at 1.0 mg/l when 80% of the explants formed an average of 1 shoot and 2.2 roots per explant with an average length of about 5 and 20 mm respectively (Table 4).

3. 1. 1. 2. Internodal explants

Internodal explants of *C. suhuiensis* regenerated shoots at 0.1 and 1 mg/l NAA and the shoots were comparatively shorter (1-2 mm). Root formation was induced at 0.5 mg/l NAA and above. The optimal concentration for root formation was at 5 mg/l NAA when 50% of the explants produced an average of 2.1 roots each at 30 ± 5.5 mm long (Table 4). The mean number of roots per explant increased with the increase in NAA concentration. At high levels of NAA the regenerated roots were thick and short, about 1.5 mm long.

Table 4: Shoot and root regeneration from nodal and internodal explants of *C. suhuiensis* after 8 weeks in culture on MT media supplemented with various concentrations of NAA.

		Root regeneration			Shoot regeneration		
NAA mg/l	No. of explants	No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of expl with shoots (%)	Mean no. of shoots ± SE	Mean length ± SE (mm)
Nodal explants							
0	13	0	-	-	0	-	-
0.1	11	16.0	1.0	10.4 ± 1.20	33.0	1.2 ± 0.20	15.20 ± 3.51
0.5	10	16.5	1.3 ± 0.21	30.5 ± 5.42	30.2	1.0	6.54 ± 1.32
1.0	11	80.2	2.2 ± 0.11	20.0 ± 6.22	80.5	1.1 ± 0.10	5.0 ± 0.10
2.0	12	70.2	3.0 ± 0.42	13.4 ± 5.00	33.2	1.0 ± 0.50	2.0 ± 0.32
3.0	10	60.0	2.0 ± 0.21	20.3 ± 3.51	33.0	1.2 ± 0.21	5.0 ± 0.70
5.0	13	60.3	3.0 ± 0.12	10.2 ± 1.50	33.1	1.0 ± 0.30	5.1 ± 0.15
10.0	11	50.0	3.4 ± 0.52	35.0 ± 5.55	0	-	-
Internodal explants							
0	13	0	-	-	0	-	-
0.1	14	0	-	-	12.2	1.3 ± 0.42	15.2 ± 3.51
0.5	13	12.0	1.2 ± 0.31	10.0 ± 1.51	0	-	-
1.0	12	20.2	1.4 ± 0.20	10.3 ± 2.00	25.4	1.2 ± 0.20	5.0 ± 0.10
2.0	12	37.4	2.0 ± 0.23	25.0 ± 2.50	0	-	-
3.0	13	40.4	2.3 ± 0.50	30.0 ± 4.02	0	-	-
5.0	14	50.3	2.1 ± 0.41	30.0 ± 5.57	0	-	-
10.0	15	30.0	4.0 ± 1.50	15.0 ± 4.26	0	-	-

Mean number of roots per explant and length of roots in (mm) ± SE.

3. 1. 1. 3. Leaf explants

Leaf explants of *C. suhuiensis* responded to all concentrations of NAA tested forming roots only. The optimal concentration for root formation was at 2.0 mg/l NAA when 69.3% of the leaf explants formed an average of approximately 10.2 roots per explant at about 25.5 mm long. Neither shoot nor callus were formed (Table 5).

3. 1. 1. 4. Root explants

Root explants exhibited root formation at 0.5 mg/l NAA and a higher concentration. The optimal NAA concentration for root formation was at 2.0 mg/l when 50 % of the explants formed an average of 2.3 roots per explant with mean length of approximately 25.5 mm (Table 6). A few number of short shoots were regenerated at 1.0 mg/l NAA from 12.0 % of the explants.

Table 5: Root regeneration from leaf explants of *C. suhuiensis* after 8 weeks in culture on MT media with various concentrations of NAA.

NAA mg/l	No. of explants	Root regeneration		
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)
0	13	0	-	-
0.1	12	0	-	-
0.5	14	18.5	1.0 ± 0.20	20.0 ± 4.52
1.0	14	33.3	2.0 ± 0.31	36.0 ± 7.30
2.0	11	69.3	10.2 ± 3.15	25.5 ± 2.51
3.0	10	30.0	4.0 ± 1.56	15.0 ± 2.50
5.0	15	30.2	6.5 ± 3.00	30.0 ± 5.13
10.0	12	12.0	3.0 ± 1.22	5.0 ± 1.50

- Mean number of roots per explant and length of roots in mm ± SE.

Table 6: Root regeneration formation from root explants of *C. suhuiensis* after 8 weeks in culture on MT media with various concentrations of NAA.

NAA mg/l	No. of explants	Root regeneration		
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)
0	13	0	-	-
0.1	14	0	-	-
0.5	14	8.7	1.0	30.0 ± 3. 42
1.0	14	37.5	2.2 ± 0.31	10.0 ± 2.5
2.0	11	50.4	2.3 ± 0.25	25.0 ± 1.31
3.0	14	6.3	2.1 ± 0.46	20.1 ± 2.4
5.0	15	100.0	3.0 ± 0.50	20.3 ± 4.17
10.0	15	28.0	2.0 ± 0.52	5.0 ± 1.0

- Mean number of roots per explant and length of roots in mm ± SE.

3. 1. 2. Effect of BAP

3. 1. 2. 1. Nodal explants

With nodal explants, the axillary buds started to develop within 1 week in culture. Buds developed on explants cultured in BAP enriched media with optimal concentration at 3 mg/l with an average of about 5.6 shoots per explant (Table 7, Plate 2 a). Growth of the shoots was somewhat stunted with high levels of BAP at 5 and 10 mg/l. Green and friable callus was obtained with media containing BAP after 4 weeks in culture. No roots were induced in basal medium or in the medium enriched with BAP.

3. 1. 2. 2. Internodal explants

The explants appeared greener than original explant and slightly swollen from both sides after 2 weeks followed by callus formation in the third week of culture with BAP concentrations at 1 to 10 mg/l.

Shoots were regenerated at concentrations from 1 to 5 mg/l BAP. At 3 mg/l BAP 100 % of the explants regenerated an average of 3.4 shoots per explant with 5 mm height (Table 7, Plate 2 b). At high levels of BAP only callus was produced . No root was observed after 8 weeks in culture for all the explants.

3. 1. 2. 3. Leaf explants

In BAP enriched media the explants turned light brown after 8 weeks in culture and no organogenesis was observed.

3. 1. 2. 4. Root explants

No shoot regeneration was observed in explants cultured on MT basal medium without the addition of hormone even after 6 months. In all BAP enriched media tried, the explants became swollen after 2 weeks and by the fourth week adventitious shoots were produced from callus. The optimal for shoot regeneration obtained with 2 and 5 mg/1 BAP where 42% of the explants produced an average of 5 shoots per explant by eight week. The regenerated shoots were small and failed to grow when subcultured in rooting media. Whitish green calli proliferated in all concentrations of BAP tested. The calli increased in size with the increase in BAP concentrations, and failed to regenerate when sub cultured in the same media. Only callus was regenerated at 10 mg/l BAP (Table 7).

3. 1. 2. 3. Leaf explants

In BAP enriched media the explants turned light brown after 8 weeks in culture and no organogenesis was observed.

3. 1. 2. 4. Root explants

No shoot regeneration was observed in explants cultured on MT basal medium without the addition of hormone even after 6 months. In all BAP enriched media tried, the explants became swollen after 2 weeks and by the fourth week adventitious shoots were produced from callus. The optimal for shoot regeneration obtained with 2 and 5 mg/l BAP where 42% of the explants produced an average of 5 shoots per explant by eight week. The regenerated shoots were small and failed to grow when subcultured in rooting media. Whitish green calli proliferated in all concentrations of BAP tested. The calli increased in size with the increase in BAP concentrations, and failed to regenerate when sub cultured in the same media. Only callus was regenerated at 10 mg/l BAP (Table 7).

Table 7: Shoots and callus formation from nodal, internodal and root explants of *C. suhuiensis* after 8 weeks in culture on MT media supplemented with various concentrations of BAP.

BAP mg/l	No. of explants	Shoot regeneration: No. of expl. with shoots (%)	Shoot regeneration: Mean no. of shoots ± SE	Shoot regeneration: Mean height ± SE (mm)	Callus formation: No. of explants with callus (%)	Callus formation: Degree of callusing
Node explants						
0	13	33.4	1.30 ± 0.35	15.50 ± 2.40	0	-
1.0	14	100.0	2.20 ± 0.19	5.10 ± 0.24	0	-
2.0	14	100.0	2.10 ± 0.12	6.30 ± 0.30	0	-
3.0	14	100.0	5.62 ± 0.55	5.0 ± 0.21	100.0	+
5.0	11	60.6	2.0 ± 0.10	5.10 ± 0.52	100.0	++
10.0	10	50.0	1.0	2.0 ± 0.11	60.5	++
Internodal explants						
0	13	0	-	-	0	-
1.0	14	40.0	1.10 ± 0.20	15.3 ± 0.72	40	+
2.0	14	44.0	2.0 ± 0.10	5.0 ± 0.21	44.0	++
3.0	14	100.0	3.41± 0.50	5.2 ± 0.43	100.0	+++
5.0	12	60.3	1.0	3.0 ± 0.42	100.0	++
10.0	13	0	0	-	60.0	++
Root explants						
0	13	0	-	-	0	-
1.0	14	30.4	1.42 ± 0.41	3.4 ± 0.50	92.6	+
2.0	14	42.8	5.20 ± 0.30	5.20 ± 0.31	100.0	++
3.0	15	6.5	2.15 ± 0.11	3.31 ± 0.22	100.0	++
5.0	13	42.0	5.15 ± 0.51	5.41 ± 0.33	100.0	++
10.0	14	0	-	-	60.3	+++

-Mean number of shoots per explant and height of shoots in mm± SE.

3. 1. 3. Effect of NAA and BAP in combinations

The four explants of the citrus species were cultured on MT media with combinations of NAA (0.5 to 5 mg/l) and BAP (1 to 3 mg/l) (Appendix A). The response of the explants were found to be dependent on the species and the type of explants.

3. 1. 3. 1. Nodal explants

Nodal explants of *C. suhuiensis* exhibited the most vigorous response among the other explants regenerating axillary shoots from the buds and formed small to medium compact callus in all the combinations of phytohormones tried. Up to 6 shoots per explant were regenerated on media with 1 mg/l NAA and 3 mg/l BAP with a regeneration frequency of 78% (Table 8). The highest percentage of explants that regenerated shoots was on media with 2 mg/l NAA and 3 mg/l BAP (91%). Shoot growth was faster in the former (Table 8). All shoots were developed directly from the axillary buds within 4 to 6 weeks in culture. Small to medium green compact callus was proliferated at the base of the explants in all the combinations of phytohormones tried (Plate 2 c). The mean height of shoots was significantly reduced when the explants were cultured in NAA and BAP enriched media compared to shoots regenerated in MT basal medium. Roots were completely inhibited in media with added NAA and BAP in combinations.

3. 1. 3. Effect of NAA and BAP in combinations

The four explants of the citrus species were cultured on MT media with combinations of NAA (0.5 to 5 mg/l) and BAP (1 to 3 mg/l) (Appendix A). The response of the explants were found to be dependent on the species and the type of explants.

3. 1. 3. 1. Nodal explants

Nodal explants of *C. suhuiensis* exhibited the most vigorous response among the other explants regenerating axillary shoots from the buds and formed small to medium compact callus in all the combinations of phytohormones tried. Up to 6 shoots per explant were regenerated on media with 1 mg/l NAA and 3 mg/l BAP with a regeneration frequency of 78% (Table 8). The highest percentage of explants that regenerated shoots was on media with 2 mg/l NAA and 3 mg/l BAP (91%). Shoot growth was faster in the former (Table 8). All shoots were developed directly from the axillary buds within 4 to 6 weeks in culture. Small to medium green compact callus was proliferated at the base of the explants in all the combinations of phytohormones tried (Plate 2 c). The mean height of shoots was significantly reduced when the explants were cultured in NAA and BAP enriched media compared to shoots regenerated in MT basal medium. Roots were completely inhibited in media with added NAA and BAP in combinations.

Table 8: Shoots and callus formation from nodal explants of *C. suhuiensis* after 8 weeks in culture on MT media supplemented with various concentrations of NAA and BAP.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration			Callus formation		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	13	33.4	1.0	15.40 ± 1.64	0	-	-
0.5	1.0	12	70.1	4.90 ± 0.20	3.25 ± 0.32	100.0	G	+ +
1.0	1.0	12	55.0	4.35 ± 0.45	9.85 ± 0.34	88.0	G	+ +
1.0	2.0	15	60.0	3.50 ± 0.41	3.23 ± 0.22	86.4	G	+ +
1.0	3.0	13	78.6	6.30 ± 0.32	4.10 ± 0.10	92.2	G	+ +
2.0	1.0	10	80.2	3.10 ± 0.20	5.00 ± 1.20	90.0	G	+ +
2.0	2.0	13	76.4	4.22 ± 0.25	3.30 ± 0.11	100.0	G	+ +
2.0	3.0	12	91.0	3.30 ± 0.50	3.60 ± 0.35	91.3	G	+ +
5.0	1.0	12	75.5	2.23 ± 0.20	4.50 ± 0.20	100.0	G	+
5.0	2.0	12	75.4	4.00 ± 0.98	3.14 ± 0.16	100.0	G	+
5.0	3.0	15	50.0	3.00 ± 0.25	2.50 ± 0.75	100.0	G	+

Mean number of shoots per explant and height of shoots in mm± SE.

