CHAPTER 4

RESULTS

RESULTS

The present chapter deal with the results obtained during the course of study and is divided into three sections which describe regeneration, metabolite and mutation studies respectively of the two species *P oleracea* and *P grandiflora*.

4.1 SECTION I REGENERATION STUDIES

The regeneration protocol was developed to generate large number of plants which could be utilized for metabolite and mutation studies. The leaf was selected as an explant for both the species because it is known to have regenerative potency to generate large number of shoots. In *P grandiflora* the regenerative capacity of nodal plants was also assessed. The explants were exposed to PGRs which stimulate morphogenic response and hence generate a large number of shoots. This response of the PGR's can be assessed by studying the effect of cytokinin and auxins individually and synergistic effect of cytokinin with auxin in both the species

4.1.1*P oleracea*

Regeneration from in vivo leaf explants of *P oleracea* was initiated and the results described below were obtained from individual concentrations of cytokinins (BA and Kin), auxins (IAA, NAA and 2,4D) and a synergistic effect of both the cytokinins and auxins. A control devoid of PGR's was maintained which formed 1-2 shoot bud in the 5^{th} week and a single incipient shoot at the end of 8^{th} week (Fig. 5a).

4.1.1.1 Effect of cytokinins

Cytokinins are known to differentiate shoot bud in a number of plants and for the present studies BA and Kin were selected as they are commonly available. Hence the effect of BA and Kin (0.5-20 µM) individually was evaluated on leaf explants.

(i) BA

A wide range of BA concentrations (0.5-20µM) were evaluated to determine their effect on leaf explants. The response was evaluated on the basis of parameters like percent response and shoot number. A low concentration of BA (0.5 µM) failed to evoke any response and the explants turned brown after 6 weeks. Increasing the concentration to 2.5 µM the response

was slow as only shoot buds were formed in 4th week from the abaxial surface which failed to elongate into in vitro shoots even after 8 weeks (Fig. 5b). Raising the concentration to 5 µM was effective in differentiating in vitro shoot buds. The swelling of the explants started from the 1stweek and shoot buds appeared from the proximal adaxial surface of explant during the 3rdweek of incubation. These shoot buds gradually progressed to the entire margins of the explant and by fourth week the entire margin was covered with shiny shoot buds which proliferated into shoots in six weeks. The percentage of explants forming shoots was noted to be 80% and optimum number of shoots (5.5±0.99) for individual BA was obtained at 5 µM(Table 1, Fig. 5c).Increase in concentration to 10 µM delayed the induction of shoot buds to 3rdweek which was coupled by a decrease in number of shoots which elongated in 6th week. BA concentration of 15 µM and 20 µM resulted in further decrease in the number of shoot buds regeneration which was accompanied with decrease in percent response (70%). At this high concentration along with shoot buds a green compact callus was also formed by the end of eight weeks (Table 3 and Fig. 5d and e).

(ii)Kin

BA when replaced with Kin revealed that low concentration of Kin (0.5-2.5 µM) failed to exhibit any response and the leaf turned brown after six weeks. At 5 µM, root differentiation was observed in 20% of the explants while at 10 µM the leaf explant swelled slowly and formed shoot buds on the abaxial surface followed by adaxial surface of the explants (Fig. 6a). Raising the concentration of Kin to 15 µM induced shoot buds and shoot (1.1±0.34) both after 6thweek (Table 3, Fig. 6b) while at 20 µM Kin there was a late induction of shoot buds at 6th week which failed to elongate into shoots.

Thus it was concluded that BA was effective in inducing in vitro shoot formation as compared to Kin. Within different concentrations of BA, highest shoot number was obtained at 5 µM (5.5±0.99) as compared to the rest of the concentrations.

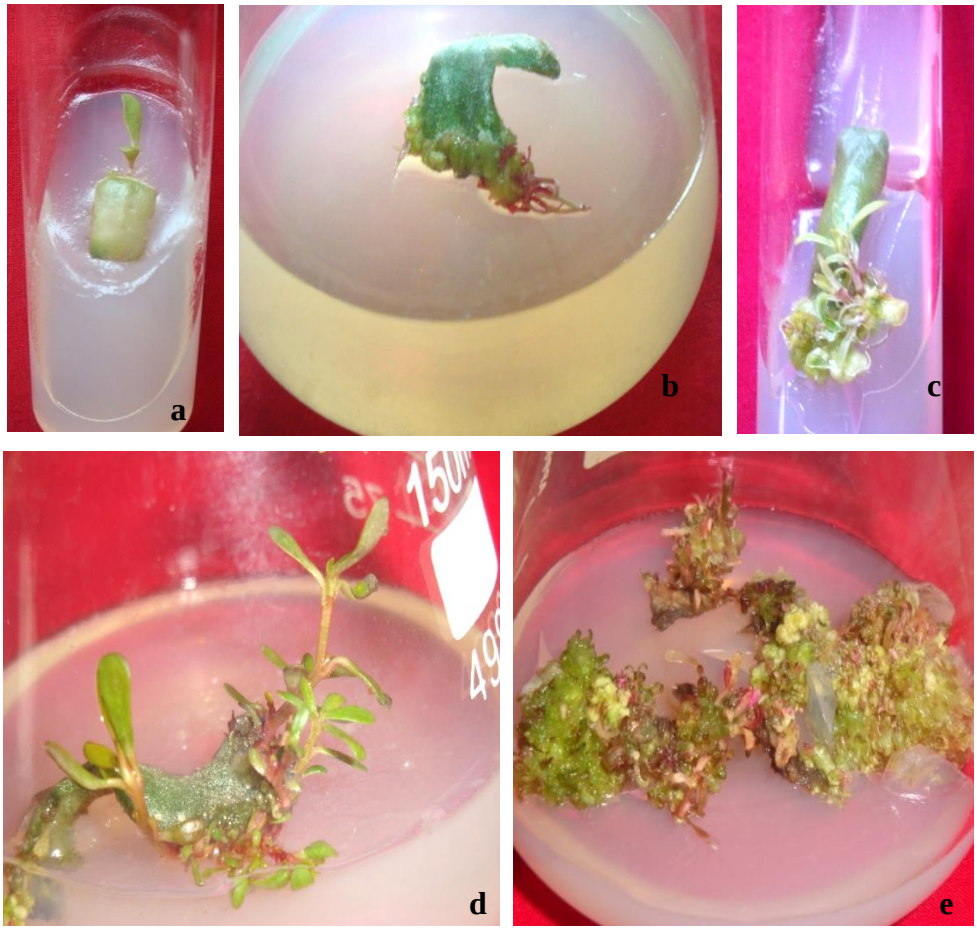


Figure5: Effect of BA on leaf explant of *Poleracea* (a) Single incipient shoot in control(b) Shoot bud formation at 2.5µM BA after 8 weeks (c) Shoot buds and shoot formation at 5 µM BA after 4 weeks (d) Development of shoot at 5 µM BA after 8 weeks (e) Differentiation of shoot buds from green compact callus at 20 µM BA.

Table 3: Effect of individual cytokinins (BA/Kin) on leaf explants of *P oleracea*

Cytokinin (µM)		Number of shoots/explant (Mean± S.E.)*	% Explants forming shoots
BA	Kin		
0.5	-	-	-
2.5	-	-	-
5	-	5.5 ± 0.99^{c}	80
10	-	3.7 ± 0.53^{bc}	90
15	-	2.6 ± 0.4^{ab}	70
20		1.5 ± 0.4^{ab}	70
-	0.5	-	-
-	2.5	-	-
-	5	-	-
-	10	-	-
-	15	1.1 ± 0.34^{a}	20
-	20	-	-

*Means followed by the same letters in a column are statistically not significant with Tukey's HSD test (n=10) and value of $p \leq 0.05$, S.E. = Standard error.

4.1.1.2 Effect of auxins

Individual auxins are known to induce shoot buds but are less commonly used for this purpose. In the present study the effect of different auxins (IAA/NAA/2,4D) was tried individually (0.5-10µM) for shoot bud differentiation and callus induction as the later was utilized in metabolite estimation.