Plate 2 . *C. suhuiensis* shoot regeneration from nodal and internodal explants.

a Shoot regeneration from nodal explants after 6 weeks in culture on MT supplemented with 2 mg/l BAP.

b Shoot regeneration from internodal explants after 8 weeks in culture on MT supplemented with 1 mg/l BAP, bar = 0.5 cm.

c Shoot regeneration from nodal explants after 8 weeks in culture on MT supplemented with NAA (2 mg/l) and BAP (3 mg/l), bar = 0.5 cm.


a
b
c

3. 1. 3. 2. Internodal explants

Addition of NAA and BAP to the medium completely suppressed root formation, but encouraged shoot regeneration at low and equal NAA to BAA ratio (Table 9). The highest percentage of shoot regeneration was at concentrations 2 mg/l NAA and 3 mg/l BAP. As the ratio of BAP to NAA increased in the medium the highest number of shoots per explants was obtained Medium to large green calli were induced at low levels of NAA, and when NAA in the medium was increased to 5 mg/l shoot regeneration was inhibited and only whitish green calli proliferated.

3. 1. 3. 3. Leaf explants

Shoots were regenerated only in media with 1 mg/l NAA and 3 mg/l BAP (Table 10). The percentage of shoot regeneration was low and the mean number of shoots per explant was approximately 2.2 at of 1 mm height. Small white green callus was produced at concentrations of NAA from 0.5 to 2 mg/l and no response was observed at high levels (5 mg/l) of NAA in the medium. No roots were produced at all the concentrations of NAA and BAP tried

3. 1. 3. 4. Root explants

Root explants exhibited low percentages of response to auxin and cytokinin combinations tested forming shoots and green callus. The number of shoots was from 1.3 to 4 and the height of shoots regenerated was very small less than (1 mm). No rhizogenesis was observed. The results are tabulated in Table 11.

Table 10: Shoots and callus formation from leaf explants of *C. suhuiensis* after 8 weeks in culture on MT media with various concentrations of NAA and BAP in combinations.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration			Callus formation		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	10	0	-	-	0	-	-
0.5	1.0	12	0	-	-	35.0	WG	+
1.0	1.0	9	0	-	-	30.4	WG	+
1.0	2.0	9	0	-	-	38.0	WG	+
1.0	3.0	11	15.0	2.20 ± 0.45	1.0 ± 0.22	38.4	WG	+
2.0	1.0	12	0	-	-	30.0	WG	+
2.0	2.0	10	0	-	-	41.5	WG	+
2.0	3.0	11	0	-	-	11.0	WG	+
5.0	1.0	10	0	-	-	0	-	-
5.0	2.0	10	0	-	-	0	-	-
5.0	3.0	10	0	-	-	0	-	-

Mean number of shoots per explant and height of shoots in mm± SE.

Table 9: Shoots and callus formation from internodal explants of *C. suhuiensis* after 8 weeks in culture on MT media with various concentrations of NAA and BAP in combinations.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	13	0	-	-	0	-	-
0.5	1.0	12	16.6	2.10 ± 0.52	1.0 ± 0.31	83.3	G	+ +
1.0	1.0	10	22.2	2.00 ± 0.41	1.0 ± 0.11	100.0	G	+ +
1.0	2.0	10	22.5	2.20 ± 0.30	1.20 ± 0.21	77.8	G	+ +
1.0	3.0	13	46.0	5.98 ± 0.64	0.10 ± 0.22	92.3	G	+ + +
2.0	1.0	11	0	-	-	77.7	G	+ +
2.0	2.0	11	33.5	2.10 ± 0.34	1.0	100.0	G	+ +
2.0	3.0	12	83.3	4.90 ± 0.50	1.0 ± 0.50	83.3	G	+ +
5.0	1.0	12	0	-	-	50.0	WG	+
5.0	2.0	10	0	-	-	66.6	WG	+
5.0	3.0	13	0	-	-	28.0	W G	+

-Mean number of shoots per explant and height of shoots in mm± SE.

Table 10: Shoots and callus formation from leaf explants of *C. suhuiensis* after 8 weeks in culture on MT media with various concentrations of NAA and BAP in combinations.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	10	0	-	-	0	-	-
0.5	1.0	12	0	-	-	35.0	WG	+
1.0	1.0	9	0	-	-	30.4	WG	+
1.0	2.0	9	0	-	-	38.0	WG	+
1.0	3.0	11	15.0	2.20 ± 0.45	1.0 ± 0.22	38.4	WG	+
2.0	1.0	12	0	-	-	30.0	WG	+
2.0	2.0	10	0	-	-	41.5	WG	+
2.0	3.0	11	0	-	-	11.0	WG	+
5.0	1.0	10	0	-	-	0	-	-
5.0	2.0	10	0	-	-	0	-	-
5.0	3.0	10	0	-	-	0	-	-

Mean number of shoots per explant and height of shoots in mm± SE.

Table 11: Shoots and callus formation from root explants of *C. suhuiensis* after 8 weeks in culture on MT media supplemented with various concentrations of NAA and BAP.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	14	0	-	-	0	-	-
0.5	1.0	14	8.0	3.2 ± 0.52	1.0 ± 0.31	42.8	G	+ +
1.0	1.0	15	0	-	-	6.0	G	+ +
1.0	2.0	15	7.0	1.3 ± 0.33	0.89	14.2	G	+
1.0	3.0	14	0	-	-	21.4	G	+
2.0	1.0	14	0	-	-	5.0	WG	+
2.0	2.0	14	0	-	-	0	-	-
2.0	3.0	14	7.0	2.1± 0.50	0.70	28.5	G	+
5.0	1.0	17	5.8	4.0 ± 0.12	1.0	23.3	G	+
5.0	2.0	17	0	-	-	29.9	WG	+
5.0	3.0	16	18	2.21 ± 0.10	0.85	37.5	G	+ +

-Mean number of shoots per explant and height of shoots in mm± SE.

3. 2. Citrumelo

3. 2. 1. Effect of NAA

3. 2. 1. 1. Nodal explants

Nodal explants of citrumelo showed good response to all NAA concentration tested, regenerating shoots and roots. Shoots were also formed on MT basal medium. Adding NAA proliferated root and shoot formations (Table 12). The percentage of root regeneration was proportional to the NAA supplementation from 2 to 5 mg/l, and decreased to 50% at 10 mg/1. The number and length of roots were significantly different according to changes of NAA in the media (Table 12). Shoot regeneration was suppressed by the addition of NAA to MT basal medium. While 100% of the explants formed shoots in MT basal medium, the regeneration percentage was reduced when NAA was added (Table 12). The number of shoots regenerated per explant was approximately the same.

3. 2. 1. 2. Internodal explants

Root regeneration started after 3 weeks in culture at concentrations 0.5 mg/1 NAA and above. Optimal NAA concentration for root regeneration was at 3 mg/1 with a frequency of 90.3% (5.3 roots per explant) (Table 13).

Green compact callus was produced at 3 mg/1 NAA and above, but no shoot regeneration was observed.

Table 12: Shoot and root regeneration from nodal explants of citrumelo after 8 weeks in culture on MT media supplemented with various concentrations of NAA.

NAA mg/l	No. of explants	Root regeneration			Shoot regeneration		
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of expl with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)
0	13	33.4	1.0	20.2 ± 1.0	100.0	1.5 ± 0.52	15.20 ± 2.31
0.1	12	40.0	1.0	10.8 ± 2.10	50.0	1.0 ± 0.20	5.21 ± 1.30
0.5	14	60.5	1.0 ± 0.20	10.0 ± 0.56	50.2	1.31 ± 0.32	3.00 ± 1.50
1.0	15	83.8	1.30 ± 0.50	34.0 ± 4.22	50.1	1.00 ± 0.10	1.00 ± 0.12
2.0	15	100.0	5.50 ± 0.42	25.0 ±1.50	50.0	1.0	2.0 ± 0.52
3.0	15	100.0	6.10 ± 0.10	30.4 ± 6.34	16.3	1.12 ± 0.10	1.00 ± 0.21
5.0	13	100.0	7.20 ± 1.0	25.10 ± 2.0	16.7	2.0 ± 0.50	5.30 ± 2.10
10.0	11	50.5	6.30 ± 0.52	15.5 ± 1.25	16. 0	1.0 ± 0.32	2.2 ± 1.20

- Mean number of roots, roots per explant and height shoots and length of roots in mm ± SE.

3. 2. 1. 3. Leaf explants

Roots were regenerated from the cut edges and mid-rib of leaf explants on media supplemented with 1 mg/l NAA and above. All the 1 explants formed an average of 9.2 roots each with approximately 30 mm length (Table 14) on medium with NAA at 2.0 mg/l. The roots were regenerated directly from the explants. Neither shoot nor callus was formed.

3. 2. 1. 4. Root explants

Roots were regenerated in the range of 0.5 to 2 mg/l NAA, whereas no response was observed at concentrations above and below this level. The percentage and number of roots regenerated per explants were low. On 1 mg/l NAA, 33.5% of the explants regenerated an average of about 2.2 roots with about 61 mm length (Table 13). Medium white green callus was produced at 3 and 5 mg/l NAA and no shoot regeneration was observed.

Table 13: Effect of NAA on internodal and root explants of citrumelo after 8 weeks in culture.

NAA mg/l	No. of explants	Root regeneration			Callus formation	
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of explants with callus (%)	Degree of callusing
Internodal explants						
0	13	0	-	-	0	-
0.1	13	0	-	-	0	-
0.5	13	70.3	2.2 ± 0.41	15.0 ± 1.52	0	-
1.0	15	80.5	2.5 ± 0.60	35.2 ± 2.43	0	-
2.0	13	83.0	3.0 ± 0.22	30.5 ± 3.30	0	-
3.0	11	90.3	5.31 ± 0.25	30.21± 2.32	100.0	+
5.0	14	80.3	4.41 ± 1.00	20.25 ± 2.30	40.0	+ + +
10.0	10	60.0	4.0 ± 0.45	15.30 ± 1.50	100.0	+ + +
Root explants						
0	13	0	-	-	0	-
0.1	14	0	-	-	0	-
0.5	14	28.5	2.0 ± 0.10	1.00 ± 0.11	0	-
1.0	12	33.4	2.20 ± 0.25	6.10 ± 0.51	0	-
2.2	14	14.6	1.53 ± 0.57	5.40 ± 0.20	0	-
3.0	15	0	-	-	12.0	+ +
5.0	15	0	-	-	0	-
10.0	10	0	-	-	75.0	+ +

- Mean number of roots per explant and length of roots in (mm) ± SE.

Table 14: Effect of NAA on leaf explants of citrumelo after 8 weeks in culture.

NAA mg/l	No. of explants	Root regeneration		
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)
0	13	0	-	-
0.1	13	0	-	-
0.5	12	0	-	-
1.0	11	33.3	2.20 ± 0.51	28.1 ± 3.50
2.0	12	100.0	9.21 ± 1.55	30.4 ± 1.00
3.0	15	44.5	8.5 ± 1.20	10.21± 1.21
5.0	14	44.0	7.0 ± 1.10	13.0 ± 2.40
10.0	14	88.5	8.3 ± 2.25	22.80 ± 3.10

- Mean number of roots per explant and length of roots in mm ± SE.

3. 2. 2. Effect of BAP

3. 2. 2. 1. Nodal explants

With nodal explants, the axillary buds started to develop within 10 days in culture. Multiple buds developed on explants cultured in BAP enriched media after 2 weeks in culture with optimal concentration at 2 mg/l BAP with an average of 5.2 shoots regenerated per explant (Table 15, Plate 3 a).

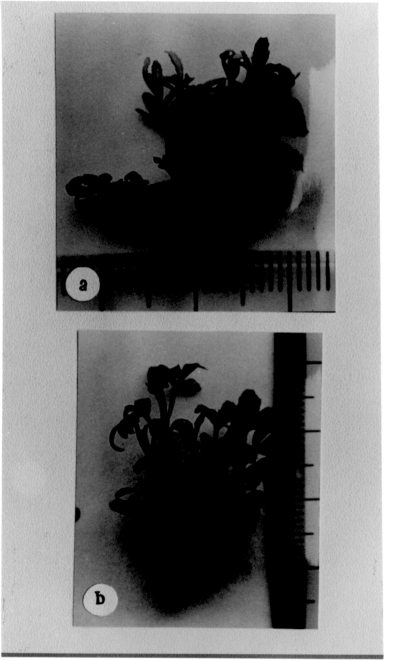
Green and friable callus was obtained in media containing BAP after 3 weeks in culture (Plate 3 b). The number and height of shoots per explants were significantly reduced at high levels of BAP (5 and 10 mg/l).

Root was induced only in basal medium up to 33% where 1 root was produced per explant with a mean length of approximately 40 mm after 8 weeks in culture.