(i) IAA

Medium fortified with different concentrations of IAA initiated a dual morphogenic response in leaf explants as both root and shoot were formed. At 0.5 µM root was initiated in all the explants while 33.33% cultures were also accompanied with shoot formation (Fig. 7a). The number of roots per explant declined at 2.5-10µM with a decrease in percent response (Table 4). At 5µM IAA 25% cultures formed shoots which were stout and healthy (Fig. 7b) while their growth decreased at 10 µM. Thus the explants in presence of IAA induced roots at all concentration and their number decreased with increase in concentration. Explants at low concentrations formed shoots.

(ii) NAA

When the leaf explants were placed on media fortified with NAA, it induced vigorous root formation with 100% response at all concentrations. At 0.5 µM NAA besides roots, 41% of the explants formed single micro shootwith few leaves. Growth of the shoots was suppressed at 2.5 µM with 16.66% response while the rest of explants formed roots (Fig. 7c). Large number of roots(16.2±0.85) were formed at 5µM (Fig. 7d) while at 10 µM a decline in their number was noted (Table 4). It was thus deduced that low concentration of NAA (0.5-2.5 µM) has the potency of forming in vitro shoots and roots. When the concentration was increased to 5-10 µM there was a shift in morphogenic response as shoot inducing capacity was lost and the explants formed profuse roots.

(iii) 2,4D

Another auxin 2,4D was employed to induce callus from leaf explant. This callus was utilized for fatty acid analysis. It was observed that individual concentration of 2,4D at 0.5µM initiated roots while higher concentrations (2.5, 5 and 10 µM) induced only white friable callus on the margins of the explants originating from the proximal end of the explant (Table , Fig. 7e).

The study of individual effect of the auxins on leaf explants revealed that they varied significantly in morphogenetic response. IAA formed healthy shoots and less number of roots whereas NAA differentiated increased number of roots as compared to shoots. 2,4D induced callus from the explants.

The experiments with individual concentration of cytokinin and auxins revealed that individual auxins like IAA and NAA too showed the potency to form shoots. BA has the potential of forming in vitro shoots while Kin induced shoot buds. Therefore the coupling effect of the two cytokinins and cytokinins and auxins has to be evaluated.

The explants responded in presence of all the individual PGR's tried but the aim of achieving large number of shoots was not attained. Therefore further studies were conducted with synergistic combinations of two cytokinin and cytokinin with auxin.

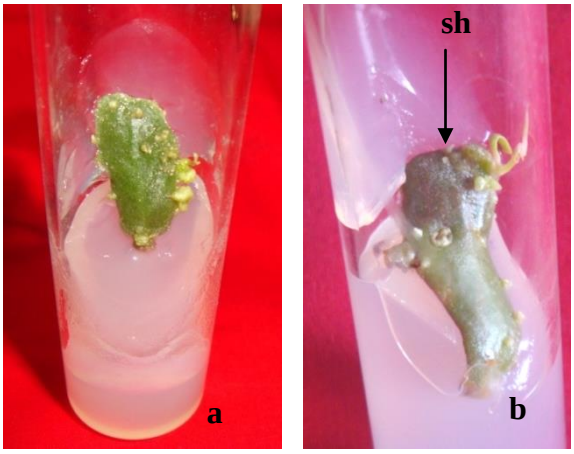


Figure6: Effect of Kin on leaf explants of *P oleracea* (a) Shoot bud formation on the adaxial surface at 10 µM. (b) Shoot formation (sh) at 15 µM.