Plate 3. Citrumelo shoot regeneration from nodal explants

a Shoot regeneration after 9 weeks in culture on MT with 2 mg/l BAP.

b Shoot regeneration after 8 weeks in culture on MT supplemented with 3 mg/l BAP.


a
b

3. 2. 2. 2. Internodal explants

In BAP enriched media, greenish and friable or nodular calli were produced from the cut edges of the segments within 3 weeks. Calli were usually observed at concentrations 2 mg/l BAP and higher, when 100% of the explants formed green nodular callus (Table 15).

Beside callus formation, the internodal explants also produced shoots directly from the cut ends (Plate 4 a). BAP stimulated shoot regeneration and exhibited a concentration effect with an optimum concentrations being 2 and 3 mg/l BAP, where 100% of the explants regenerated approximately 5.6 and 8.7 shoots per explants respectively (Table 15, Plate 4 b). Increase in BAP concentration in the medium at 5 and 10 mg/l significantly increased the number of shoots regenerated per explant, up to approximately 15 shoots per explant. However, the height of shoots was inversely proportional to the BAP concentration. Shoots produced were readily rooted on MT basal medium with 1 to 3 mg/l NAA and successfully transplanted in soil (Plate 4 c).

Plate 4 . Citrumelo shoot regeneration and plantlet.

a Induction of shoots from internodal explants after 3 weeks in culture on MT with 3 mg/l BAP, bar = 0.25 cm.

b Shoot regeneration from internodal explants 8 weeks in culture on MT with 3 mg/l BAP.

c Plantlet established in soil, bar = 0.9 cm .

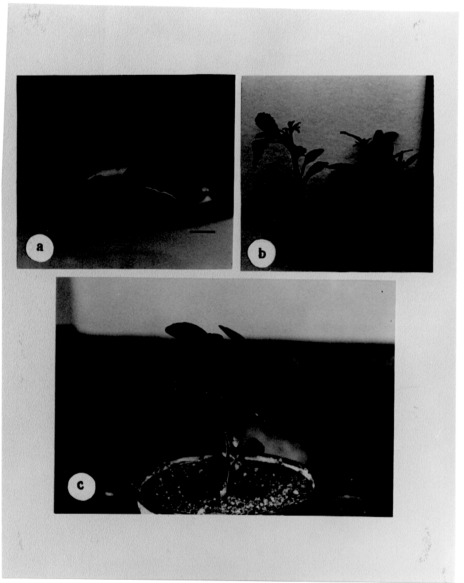


Table 15: Shoots and callus formation from nodal and internodal explants of citrumelo after 8 weeks in culture on MT media with various concentrations of BAP.

BAP mg/l	No. of explants	Shoot regeneration			Callus formation	
		No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Degree of callusing
nodal explants						
0*	13	100.0	1.50 ± 0.24	15.20 ± 2.31	0	-
1.0	15	100.0	5.20 ± 0.31	54.20 ± 5.51	0	-
2.0	12	100.0	6.41 ± 0.51	10.20 ± 1.52	25.0	+
3.0	14	100.0	5.0 ± 0.82	5.33 ± 0.43	100.0	+
5.0	10	100.0	3.12 ± 0.22	3.20 ± 0.11	100.0	+ + +
10.0	11	100.0	2.10 ± 0.10	1.50 ± 0.45	100.0	+ + +
internodal explants						
0	13	0	-	-	0	-
1.0	14	100.0	4.51 ± 0.42	6.42 ± 0.22	0	-
2.0	14	100.0	5.60 ± 0.41	5.32 ± 0.31	100.0	+
3.0	14	100.0	8.70 ± 0.30	3.51 ± 0.12	100.0	+ +
5.0	10	100.0	15.20 ± 2.21	2.50 ± 0.21	100.0	+ +
10.0	12	100.0	15.33 ± 1.25	2.00 ± 0.33	100.0	+ +

Mean number of shoots per explant and height of shoots in (mm) ± SE.

* 33 % of the explants formed 1 root each with 20.2 ± 1 mm height.

3. 2. 2. 3. Leaf explants

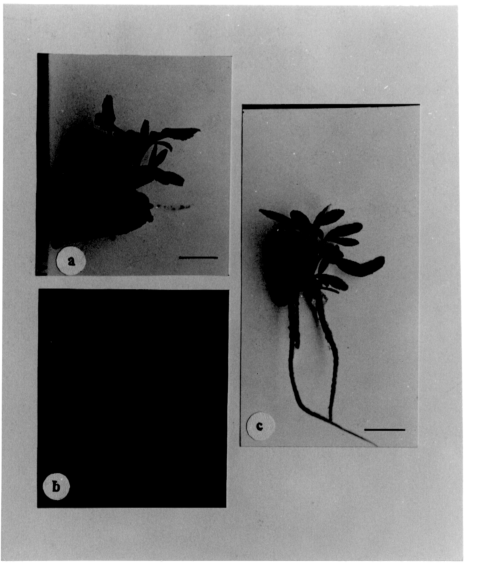
On basal medium the leaf explants did not show any significant response. BAP stimulated direct shoot regeneration and exhibited an optimum at 3 mg/l (Plate 5 a). High BAP of 10 mg/l proved to be inhibitory for shoot regeneration where only calli were produced (Table 16). Similarly, callus production also decreased as BAP levels increased. In the optimum media the explants maintained their totipotency by producing a considerable number of shoots for more than 18 months by subculturing in the same media every 6 to 8 weeks. All of the adventitious shoots initially emerged from the cut edges, the petiole, mid-rib and later from a small mass of callus in the center of the explants like a cluster of shoots (Plate 5 b). Shoots produced were readily rooted on MT basal medium with 1 to 3 mg/l NAA (Plate 5 c).

Plate 5. Citrumelo leaf culture.

a Shoot regeneration from leaf explants of citrumelo after 6 weeks in culture on MT with 3 mg/l BAP, bar = 0.5 cm.

b Shoots regenerated from leaf explants 9 weeks in culture on MT supplemented with 3 mg/l BAP, bar = 0.5 cm.

c Shoots originated from leaf explants formed roots on MT supplemented with 2 mg/l NAA, bar = 0.6 cm.


a
b
c

3. 2. 2. 4. Root explants

Root explants of citrumelo responded poorly to BAP at all concentrations and produced moderate callus at concentration 1 mg/l and above. Shoots were regenerated from callus at 2 and 3 mg/l BAP.

The optimal concentration for shoot regeneration from root explants was at 2 mg/l when 60.8 % of the 12 explants regenerated about 7.3 shoots per explant with an average of 10.3 mm height (Table 16).

Table 16: Shoots and callus formation from leaf and root explants of citrumelo after 8 weeks in culture on MT media with various concentrations of BAP.

BAP mg/l	No. of explants	Shoot regeneration			Callus formation	
		No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Degree of callusing
leaf explants						
0	13	0	-	-	0	-
1.0	14	16.4	2.50 ± 0.32	15.20 ± 0.23	8.0	+
2.0	14	55.6	3.41 ± 0.22	10.40 ± 1.22	22.0	+
3.0	13	41.0	5.80 ± 0.21	10.60 ± 0.61	50.8	+
5.0	14	33.0	2.60 ± 0.31	5.40 ± 0.31	44.5	+
10.0	10	0	-	-	33.0	+
root explants						
0	13	0	-	-	0	-
1.0	14	0	-	-	100.0	+
2.0	12	60.8	7.31 ± 0.42	10.32 ± 1.41	100.0	+ +
3.0	13	37.5	4.22 ± 0.34	5.22 ± 1.21	100.0	+ +
5.0	12	0	-	-	75.0	+
10.0	11	0	-	-	45.0	+

- Mean number of shoots per explant and height of shoots in (mm) ± SE.

3. 2. 3. Effect of NAA and BAP in combinations

3. 2. 3. 1. Nodal explants

Nodal explants produced 100% shoot regeneration in all the media tried except the ones with higher concentration of auxin to cytokinin ratio. However, the number of shoots per explant fluctuated throughout the experiment (Table 17). It was observed that MT media supplemented with 2 mg/l NAA and 3 mg/l BAP produced the most number of shoots and with good vigour (12.3 mm high). Shoots were regenerated through callus intermediary after 3 to 4 weeks in culture.

The callus tissue were maintained in MT medium supplemented with 2 mg/l BAP. The callus regenerated shoots and maintained its totipotency for more than 18 months.

Table 17: Shoots and callus formation from nodal explants of citrumelo after 8 weeks in culture on MT media supplemented with various concentrations of NAA and BAP.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
node explants								
0	0	13	100.0	1.5 ± 0.25	10.10 ± 1.51	0	-	-
0.5	1.0	10	100.0	2.10 ± 0.31	6.31 ± 0.21	100.0	G	+
1.0	1.0	10	100.0	6.21 ± 0.45	15.22 ± 1.34	100.0	G	+
1.0	2.0	11	100.0	6.10 ± 0.20	4.20 ± 0.26	25.0	G	+
1.0	3.0	9	100.0	5.8 ± 0.75	4.0 ± 0.15	20.4	G	+ +
2.0	1.0	10	83.3	6.20 ± 0.84	10.52 ± 1.30	16.6	G	+
2.0	2.0	13	100.0	8.32 ± 0.35	8.23 ± 0.10	100.0	G	+ + +
2.0	3.0	12	100.0	15.4 ± 2.50	12.33 ± 2.21	100.0	G	+ +
5.0	1.0	10	66.6	2.0 ± 0.30	3.42 ± 0.32	100.0	WG	+ +
5.0	2.0	10	60.0	15.10 ± 3.20	7.50 ± 0.41	75.5	G	+ +
5.0	3.0	11	40.0	6.10 ± 0.95	5.12 ± 1.50	40.0	G	+

- Mean number of shoots per explant and height of shoots in (mm) ± SE.

Key : G : green ; WG : light green

3. 2. 3. 2. Internodal explants

Shoot and green calli were produced after 4 to 6 weeks in culture (Table 18, Plate 6 a). Compact or nodular, greenish in colour calli were produced from the cut surfaces of all the explants tried. Upon subculture, they formed shoots (3 to 6 shoots per explant). It was observed that shoot regeneration increased from 33.4% to 100% when the ratio of BAP to NAA was higher in the media (Plate 6 b).

Plate 6. Citrumelo internodal culture

a Shoot and green compact callus regenerated from internodal explants after 4 weeks in culture on MT supplemented with 1 mg/l NAA and 2 mg/l BAP.

b Shoot regeneration from internodal explants on MT supplemented with NAA 1 mg/l and BAP 3 mg/l 9 weeks in culture, bar = 0.3 cm.

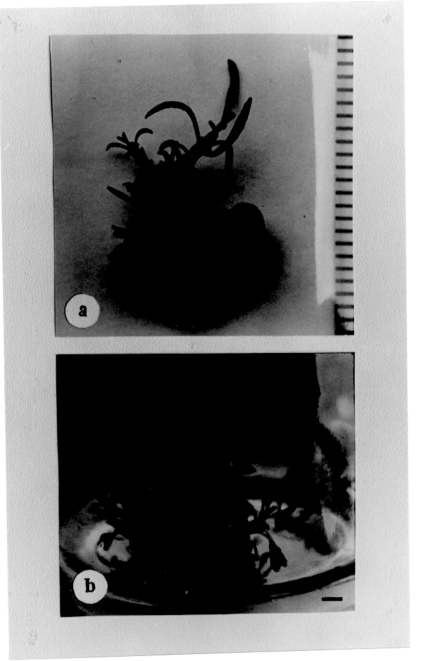

a
b

Table 18: Shoots and callus formation from internodal explants of citrumelo after 8 weeks in culture on MT media supplemented with various concentrations of NAA and BAP.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	14	0	-	-	0	-	-
0.5	1.0	14	66.5	3.21 ± 1.50	3.21 ± 1.50	100.0	G	+
1.0	1.0	14	33.4	5.88 ± 0.71	5.0 ± 0.31	100.0	G	+
1.0	2.0	13	62.3	4.50 ± 0.54	4.30 ± 0.40	100.0	G	+
1.0	3.0	11	100.0	6.0 ± 0.21	2.10 ± 0.11	100.0	G	+ + +
2.0	1.0	10	50.0	2.30 ± 0.40	3.21± 0.25	100.0	G	+ +
2.0	2.0	11	100.0	4.21 ± 0.11	5.0 ± 0.31	100.0	G	+ +
2.0	3.0	14	100.0	5.40 ± 2.30	6.10 ± 0.11	100.0	G	+ + +
5.0	1.0	15	33.0	2.50 ± 0.46	1.0	100.0	G	+
5.0	2.0	11	40.5	3.11 ± 0.13	1.90 ± 0.45	100.0	G	+ +
5.0	3.0	10	40.6	3.12 ± 0.20	3.12 ± 0.11	100.0	G	+ +

Mean number of shoots per explant and height of shoots in (mm) ± SE.

Key: G: green

3. 2. 3. 3. Leaf explants

Shoots were regenerated directly from the mid-rib of the leaf explants after 3 weeks in culture on MT media with various NAA and BAP combinations (Plate 7 a). The optimal concentration for shoot regeneration was at 1 mg/l NAA and 2 mg/l BAP where 83.3% of the explants regenerated approximately $\leq$ shoots per explants at 6.3 mm height (Table 19, Plate 7b). Higher concentrations of NAA in the medium appeared to be inhibitory for shoot regeneration. Shoots regenerated produced roots readily in rooting media MT containing 1 to 3 mg/l NAA and the plantlets obtained successfully transferred to soil (Plate 7 c).

Plate 7. Citrumelo leaf explants culture and plantlet.

a Shoot regeneration after 3 weeks in culture on 1 mg/l NAA and 2 mg/l BAP, bar = 0.25 cm.

b Shoot regeneration from leaf explants on MT supplemented with NAA 1 mg/l and 2 mg/l 6 weeks in culture, bar = 0.3 cm.

c Plantlet established in soil, bar = 1 cm.

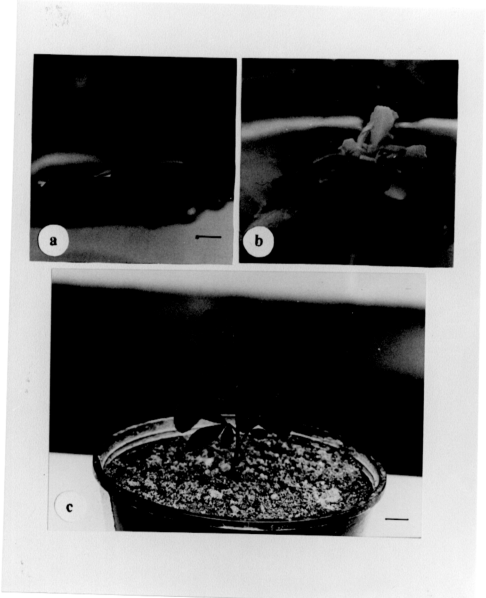

a
b
c

Table 19: Shoot regeneration from leaf explants of citrumelo after 8 weeks in culture on MT media supplemented with various concentrations of NAA and BAP.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)
0	0	13	0	-	-
0.5	1.0	10	80.0	3.80 ± 0.72	5.00 ± 0.15
1.0	1.0	13	8.0	2.0 ± 0.10	1.0
1.0	2.0	12	83.3	5.10 ± 0.23	6.30 ± 0.40
1.0	3.0	12	40.0	3.50 ± 0.46	2.50 ± 0.31
2.0	1.0	10	0	-	-
2.0	2.0	13	0	-	-
2.0	3.0	13	63.6	3.35 ± 0.25	5.00 ± 0.18
5.0	1.0	14	0	-	-
5.0	2.0	15	11.7	2.36 ± 0.37	1.50 ± 0.20
5.0	3.0	15	64.0	3.20 ± 0.15	4.87 ± 0.88

- Mean number of shoots per explant and height of shoots in mm± SE.

3. 2. 3. 4. Root explants

No response was observed in basal medium. However, shoots were regenerated after 6 weeks in culture through intermediate callus stage in MT media supplemented with various concentration ratios of NAA and BAP (Table 20). No significant pattern of hormone concentrations seemed to influence the regeneration frequency. No root regeneration was observed on the explants.

Table 20: Shoots and callus formation from root explants of citrumelo after 8 weeks in culture on MT media supplemented with various concentrations of NAA and BAP.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
root explants								
0	0	15	0	-	-	0	-	-
0.5	1.0	10	66.6	3.0 ± 0.21	3.80 ± 0.33	100.0	G	+ +
1.0	1.0	15	6.6	2.00 ± 0.10	1.0 ± 0.31	100.0	G	+ +
1.0	2.0	11	36.3	2.32 ± 0.53	1.0	100.0	G	+
1.0	3.0	16	43.3	2.50 ± 0.14	0.89	100.0	G	+ +
2.0	1.0	13	16.0	3.00 ± 0.60	32.40 ± 0.35	100.0	G	+ +
2.0	2.0	13	15.3	2.00 ± 0.10	3.20 ± 0.20	100.0	G	+ + +
2.0	3.0	13	6.0	1.0	3.50 ± 0.56	86.0	G	+ + +
5.0	1.0	13	0	-	-	100.0	WG	+ + +
5.0	2.0	14	10.5	4.21 ± 0.31	2.50 ± 0.42	100.0	G	+ + +
5.0	3.0	16	22.6	4.30 ± 0.40	2.00 ± 0.30	93.0	G	+ + +

- Mean number of shoots per explant and height of shoots in (mm) ± SE.

Key : G : green ; WG : light green

3. 3. *C. Limonia*

3. 3. 1. Effect of NAA.

3. 3. 1. 1. Nodal explants

Shoots, roots and calli were produced in various media tried. On MT basal medium both shoots and roots were formed but the former did not proliferate. Presence of NAA in culture media stimulated root formation within 3 weeks and 2 weeks for shoot regeneration (Plate 8 a). Root length and the shoot regeneration frequency were decreased when the concentrations of NAA in the medium was increased to a levels higher than 1 mg/l. Callus was formed only in media with NAA concentrations of higher than 3 mg/l (Table 21).

3. 3. 1. 2. Internodal explants

Callus was proliferated on media containing 1 to 10 mg/l NAA and root primordia appeared after 4 weeks in culture. Roots were regenerated directly from explants cultured on 0.5 mg/l NAA supplementation. Root formation was optimum at 3 and 5 mg/l NAA (Table 22).

Plate 8. *C. limonia*

a Nodal explants produced shoots and roots on MT supplemented with 1 mg/l NAA after 4 weeks in culture.

b Root regeneration from leaf explants on MT with 3 mg/l NAA.

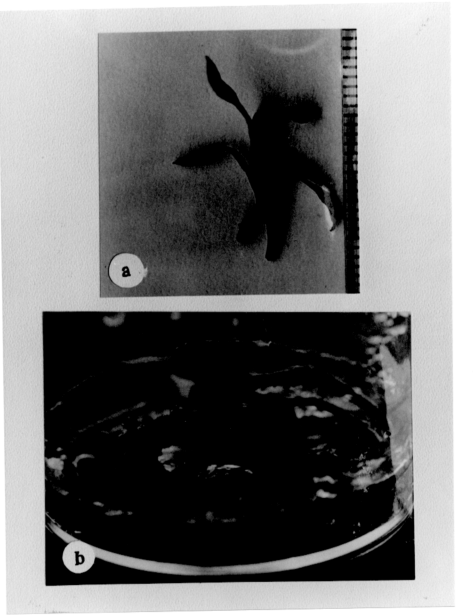

a
b

Table 21: Effect of NAA in MT basal medium on nodal explants of *C. limonia* after 8 weeks in culture.

NAA mg/l	No. of explants	Root regeneration			Shoot regeneration		
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of explants with shoots (%)	Mean no. of shoots ± SE	Mean length ± SE (mm)
0	13	60.4	1.1 ± 0.20	10.2 ± 1.30	100.0	1.6 ± 0.21	15.4 ± 2.40
0.1	13	66.2	2.5 ± 0.51	24.3 ± 3.10	100.0	1.0 ± 0.20	10.3 ± 1.31
0.5	14	66.5	2.3 ± 0.23	20.4 ± 1.52	100.0	1.4 ± 0.32	10.5 ± 2.20
1.0	14	100.0	1.8 ± 0.21	60.0 ± 8.20	100.0	1.4 ± 0.50	5.2 ± 1.11
2.0	13	70.0	3.2 ± 0.30	4.30 ± 0.21	66.0	0.9 ± 0.30	3.0 ± 0.62
3.0	12	60.5	1.3 ± 0.40	2.52 ± 0.45	16.0	0.8 ± 0.21	2.0 ± 0.40
5.0	15	60.3	1.5 ± 0.45	8.71 ± 1.30	50.0	1.0 ± 0.20	2.1 ± 0.35
10.0	10	16.0	3.0 ± 0.21	7.50 ± 0.65	30.4	1.1 ± 0.32	1.0

Mean number of roots per explant and length of shoots in mm ± SE.

Table 22: Effect of NAA in MT basal medium on internodal explants of *C. limonia* after 8 weeks in culture.

NAA mg/l	No. of explants	Root regeneration			Callus formation	
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of explants with callus (%)	Degree of callusing
0	13	0	-	-	0	-
0.1	14	0	-	-	0	-
0.5	14	11.6	1.2 ± 0.80	5.0 ± 1.3	0	-
1.0	14	33.4	2.4 ± 1.51	2.0 ± 0.3	66.0	+
2.0	11	83.4	2.5 ± 0.30	15.0 ± 2.2	100.0	+
3.0	14	100.0	2.6 ± 0.57	10.0 ± 3.0	100.0	+ +
5.0	13	100.0	2.4 ± 0.32	5.0 ± 1.2	100.0	+ +
10.0	12	16.0	2.0 ± 0.45	2.6 ± 0.5	100.0	+ +

- Mean number of roots per explant and length of roots in (mm) ± SE.

3. 3. 1. 3. Leaf explants

Roots were produced directly from leaf explants on media with concentration higher than 0.5 mg/l NAA. Yellow compact callus was observed from the cut edges of the explants after 3 to 4 weeks in media with 1 mg/l NAA and at higher concentrations (Table 23, Plate 8 b). Shoots were not regenerated on any of the media tried.

3. 3. 1. 4. Root explants

Roots were regenerated directly from explants after 4 weeks in culture in media added with 0.1 and 1 mg/l NAA at 12 and 28 % respectively. Callus was produced at high levels of NAA (3 and 10 mg/l) (Table 23). No shoot regeneration was observed.

Table 23: Effect of NAA in MT basal medium on leaf and root explants of C. *limonia* after 8 weeks in culture .

NAA mg/l	No. of explants	Root regeneration			Callus formation	
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of explants with callus (%)	Degree of callusing
Leaf explants						
0	13	0	-	-	0	-
0.1	10	0	-	-	0	-
0.5	12	0	-	-	0	-
1.0	11	33.3	1.2 ± 0.55	60.0 ± 3.3	0	-
2.0	10	33.6	4.3 ± 0.21	40.12 ± 5.6	16.0	+
3.0	11	50.0	5.1 ± 0.24	45.0 ± 9.50	25.0	+ +
5.0	12	40.0	4.4 ± 0.54	35.0 ± 0.74	33.9	+ +
10.0	11	33.2	4.2 ± 0.64	23.8 ± 1.30	33.1	+ +
Root explants						
0	13	0	-	-	0	-
0.1	11	12.0	3.4 ± 1.20	1.0	0	-
0.5	10	0	-	-	0	-
1.0	12	28.0	2.2 ± 0.13	2.0 ± 0.3	0	-
2.0	13	0	-	-	0	-
3.0	12	0	-	-	12.0	+
5.0	11	0	-	-	0	-
10.0	10	0	-	-	75.0	+

- Mean number of roots per explant and length of shoots in mm ± SE.

3. 3. 2. Effect of BAP

3. 3. 2. 1. Nodal explants

In the basal medium, axillary buds continued to grow with good vigour (15.2 mm height) but without proliferation. All the nodal explants produced multiple shoot buds after 3 weeks in culture on BAP enriched media (Plate 9 a). The response exhibited a concentration effect where high regeneration was observed on BAP added media at 1 , 2 , 3 mg/l and decreased at 5 and 10 mg/l (Table 24). Shoots produced were readily rooted on MT basal medium.

3. 3. 2. 2. Internodal explants

Internodal explants exhibited poor response to BAP concentrations tested where shoot regeneration was only observed in media with 2 mg/l BAP وhere≥ 16% of the explants regenerated 2.5 shoots each after 6 weeks in culture (Table 24).

Plate 9. *C. limonia*

a Shoot regeneration from nodal explants after 3 weeks in culture on MT supplemented with 2 mg/l BAP.

b Shoot regeneration from nodal explants on MT supplemented with 2 mg/l NAA and 3 mg/l BAP, 8 weeks in culture.

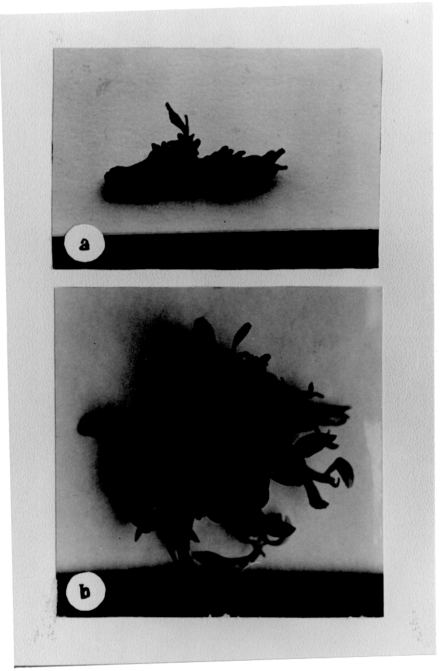

a
b

Table 24: Shoot regeneration from nodal and internodal explants of *C. limonia* after 8 weeks in culture on MT media supplemented with various concentrations of BAP.

BAP mg/l	No. of explants	Shoot regeneration		
		No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)
nodal explants				
0	13	100.0	1.0	15.20 ± 3.50
1.0	12	100.0	4.0 ± 0.30	15.40 ± 2.11
2.0	10	100.0	3.51 ± 0.40	10.00 ± 2.10
3.0	11	100.0	3.24 ± 0.10	10.50 ± 3.40
5.0	12	80.3	3.10 ± 0.32	5.71 ± 2.00
10.0	12	16.4	1.50 ± 0.21	4.52 ± 0.31
internodal exp.				
0	13	0	0	0
1.0	12	0	0	0
2.0	10	16.0	2.51 ± 0.41	1.2
3.0	11	0	0	0
5.0	12	0	0	0
10.0	12	0	0	0

- Mean number of roots per explant and length of shoots in mm ± SE.

3. 3. 2. 3. Leaf explants

No significant results were observed except for poor callus formation in media with 1 and 3 mg/l BAP.

3. 3. 2. 4. Root explants

No significant response were observed from the explants cultured.

3. 3. 3. Effect of NAA and BAP supplemented media on explants

3. 3. 3. 1. Nodal explants

Axillary buds developed directly from explants within 2 to 3 weeks in culture. Although all of the explants produced shoots on basal media in comparison to the decreasing frequency in the NAA and BAP added media, the number of shoots had significantly increased throughout. The highest number of shoots were produced from media with 2 mg/l NAA and 3 mg/l BAP (Plate 9 b). The growth of shoots were however faster in the basal medium (Table 25).

3. 3. 3. 2. Internodal explants

Explants responded poorly to the medium tried except in media with 5 mg/l NAA and 3 mg/l BAP, 10% of the explants regenerated approximately 4 shoots per explant after 6 weeks in culture (Table 26).

3. 3. 3. 3. Leaf explants

No response was observed from leaf explants at all combinations tested.

3. 3. 3. 4. Root explants

Root explants exhibited very poor response to NAA and BAP combinations. Only 7 % of the explants regenerated shoots at 1 mg/l NAA and 3 mg/l BAP (1 shoot per explant) (Table 27).

Table 25: Shoot regeneration from nodal explants of *C. limonia* after 8 weeks in culture on MT media with various concentrations of NAA and BAP.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)
0	0	11	100	1.0	15.0 ± 1.50
0.5	1.0	13	69.2	4.23 ± 0.25	3.40 ± 0.25
1.0	1.0	15	73.3	3.50 ± 0.22	5.70 ± 0.65
1.0	2.0	12	33.3	3.22 ± 0.12	5.41± 0.10
1.0	3.0	13	69.2	4.80 ± 0.55	6.63 ± 0.71
2.0	1.0	12	66.6	2.40 ± 0.30	10.0 ± 1.30
2.0	2.0	12	33.3	2.20 ± 0.12	5.14 ± 0.16
2.0	3.0	13	66.6	5.58 ± 0.62	5.10 ± 0.12
5.0	1.0	12	58.3	3.15 ± 0.10	5.60 ± 0.50
5.0	2.0	12	50.0	3.80 ± 0.90	3.76 ± 0.81
5.0	3.0	12	50.0	2.40 ± 0.10	2.6 ± 0.30

- Mean number of shoots per explant and height of shoots in mm± SE.

Table 26: Effect of NAA and BAP on internodal explants of *C. limonia* after 8 weeks in culture on MT supplemented with various concentrations of NAA and BAP.

			Shoot regeneration			Callus formation		
NAA mg/l	BAP mg/l	No. of explants	No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
internedal explants								
0	0	12	0	-	-	0	-	-
0.5	1.0	15	0	-	-	33.0	G	+
1.0	1.0	13	0	-	-	30.0	G	+
1.0	2.0	13	0	-	-	-	-	-
1.0	3.0	12	0	-	-	-	-	-
2.0	1.0	12	0	-	-	-	-	-
2.0	2.0	12	0	-	-	33.0	G	++
2.0	3.0	12	0	-	-	-	-	-
5.0	1.0	12	0	-	-	75.0	G	+
5.0	2.0	12	0	-	-	66.0	G	+
5.0	3.0	10	10.0	4.30 ± 0.12	1.0	30.0	G	++

- Mean number of shoots per explant and height of shoots in mm± SE.

Key : G : green

Table 27: Effect of NAA and BAP on root explant of *C. limonia* after 8 weeks in culture on MT media with various concentrations of NAA and BAP.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration			Callus formation		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	height ± SE (mm)	No. of explants with callus (%)	Color of callus	Degree of callusing
0	0	13	0	-	-	0	-	-
0.5	1.0	10	0	-	-	12.6	WG	+
1.0	1.0	10	0	-	-	0	-	-
1.0	2.0	12	0	-	-	0	-	-
1.0	3.0	13	7.0	1.0	2.3 ± 0.2	0	-	-
2.0	1.0	12	0	-	-	0	-	-
2.0	2.0	11	0	-	-	0	-	-
2.0	3.0	10	0	-	-	0	-	-
5.0	1.0	10	0	-	-	0	-	-
5.0	2.0	12	0	-	-	0	-	-
5.0	3.0	12	0	-	-	6.0	WG	++

Mean number of roots per explant and length of shoots in mm ± SE.

Key : WG : light green

3.4. *C. reticulata*

3. 4. 1. Effect of NAA

3. 4. 1. 1. Nodal explants

Root primordia started to develop within 3 weeks in culture on media with NAA concentrations from 1 to 3 mg/1. The axillary buds started to develop after 2 weeks in culture in either MT basal medium or when NAA was added. The number and height of shoots were not affected by the NAA concentration in the medium. At 10 mg/1 NAA media only callus was proliferated (Table 28).

The callus tissues obtained were subcultured onto MT media containing 2 mg/1 BAP where the shoot organogenesis was only observed after 8 months in culture.

Table 28: Roots, axillary buds and callus formation from nodal explants of *C. reticulata* after 8 weeks on MT media with various concentrations of NAA.

NAA mg/l	No. of explants	Root regeneration			Shoot regeneration		
		No. of expl. with roots (%)	Mean no. of roots ± SE	Mean length ± SE (mm)	No. of expl with shoots (%)	Mean no. of shoots ± SE	Mean length ± SE (mm)
0	13	0	-	-	50.0	2.30 ± 0.21	5.4 ± 0.50
0.1	15	0	-	-	75.0	1.0 ± 0.20	15.3 ± 2.50
0.5	15	0	-	-	40.0	1.30 ± 0.21	10.2 ± 2.20
1.0	14	40.0	1.2 0± 0.50	1.0	37.2	1.0	15.3 ± 1.50
2.0	15	55.0	1.0 0± 0.20	1.50 ± 0.30	40.6	0.9	10.2 ± 1.32
3.0	12	60.5	3.23 ± 0.23	2.12 ± 0.20	25.1	1.0 ± 0.50	10.1 ± 2.20
5.0	15	0	-	-	50.0	1.0 ± 0.20	10.0 ± 1.00
10.0	11	0	-	-	0	-	-

Mean number of roots, shoots per explant and length of roots and height of shoots in (mm) ± SE.

3. 4. 1. 2. Internodal explants

Root primordia started to emerge from the sides of the explants at concentrations higher than 2 mg/l NAA after 3 weeks in culture. Callus was produced only at high levels of NAA (5 and 10 mg/l). No shoots were regenerated in these media (Table 29).

3. 4. 1. 3. Leaf explants

Leaf explants remained green in basal medium and gradually turned light brown with NAA added media. No organogenesis was observed.

3. 4. 1. 4. Root explants

On MT basal medium and medium containing 0.1 mg/l NAA, root explants failed to show any significant response (Table 29). On NAA at 0.5 to 5 mg/l root primordia was initiated directly from the explants after 2 to 3 weeks in culture. The average of root length increased with increase in NAA concentration to 2 mg/l, but decreased with 5 mg/l while at 10 mg/l root initiation was completely inhibited. NAA stimulated root production and exhibited a concentration effect with optimum concentration at 2 mg/l, where 75.5% of the explants produced an average of approximately 4 roots per explant (Table 29). The root length varied from 1 to 5 cm.

Table 29: Root formation from internodal and root explants of *C. reticulata* after 8 weeks on MT media with various concentrations of NAA.

NAA mg/l	No. of explants	root regeneration: No. of expl. with roots (%)	Mean no. of roots ± SE	Mean height ± SE (mm)
internodal explants				
0	13	0	-	-
0.1	12	0	-	-
0.5	12	0	-	-
1.0	13	0	-	-
2.0	13	70.0	2.2 0± 0.41	10.0 ± 1.21
3.0	12	70.8	2.83 ± 0.55	15.0 ± 2.30
5.0	11	40.4	2.0 ± 0.12	10.0 ± 3.32
10.0	13	40.0	5.10 ± 0.24	10.0 ± 3.50
root explants				
0	14	0	-	-
0.1	13	0	-	-
0.5	12	75.0	1.5 ± 0.61	20.4 ± 1.30
1.0	14	44.9	2.0 ± 0.21	20.5 ± 2.50
2.0	11	75.5	3.28 ± 0.21	10.2 ± 1.40
5.0	14	28.0	1.40 ± 0.50	5.0 ± 1.22
10.0	14	0	-	-

- Mean number of roots per explant and length of roots in (mm) ± SE.

3. 4. 2. Effect of BAP

3. 4. 2. 1. Nodal explants

Axillary buds started to develop after 2 weeks in culture either from the main node or through callus. Addition of BAP (1 to 5 mg/l) to the medium enhanced the percentage of shoot regeneration per explant although the number of shoots fluctuated (Table 30). The highest number of shoots were regenerated from media with 3 mg/l BAP at a frequency of 66% (4 shoots per explant). Media with BAP at 10 mg/l inhibited the response of nodal explants.

Nodular light green callus were proliferated in media with BAP levels from 1 to 5 mg/1. The callus obtained was maintained in MT media containing 3 mg/1 BAP for more than a year and exhibited totipotency. The number of shoots regenerated per explant was observed to increase four to five times.

3. 4. 2. 2. Internodal explants

Internodal explants of *C. reticulata* failed to show any response on MT basal medium or on BAP enriched media.

3. 4. 2. 3. Leaf explants

The leaf explants did not show any response in the MT basal medium or in the presence of 1 to 10 mg/1 BAP supplemented media after 8 weeks in culture.

3. 4. 2. 4. Root explants

Root explants were responsive to all media with added BAP concentrations forming green nodular callus ~~and~~ which subsequently regenerated into a considerable number of shoots (Plate 10 a, b). The highest number of regenerated shoots was from the media supplemented with 10 mg/l BAP giving 90 % regenerated frequency forming 15 shoots per explant (Table 30). However, growth of the shoots were slower compared to explants with less number of shoots.

The green compact callus obtained from all the media tried were maintained in 2 mg/1 BAP enriched media with regular subculture every 6 to 8 weeks. Totipotency was observed for more than 18 months (Plate 10 b).

Shoots regenerated produce roots readily in MT basal medium supplemented with 1 to 3 mg/1 NAA and were successfully transferred to soil (Plate 10 c).

Plate 10. *C. reticulata* shoot regeneration and plantlet.

a Callus formation from root explants after 4 weeks in MT with 3 mg/l BAP, bar = 0.2 cm.

b Shoot regenerated from root explants derived callus on MT with 3 mg/l BAP, 8 weeks in culture, bar = 0.5 cm.

c Plantlet established in soil, bar + 1.3 cm.

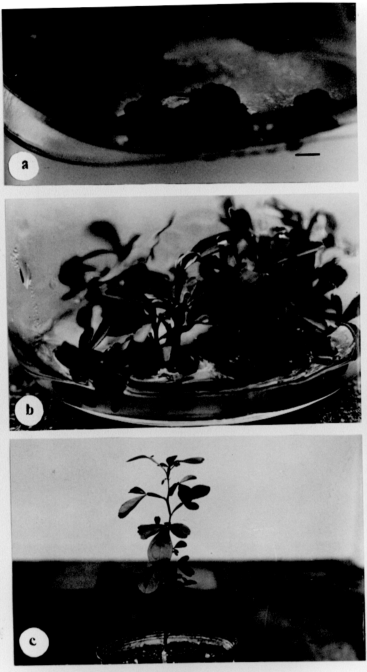

a
b
c

Table 30: Shoots and callus formation from nodal and root explants of *C. reticulata* after 8 weeks in culture on MT media supplemented with various concentrations of BAP.

BAP mg/l	No. of explants	Shoot regeneration: No. of expl. with shoots (%)	Shoot regeneration: Mean no. of shoots ± SE	Shoot regeneration: Mean height ± SE (mm)	Callus formation: No. of explants with callus (%)	Callus formation: Degree of callusing
nodal explants						
0	13	50.6	1.0	10.50 ± 1.30	0	-
1.0	12	85.8	1.40 ± 0.32	10.40 ± 3.21	85. 0	+ +
2.0	11	75.0	2.51 ± 0.51	5.20 ± 1.50	100.0	+ +
3.0	14	66.0	4.0 ± 0.15	5.50 ± 1.40	66.0	+ +
5.0	15	60.3	2.20 ± 0.32	10.21 ± 3.50	60.0	+ + +
10.0	14	0	-	-	0	-
root explants						
0	13	0	-	-	0	-
1.0	14	100.0	3.42 ± 0.31	10.52 ± 1.45	100.0	+ + +
2.0	14	100.0	6.10 ± 0.52	20.24 ± 0.22	100.0	+ + +
3.0	14	95.0	3.52 ± 0.15	25.10 ± 2.13	100.0	+ + +
5.0	13	80.0	5.52 ± 0.51	10.40 ± 0.52	100.0	+ + +
10.0	11	90.0	15.63 ± 2.11	1.20	100.0	+ + +

- Mean number of roots per explant and length of shoots in mm ± SE.

3. 4. 3. Effect of NAA and BAP

3. 4. 3. 1. Nodal explants

Explants on the basal medium produced single axillary buds at a frequency of 50 % with a mean height of 10.5 mm. When NAA (0.5 to 5 mg/l) and BAP (1 to 3 mg/l) were added to MT basal medium, the axillary buds developed multiple shoots after 2 to 3 weeks in culture. The highest shoot number was obtained in media with 1 mg/l NAA and 3 mg/l BAP with an average of 4 shoots per explant (Table 31). Comparatively shoot growth was most vigorous in the basal medium.

Table 31: Effect of NAA and BAP on nodal explants of *C. reticulata* after 8 weeks in culture on MT medium.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)
0	0	11	50.0	1.0	10.50 ± 0.67
0.5	1.0	12	70.3	2.10± 0.18	3.80 ± 0.91
1.0	1.0	14	30.8	1.90 ± 0.53	5.41± 0.26
1.0	2.0	14	68.6	2.40	3.25 ± 0.33
1.0	3.0	12	80.0	3.86 ± 0.21	4.0 ± 0.70
2.0	1.0	11	30.3	1.58 ± 0.52	516 ± 0.28
2.0	2.0	13	35.7	1.72 ± 0.46	5.38 ± 0.14
2.0	3.0	15	60.6	2.0 ± 0.15	3.0 ± 0.30
5.0	1.0	15	50.0	1.46 ± 0.30	5.89 ± 0.60
5.0	2.0	10	50.2	1.55 ± 0.60	2.15 ± 0.23
5.0	3.0	12	40.0	4.30 ± 0.12	2.10 ± 0.11

Mean number of roots per explant and length of shoots in (mm) ± SE.

3. 4. 3. 2. Internodal explants

Explants did not show any significant response to the media tried.

3. 4. 3. 3. Leaf explants

Leaf explants did not show any response.

3. 4. 3. 4. Root explants

No response was observed on MT basal medium. In NAA and BAP added media, nodular callus were formed along the explants after 2 to 3 weeks (Plate 11 a). Adventitious shoots were produced through after 4 weeks in culture. The optimal response was obtained from media with 1 mg/l NAA and 3 mg/l BAP where 80.2 % of the explants produced an average of 4 buds per explant after 8 weeks in culture (Table 32). At high NAA to BAP ratio shoot regeneration was inhibited. Green compact callus was produced from all the combinations of NAA and BAP tested media. The callus was subcultured in 1 and 2 mg/l BAP media and totipotency was observed for more than 18 months (Plate 11 b).

The shoots excised formed roots in 1 to 3 mg/l NAA and were successfully transferred to soil (Plate 11 c).

Plate 11. *C. reticulata* shoot and root formation.

a Shoot regeneration and callus formation from root explants after 3 weeks in culture on MT with 1 mg/l NAA and 3 mg/l BAP, bar = 0.5 cm.

b Shoot regenerated from root explants subcultured on MT with 2 mg/l BAP, 6 months in culture, bar = 0.5 cm.

c Regenerated shoots from root explants formed roots on MT with 3 mg/l NAA, bar = 0.5 cm.

Table 32: Effect of NAA and BAP on root explants of *C. reticulata* after 8 weeks in culture.

NAA mg/l	BAP mg/l	No. of explants	Shoot regeneration			Callus formation		
			No. of expl. with shoots (%)	Mean no. of shoots ± SE	Mean height ± SE (mm)	No. of explants with callus (%)	Colour of callus	Degree of callusing
0	0	11	0	-	-	0	G	+ +
0.5	1.0	10	30.3	1.10 ± 0.20	3.24 ± 1.51	70.3	G	+ + +
1.0	1.0	12	20.5	1.0 ± 0.22	3.00 ± 0.21	50.0	G	+ + +
1.0	2.0	10	60.0	3.20 ± 0.35	5.12 ± 0.34	80.6	G	+ + +
1.0	3.0	12	80.2	4.30 ± 0.41	5.50 ± 1.20	90.2	G	+ + +
2.0	1.0	12	0	-	-	10.0	WG	+
2.0	2.0	12	30.0	2.0	3.21 ± 0.10	50.5	G	+ +
2.0	3.0	14	10.0	1.0	5.0 ± 0.30	50.4	G	+ +
5.0	1.0	14	0	-	-	70.0	G	+ + +
5.0	2.0	13	0	-	-	80.4	G	+ + +
5.0	3.0	14	0	-	-	70.3	G	+ +

- Mean number of roots per explant and length of shoots in mm ± SE.

Key : G : green ; WG : light green

3. 5. Induction of roots

It was found that the effect of NAA concentrations on root regeneration from shoot were species dependent.

In-vitro shoots of citrumelo and *C. limonia* regenerated roots when cultured on either MT basal media with or without NAA supplements. The number of roots per shoot varied where media without NAA supplementation formed approximately 1 root whereas on NAA added media approximately 1 - 6 roots were produced (Plate 12 a).

In citrumelo root formation on media with 1 mg/l NAA took 15 days and those with 3 mg/l NAA took 8 days. Roots formed on the latter media was formed through callus phase. Media with 2 mg/l NAA was considered optimal as 3 true roots were formed after 10 days at 100% frequency (Table 33, Plate 12 b).

For *C. limonia* the best media was on MT with 1 mg/l NAA added where 100% of shoots formed true roots after 20 days.

An alternative method of rooting was also tried for citrumelo by immersing the shoots in NAA solution before culturing in MS medium. Roots were observed after 21 days in culture. Similar observations were made when shoots were cultured on MS basal medium directly (Table 34) .

In contrast *in-vitro* shoots of *C. suhuiensis* and *C. reticulata* failed to induce roots in MT basal medium without any hormones. Addition of NAA to the MT basal medium initiated root regeneration. Shoots of *C. suhuiensis* took 22 days for root regeneration when cultured on media with 1 mg/l NAA at 80.5% rooting frequency (approximately 2 roots per shoot) (Table 33).

It was observed that *C. reticulata* shoots took 26 days for root regeneration on media with 3 mg/l NAA at a frequency of 60.2 %. When cultured on MS basal medium, 14.5% of the shoots regenerated roots after 44 days in culture. However, immersing the shoots in NAA solution before culturing in MS media increased the percentage of root regeneration (57.7 %) and in a shorter time. The number of root regenerated per shoot fluctuated as the NAA concentration in the solution differed (Table 34).

Plate 12. Root regeneration on citrumelo shoots regenerated *in-vitro* from internodal explants, after 18 days in culture, bar = 0.5 cm.

a- Shoots of citrumelo forming roots on MT basal medium supplemented with different concentrations of NAA, from left to right.

- MT basal medium without hormone supplementation.
- MT basal medium supplemented with 1 mg/l NAA.
- MT basal medium supplemented with 2 mg/l NAA
- MT basal medium supplemented with 3 mg/l NAA

b-Root regeneration on citrumelo shoots cultured on MT with 2 mg/l NAA, bar = 0.5 cm.


a

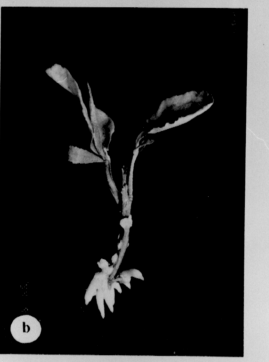

b

Table 33: Establishment of roots from *in-vitro* regenerated shoots of Citrumelo, *C. suhuiensis*, *C. limonia* and *C. reticulata*.

Species	MT basal medium + NAA mg/l	Root initiation (days)	Freq. of root regenerated (%)	No. of roots per shoot
Citrumelo	0	28	33.5	1.28 ± 0.15
	1	15	85.2	3.10 ± 0.47
	2	10	100.0	5.41 ± 0.75
	3	8	100. 0	6.11 ± 0.90
C. suhuiensis	0	-	0	-
	1	22	80.5	2.00 ± 0.50
	2	19	70.1	3.17 ± 0.61
	3	15	60.0	2.00 ± 0.31
C. limonia	0	36	60.0	1.00 ± 0.14
	1	20	100.0	1.30 ± 0.13
	2	19	70.2	3.21 ± 0.43
	3	16	60.1	1.90 ± 0.81
C. reticulata	0	-	0	-
	1	32	40.6	1.12 ± 0.35
	2	30	55.3	1.53 ± 0.20
	3	26	60.2	3.17 ± 0.65

- Mean number of roots per shoot ± SE

Table 34 : Rooting of *in-vitro* regenerated shoots of citrumelo and *C. reticulata* by immersing the shoots on solution of NAA at various concentration before culturing on MS medium.

NAA solution (mg/l)	Citrumelo			*C. reticulata*		
	Root initiation (days)	Freq. of root regeneration (%)	No. of roots per shoot	Root initiation (days)	Freq. of root regeneration (%)	No. of roots per shoot
0	21	16.4	1.1 ± 0.2	44	14.5	1.1 ± 0.3
1	18	60.2	1.2 ± 0.1	39	28.3	1.2 ± 0.1
2	16	62.5	1.3 ± 0.5	37	30.2	1.7 ± 0.2
3	15	85.2	1.0 ± 0.2	33	42.5	1.0 ± 0.1
5	12	100.0	1.5 ± 0.4	30	57.2	1.3 ± 0.5
10	10	100.0	2.3 ± 0.3	28	57.7	1.4 ± 0.3

- Mean number of roots per shoot ± SE

3. 6. Establishment of plantlets

Tissue culture plantlets regenerated from internodal and leaf explants for Citrumelo, internodal explants for *C. suhuiensis* and *C. limonia* and from root explants for *C. reticulata* were established in soil as in Section 2. 6. .Plantlets regenerated from nodal explants and respective explants mentioned for all species are also shown in Table 35.

Plantlets regenerated from nodal explants exhibited a higher percentage of success compared to the other explants for all the species studied (Table 35). Loosening jar caps in which *in-vitro* shoots were grown for 7 days prior to transfer (method **a**) (Plate 13 a) gave better results compared to the ones transferred directly to pots (method b) (Plate 13 b) in all tissue cultured plants obtained (Table 35). In all cases citrumelo plantlets exhibited the highest establishment percentages.

Plate 13. Acclimatisation methods

a Loose jar caps for 7 days prior to transfer the plantlets to pots (method **a**), bar = 1 cm.

b Covering the pots with plastic bags for 4 weeks (method **b**), bar = 1 cm.

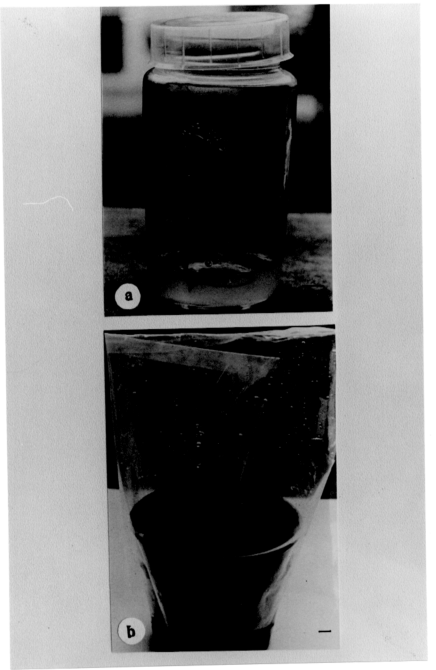

a
b

Table 35: Frequency of acclimatised plantlets for various explants cultured using 2 methods . Data was collected after 4 weeks.

Source of plantlets	Citrumelo		*C. suhuiensis*		*C. limonia*		*C. reticulata*	
	a	**b**	**a**	**b**	**a**	**b**	**a**	**b**
Nodes	94.0	90.3	60.0	50.3	90.2	55.2	93.0	90.3
*Other explants	90.2	75.2	36.0	25.2	60.2	50.0	83.0	80.8

Key:

-method **a** = The jars containing tissue culture plantlets were loosened for 7 days prior to transfer to pots.

-method **b** = The plantlets transferred directly from jars to pots covered with plastic bags and gradually exposing the plantlets to the physical environment.

-* reading of other explants included internodal and leaf explants for citrumelo, internodal explants for *C. suhuiensis* and *C. limonia* and root explants for *C. reticulata*.

3. 7. Leaf anatomy

In order for the tissue culture plants to survive the transition environment from the culture vessels to the field or greenhouse conditions, they must be slowly acclimatised or hardened-off. This is done by gradually decreasing the humidity surrounding the plants following transplanting. During this process, anatomical changes in the leaf structure of the plants occurred.

Leaf samples of citrumelo and *C. reticulata* plantlets from the leaf and root explants respectively were sectioned and compared with those of greenhouse plants.

Light microscopy transverse sections of greenhouse-grown citrus leaves of both species displayed well differentiated mesophyll cells where palisade and spongy parenchyma were well-defined and contained a high density of chloroplasts (Plate 14 d; 15 d). The mesophyll cells were very compact with few intercellular spaces in either the palisade or spongy parenchyma. All of the cells of both mesophyll types contained chloroplasts. Upper epidermal cells were of straight anticlinal walls and the lower epidermal cells were smaller and resembled adjacent mesophyll cells. Stomata were concentrated in the lower epidermis. Chloroplasts were dense and present in both mesophyll cell types. The exterior surface of the epidermal cell walls were covered with a thick cuticle.

Samples of leaves obtained directly from tissue culture plantlets had a much lower mesophyll cell density than the greenhouse plants (Plate 14 a; 15 a). Cells

were not differentiated into palisade layer. The mesophyll and parenchyma cells had irregular shapes. The adaxial epidermis consisted of oval cells, and the abaxial epidermis was similar except that the cells were smaller and thinner walled. Stomata were found on both adaxial and abaxial epidermis and the presence of chloroplast density was much lower in number in these samples compared to the ones from greenhouse grown. The cuticle is thin and not well developed compared to cuticle *in-vivo* leaves.

Leaves after 2 and 4 weeks of hardening period showed some differentiation of the mesophyll tissue into a palisade and spongy parenchyma. There was an elongation and greater density of adaxial mesophyll (Plate 14 b, c; 15 b, c). The spongy parenchyma had fewer and smaller intercellular spaces than those under tissue culture conditions, but still had numerous and larger air spaces than the leaves from greenhouse-grown plants. Adaxial epidermal cells were again larger than abaxial epidermal cells, but maintained their irregular, oval shape. The cuticle was well evident on both abaxial and adaxial epidermal surfaces.

Some variations in leaf thickness between the different conditions were observed. Greenhouse-grown and acclimated leaves were approximately 130 and 135 μm thick for citrumelo and *C. reticulata* respectively, while cultured leaves were much thicker with similar measurements of approximately 153 and 163 μm and in the same order.

Plate 14. Light micrographs of cross sections of Citrumelo leaves.

a tissue culture plantlet leaves, showing irregular shape of epidermal cells and poor differentiation of mesophyll and spongy parenchyma, with large intercellular space. × 800.

b leaf cross section of tissue culture plants after 2 weeks of hardening showing intermediate stage between tissue culture leaves and greenhouse- grown leaves. × 840.

c leaf cross section of tissue culture plants after 4 weeks of hardening showing well defined adaxial and abaxial epidermis, two layers of compact measophyll cells, and spongy parenchyma with high density of chloroplasts and little intercellular space. × 820.

d greenhouse leaf cross section showing high cell density and defined palisade and spongy parenchyma. × 810.

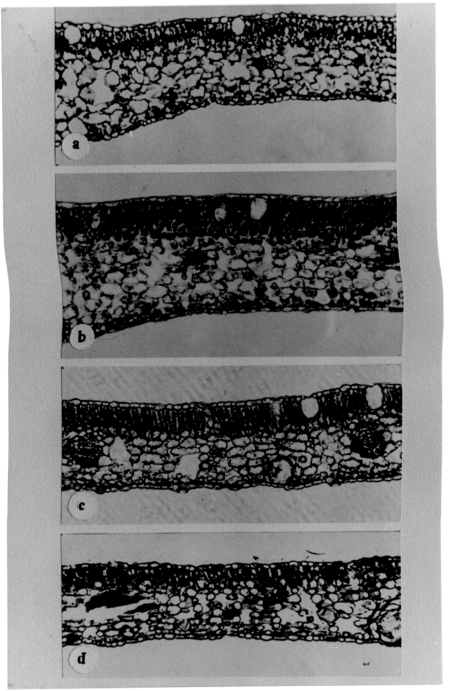

a
b
c
d

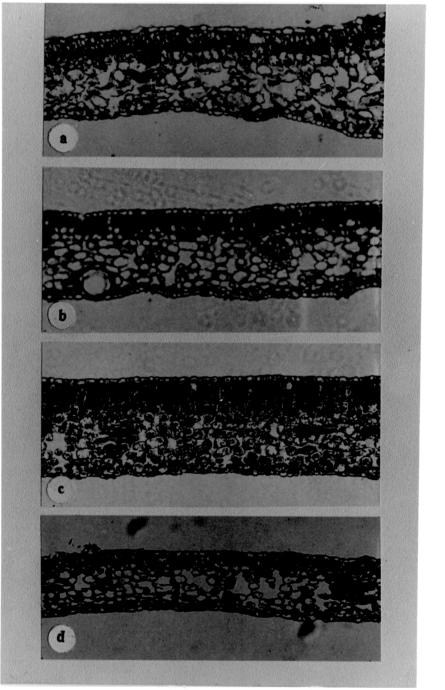
Plate 15. Light micrographs of cross sections of *C. reticulata* leaves.

a tissue culture plantlet leaves, showing irregular shape of epidermal cells and poor differentiation of mesophyll and spongy parenchyma, with large intercellular space × 800.

b leaf cross section of tissue culture plants after 2 weeks of hardening showing intermediate stage between tissue culture leaves and greenhouse-grown leaves × 810.

c leaf cross section of tissue culture plants after 4 weeks of hardening showing well defined adaxial and abaxial epidermis, two layers of compact mesophyll cells, and spongy parenchyma with high density of chloroplasts and little intercellular space × 850.

d greenhouse leaf cross section showing high cell density and defined palisade and spongy parenchyma × 790.


a
b
c
d

3. 8. Isoenzyme analysis

Isoenzyme analysis of malat dehydrogenase, peroxidase, and glutamate oxalo transminase were carried out to compare banding patterns of *in-vivo* and *in-vitro* plants germinated from seeds and those regenerated from tissue culture for citrumelo, *C. limonia* and *C. reticulata*. This was aimed to identify any nucleic variations between the plants. The isoenzymes except malat dehydrogenase responded to the systems by producing banding patterns. In all cases, similar banding patterns were observed in all the isoenzymes of both plants in comparison (Plate 16 a, b). In the absence of the variants, the isoenzyme patterns displayed could be used to characterise somatic hybrids in the future.

Plate 16. Characterization of citrumelo, *C. limonia*, and *C. reticulata* plants regenerated from seeds and tissue culture.

a Glutamate Oxalo Transminase (GOT)

1 Citrumelo from seeds grown in greenhouse

2 Citrumelo regenerated from internodal explants

3 Citrumelo regenerated from leaf explants

4 Citrumelo regenerated from root explants

5 Citrumelo from seeds grown aseptically in jars

6 *C. limonia* grown from seeds in greenhouse

7 *C. limonia* regenerated from internodal explants

8 *C. reticulata* grown from seeds in green house

9 *C. reticulata* regenerated from root explants

b Peroxidase (P)

1 Citrumelo from seeds grown in greenhouse

2 Citrumelo from internodal explants

3 Citrumelo from leaf explants

4 Citrumelo from root explants

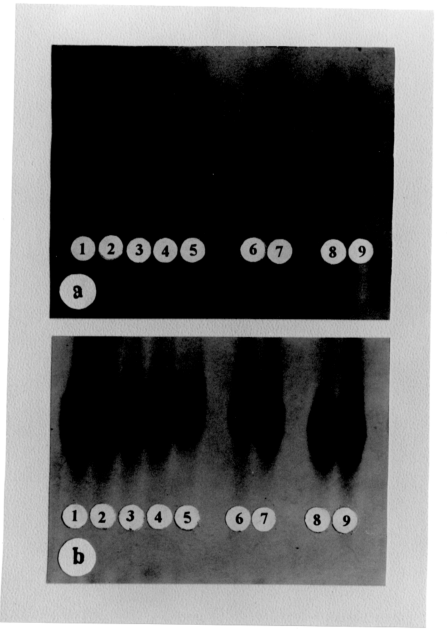
5 Citrumelo from seeds grown aseptically in jars

6 *C. limonia* grown from seeds in greenhouse

7 *C. limonia* regenerated from internodal explants

8 *C. reticulata* from seeds in green house

9 C. reticulata from root explants


1 2 3 4 5 6 7 8 9
a
1 2 3 4 5 6 7 8 9
b

3. 9. Protoplast culture

3. 9. 1. Isolation and culture of citrumelo embryo callus protoplast

3. 9. 1. 1. Enzyme treatment

Embryo callus (about 1 g) was chopped into small pieces and incubated for 16 h. in 10 ml of enzyme mixture (E_1) (Appendix D) and the yield of protoplast produced was $4.8 \pm 0.7 \times 10^5$ protoplasts/ml. Addition of 1 % driselase to the enzyme mixture E_1 (E_2) (Appendix D) increased the protoplast yield obtained (Table 3-1). When macerozyme in E_1 (Appendix D) was increased to 0.35 % (E_3), the yield was increased. On the other hand the protoplast viability ~~however,~~ was the highest comparatively, 87.6 ± 2.3 % when E_1 was used (Table 36). In this experiment the enzyme adopted throughout the project was E_1. Protoplasts isolated varied in size from 15 to 33 μm in diameter.

Table 36: Enzyme mixtures and their influence on protoplast isolation from citrumelo embryo callus. Data was collected from two experiments in two replicates each.

Enzyme code	yield protoplasts / ml	viability %	diameter μ m
E_1	$4.8 \pm 0.7 \times 10^5$	87.6 ± 2.30	14 ± 1.8 33 ± 1.5
E_2	$5.0 \pm 0.7 \times 10^5$	63.5 ± 1.35	15 ± 1.0 33 ± 1.2
E_3	$5.98 \pm 1.0 \times 10^5$	65.3 ± 1.22	15.3 ± 1.0 31.8 ± 0.7

-Enzyme mixtures are in appendix D.

3. 9. 1. 2. Protoplast culture

Callus derived protoplasts were cultured in MT basal medium solidified with 0.6 % agarose and supplemented with 0.3 M sucrose and 0.3 M mannitol without the addition of any plant growth substances. After 15 days about 2.2 ± 0.4 % of the protoplast started first cell division. Similar observation was obtained from protoplast cultured in liquid medium but, first division started after 10 days (Table 37, Plate 17 c). However, when 0.5 mg/l BAP was added to the basic media both as solid and liquid culture first division of citrumelo protoplasts was observed after 5 days.

In another series of experiments, observations were made on protoplasts cultured on MT liquid medium supplemented with 1 mg/l 2,4-D and 3 mg/l BAP. Cell elongation was observed after 7 days and subsequently first cell division occurred on the 10th. day (Plate 17 d) with a percentage of about 0.2 and 0.3 % in liquid and solidified medium respectively (Table 37). When sucrose was replaced by mannitol at 0.38 M either without growth substances or with 2 mg/l 2,4-D and 5 mg/l BAP in liquid medium only 0.1 and 1.0 % of the protoplasts divided after 12 days in culture respectively. However, division was not sustained in all cases (Table 37).

First cell division was also observed after 1 week in liquid culture on MT medium which contained 0.5 mg/l 2,4-D and 1.0 mg/l BAP at the highest frequency of 3.3 ± 0.5 % (Table 37, Plate 17 e) comparatively. However, division was not sustained. After 2 months in culture, 25% of the divided cells remained viable.

Attempts were also made to culture the protoplasts in MS liquid medium containing 0.3 M sucrose and 0.3 M mannitol without growth substances. Only cell elongation was observed after five days in culture indicating cell wall formation but there was no cell division even after 3 weeks in culture (Table 37, Plate 17 f). No division was observed when protoplasts were cultured by agarose droplets method.

In all cases, protoplast division was not sustained because mainly of the contamination problems that have encountered at each time of protoplast culture.

Plate 17. Citrumelo protoplasts

a Freshly isolated mesophyll protoplast, bar = 45 μm.

b Viable protoplast fluoresced under UV light when stained with FDA, bar = 45 μm.

c First division of embryo callus protoplast after 15 days, on MT solid medium with 0.3 M sucrose and 0.3 M mannitol, bar = 9 μm.

d Embryo callus protoplast divided after 10 days on MT liquid medium, bar = 8 μm.

e First division of protoplast on MT liquid medium with 0.5 mg/l 2,4-D and 1 mg/l BAP, bar = 15 μm.

f First division of protoplasts on MS liquid medium, bar = 7 μm.

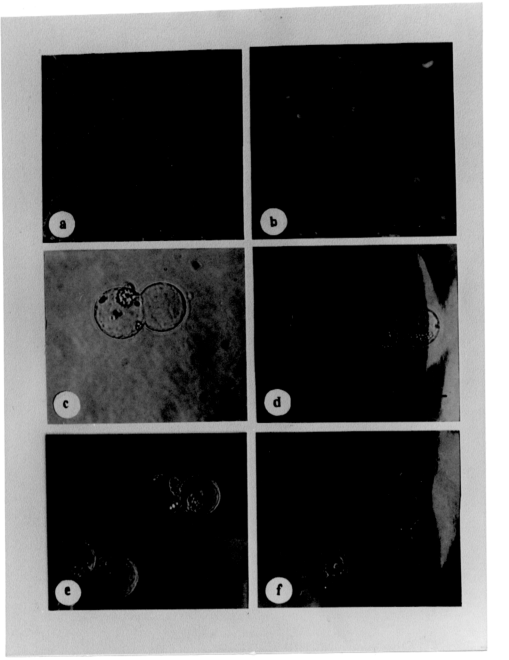

a
b
c
d
e
f

Table 37: Effect of 2,4-D and BAP on cell division of citrumelo embryo callus protoplast of citrumelo cultured on MT medium. The results are average of two experiments with two replicates each.

Basal medium	Growth substances		Culture method			Sugars (M)		Frequency of dividing cells %
	2,4-D mg/l	BAP mg/l	solid	liquid	agarose droplets	sucrose	mannitol	
MT	0	0	×			0.3	0.3	2.2 ± 0.4
MT	0	0		×		0.3	0.3	2.2 ± 0.3
MT	1	3		×		0.3	0.3	0.2
MT	0	0		×		0	0.38	0.10
MT	2	5		×		0	0.38	1.0 ± 0.3
MT	0	0	×			0	0.38	2.0 ± 0.4
MT	0.5	1.0		×		0.3	0.3	3.3 ± 0.5
MT	1	3	×			0	0.38	2.0 ± 0.4
MT	0	0.5	×			0.3	0.3	1.0 ± 0.4
MT	0	0			×	0.3	0.3	0
MS	0	0		×		0.3	0.3	0

-Enzyme mixtures are in appendix D.

3. 9. 2. Mesophyll protoplasts of citrumelo, *C. suhuiensis*, and *C. limonia*

The mesophyll protoplasts were isolated from leaves of 2 months-old *in-vitro* grown plants of citrumelo, *C. suhuiensis*, and *C. limonia* using various enzyme mixture. Protoplast yield and viability were accounted for.

The highest yield for citrumelo at $8.1 \pm 0.4 \times 10^5$ protoplasts/ml was obtained by using E_3 and the highest viability frequency was obtained by using E_2 enzyme mixture (Table 38) (Appendix D) (Plate 17 a, b). Whereas for *C. suhuiensis* the highest yield was at $6.7 \pm 0.3 \times 10^5$ protoplasts/ml and a viability frequency at 72 ± 2.5 % was obtained using E_1 enzyme mixture (Appendix D). For *C. limonia* the highest yield $7.4 \pm 0.8 \times 10^5$ protoplasts/ml, was obtained by using E_3 enzyme mixture while the highest viability frequency was at E_1 enzyme mixture (Table 38). The plating density used was 1.0×10^5 protoplasts/ml on MT basal medium containing 0.15 M sucrose and 0.45 M mannitol without any growth substances and in the dark. No cell division was observed even after 4 weeks, and eventually the protoplasts became necrotic.

Table 38: The yield and viability of mesophyll protoplasts of citrumelo, *C. suhuiensis* and *C. limonia* from *in-vitro* grown plants. Data was calculated immediately after isolation. The results were the average of two experiments with three replicates each.

Species	E_1		E_2		E_3	
	Yield proto. /ml	Viability %	Yield proto. /ml	Viability %	Yield proto. /ml	Viability %
Citrumelo	$7.2 \pm 0.2 \times 10^4$	56.6 ± 2.0	$5.5 \pm 0.3 \times 10^5$	80.6 ± 1.8	$8.1 \pm 0.3 \times 10^5$	71.2 ± 2.4
C. suhuiensis	$6.7 \pm 0.2 \times 10^5$	72.2 ± 2.4	$4.6 \pm 0.3 \times 10^4$	37.2 ± 2.2	$3.9 \pm 0.2 \times 10^5$	35.4 ± 1.9
C. limonia	$5.4 \pm 0.2 \times 10^5$	71.2 ± 3.3	$6.5 \pm 0.2 \times 10^5$	66.0 ± 2.3	$7.3 \pm 0.8 \times 10^5$	45.0 ± 2.2

-Enzyme mixtures are in appendix D.

3 . 10. Physiological studies on some citrus species

Several photosynthetic parameters were determined for the plants of citrumelo, *C. limonia* and *C. reticulata* regenerated *in-vitro* from internodal, leaf and root explants after the plantlets were acclimatized for about 2 months as stated in Section 2. 6. Their photosynthetic capabilities and efficiency were compared with those grown from seeds. As mentioned in Section 2.11 these physiological measurements were made using an infra-red gas analyzer (LCA- 4).

Plantlets and seedlings of the same age were difficult to obtain due to their different pattern of growth and development (Pospisilova, *et al.*, 1987). As such morphologically similar plants, with 10 to 12 leaves, were chosen for these experiments.

3. 10. 1. Photosynthetic rates

Maximal photosynthetic rates in the various plantlets and seedlings studied are shown in Table 39. In citrumelo, plantlets from leaf and internodal explants exhibited maximum CO_2 assimilation rates similar to that of seedlings with rates ranging from 7.1 to 7.6 μmol CO_2 m^{-2} s^{-1} . In the two other species studied, *C. limonia* and *C. reticulata* the plantlets from internodal explants achieved slightly higher photosynthetic rates compared to the seedlings, reaching rates of 7.8 - 8.2 μmol CO_2 m^{-2} s^{-1}.

Table 39. Maximum photosynthetic rates in citrus seedlings and plantlets grown *in-vitro* from leaf, internodal and root explants.

Species	Maximum photosynthetic rates ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)
Citrumelo	
seedling	7.10 ± 0.067
internodal	7.12 ± 0.053
leaf	7.66 ± 0.049
C. limonia	
seedling	6.37 ± 0.095
internodal	7.86 ± 0.035
C. reticulata	
seedling	7.03 ± 0.086
root	8.18 ± 0.173

- Mean values ± standard error.
- Each point indicates the mean value of 10 to 12 readings.

3. 10. 1. 1. Light response curve

The light response curve shows the relationship between varying light intensities and photosynthetic rates. A typical relationship between these two parameters would be a rectangular hyperbole of the Michaelis-Menten type.

This is shown in Figures 1, 2, and 3, where the light saturation curves for the plantlets and seedlings of the 3 species studied were found to be similar. In both seedlings of citrumelo photosynthetic rates saturated at a low light intensity of about 400 μmol m^{-2} s^{-1} (Figure 1). Similarly in *C. limonia* plantlets from internodal explants and the seedlings achieved light saturation at around 350 μmol m^{-2} s^{-1} (Figure 2). In *C. reticulata*, both plantlets grown from root explants and seedlings exhibited saturating net photosynthetic rates at about 500 μmol m^{-2} s^{-1} (Figure 3).

These data suggests that the plantlets were physiologically similar to the seedling in their photosynthetic capacities.

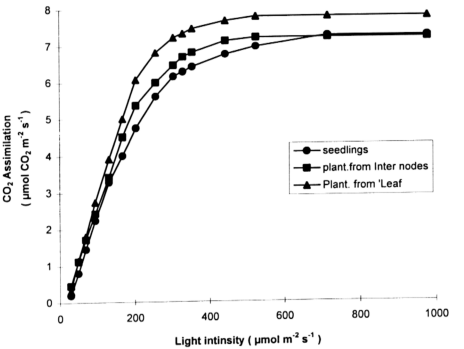


Figure 1. Light response curves of citrumelo seedlings and plantlets grown *in-vitro* from internodal and leaf explants.

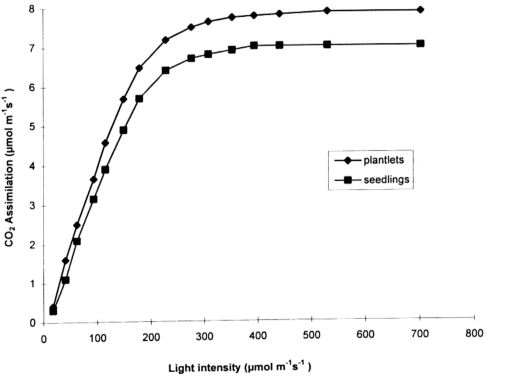


Figure 2. Light response curves of *C. limonia* seedlings and plantlets grown *in-vitro* from internodal explants.

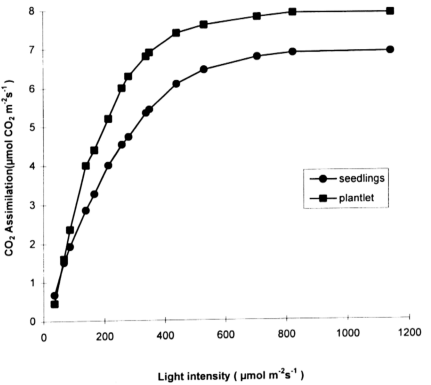


Figure 3. Light response curves of *C. reticulata* seedlings and plantlets grown *in-vitro* from root explants.

3. 10. 1. 2. Light compensation point

The light compensation point is taken from the linear phase of the light response curve. It is the point whereby the net photosynthetic rate is zero as a result of the photosynthetic rate (CO_2 intake) being balanced by the respiratory rate (CO_2 release).

As shown in Table 40, the light compensation point in the citrumelo plantlets and seedlings varied between 23.6 ± 2.3 to 27 ± 3 μmol m^{-2} s^{-1}. Both *C. limonia* plantlets and seedlings showed lower values of 10.7 ± 1.8 and 12.9 ± 2 μmol m^{-2} s^{-1} respectively. A difference in light compensation point was observed between the root plantlets and seedlings of *C. reticulata* with reading of 24 ± 2.8 and 9 ± 1.2 μmol m^{-2} s^{-1} respectively.

Table 40. The light compensation point in citrus seedlings and plantlets grown *in-vitro* taken from the light response curves.

Species	Light compensation point ($\mu mol\ m^{-2}\ s^{-1}$)
Citrumelo	
seedling	27.00 ± 3.07
internodal	25.85 ± 2.19
leaf	23.60 ± 2.34
C. limonia	
seedling	12.64 ± 2.12
internodal	10.71 ± 1.89
C. reticulata	
seedling	9.00 ± 1.29
root	24.00 ± 2.80

- Mean values ± standard error.
- Each point indicates the mean value of 10 to 12 readings.

3. 10. 1. 3. Quantum efficiency

The initial phase (slope) of the light response curve may be taken as the maximum quantum yield or efficiency. It is the efficiency by which light is used for photosynthesis. It could be expressed as amount of CO_2 fixed (mmol) per μmol of light absorbed by the leaf.

The quantum efficiency of photosynthesis for different plantlets and seedlings are shown in Table 41. In both citrumelo and *C. limonia* the seedlings and plantlets displayed similar quantum efficiency numbers whilst in *C. reticulata* the plantlets grown from root explants showed greater efficiency than the seedlings.

Table 41. Quantum efficiency in citrus seedlings and plantlets grown *in-vitro*.

Species	Quantum efficiency (mmol CO_2/μmol light)
Citrumelo	
seedling	31.58 ± 2.89
internodal	35.34 ± 3.11
leaf	38.05 ± 1.35
C. limonia	
seedling	45.49 ± 3.24
internodal	48.68 ± 2.98
C. reticulata	
seedling	20.50 ± 0.61
root	50.00 ± 2.63

- Mean values ± standard error.

- Each point indicates the mean value of 10 to 12 readings.

3. 10. 1. 4. Water use efficiency

The water use efficiency (WUE) of a plant is calculated as the ratio of the photosynthetic rate to the transpiration rate. It reflects the efficiency of the leaves in the utilization of water and is an indication of the function of the stomata.

The WUE values at different light intensities calculated from the light saturation experiments are shown in Figures 4, 5 and 6 for the various seedlings and plantlets studied.

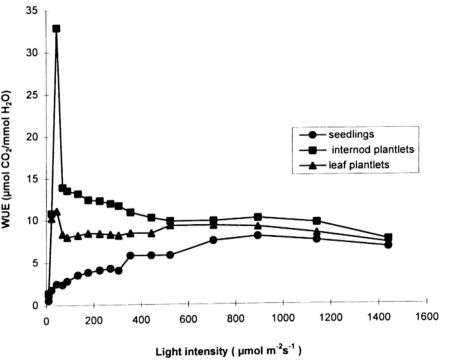


Fig. 4. WUE for citrumelo plantlets grown *in-vitro* from internodal and leaf explants and seedlings.

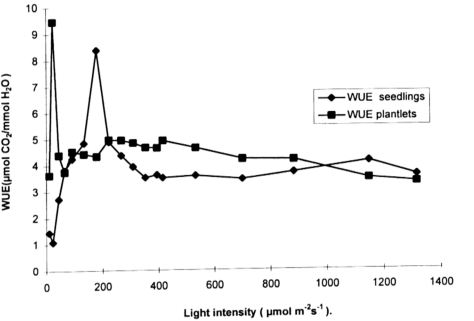


Fig.5. WUE for *C. limonia* plantlets grown *in-vitro* from internodal explants and seedlings.

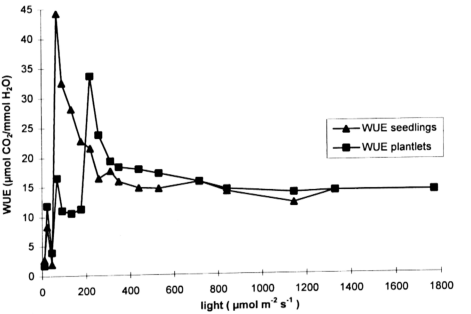


Fig.6. WUE for *C. reticulata* plantlets grown *in-vitro* from root explants and seedlings.