Figure7: Effect of auxins on leaf explants of *P oleracea* after 8 weeks (a) In vitro shoot and root formation (r) at 0.5µM IAA (b) Healthy in vitro shoots at 5µM IAA (c) Incipient shoots and root formation at 2.5 µM NAA (d) Profuse rooting at 5µM NAA (e) Induction of friable callus at 5µM 2,4D

Table 4: Effect of auxins (IAA/NAA/2,4D) on number of roots and shoots per explant from *P oleracea*

Auxins (µM)			Number of roots/explant (Mean± S.E.)*	% Explants forming roots	Number of shoots/explant(Mean ± S.E.)*	% Explant forming shoots
IAA	NAA	2,4D				
0.5	-	-	4.1±0.92[a]	83.33	0.5±0.23[a]	33.33
2.5	-	-	3.3±0.76[a]	75	1.25±0.55[a]	33.33
5	-	-	2.1±0.79[a]	50	1.83±0.69[a]	25
10	-	-	-	-	0.33±0.18[a]	25
-	0.5	-	4.8±0.64[a]	100	0.82±0.34[a]	41
-	2.5	-	10.5±0.81[b]	100	0.25±0.13[a]	16.66
-	5	-	16.2±0.85[c]	100	-	-
-	10	-	12.3±0.94[b]	100	-	-
-	-	0.5	1.4±0.61[a]	55.55	-	-
-	-	2.5	-	-	-	-
-	-	5	-	-	-	-
-	-	10	-	-	-	-

*Means followed by the same letters are statistically not significant according to Tukey's HSD test where n=12 and $p \leq 0.05$, S.E. = Standard error.

4.1.1.3 Synergistic effect of cytokinins

In the previous experiments with individual concentrations of BA and Kin it was revealed that both the cytokinins had an ability to induce shoot buds and shoots from leaf explants of *P oleracea.* Therefore synergistic combinations of the two cytokinins were tried to evaluate their dual role in enhancement of the number of shoots per explant.

(i) BA and Kin

A synergistic effect of BA (0.5-20µM) and Kin (0.5-20µM) was evaluated for the induction of shoots. Low concentration of BA (0.5µM) with Kin (20µM) induced 5-6 shoot buds in the 6th week which failed to proliferate into shoots. With an increase in BA concentration to 2.5µM and Kin (2.5-20µM) there was a faster induction of shoot buds in 2nd week (Fig. 8a) in all the combinations and at 10µM Kin the shoot buds formed cluster of incipient shoots (Fig. 8b) which failed to elongate. Further increase in Kin to 15 and 20µM differentiated shoot buds along with callus (Fig. 8c).

Increase in concentration of BA to 5 µM with Kin induced shoot formation. A combination of 5 µM BA and 0.5 µM Kin failed to evoke any morphogenetic response even after 4 weeks while with 2.5 µM Kin the shoot buds elongated into shoots during 5th week (Table 5, Fig. 9a). The response to cytokinins in terms of number of shoots increased with increase in

concentration of Kin (Table 5). At 5 µM BA and 5 µM Kin the explants induced shoot buds in two weeks on the abaxial surface which differentiated shoots during 4th week and in vitro shoots and clusters of shoots by 6th week. A similar response was observed at 5 µM BA and 10 µM Kin with slight increase in number of shoot (8.3±1.01) which was an optimum response. In this combination induction of light green shiny shoot bud clusters was observed on the abaxial surface during 2nd week (Fig. 9b) and incipient shoots formation in 3rd week (Fig. 9c). By 6th week a cluster of shoots were formed with 90% response (Fig. 9d, Table 5). There was a decline in the number of shoot formation with further increase in Kin concentration and along with shoots and shoot buds a green nodular callus also differentiated at 5 µM BA and 15-20 µM Kin after 8 weeks (Fig. 9e).

A further increase in BA to 10µM with 2.5-5µM Kin induced shoots however their number was less as compared to 5µM BA and 10µM Kin which was concluded to be an optimum concentration (Table 5, Fig. 10a, b). At 10-20µM along with shoot buds little green compact callus was also induced from the explants (Fig. 10c).

At 15-20µM BA and Kin (0.5-20µM) low concentration of Kin (0.5-5µM) induced stunted shoots (Fig. 11a, b) while at high level (10-20µM) failed to regenerate and formed whitish green compact callus (Fig. 11c).

It was thus concluded from the experiment that at low concentration of BA with Kin shoot buds were induced. In vitro shoots were formed in the concentration of 5µM BA with Kin and optimum number of shoot per explants was obtained at 5µMBA and 10 µM Kin (8.3±1.01). The number of shoot per explants declined from 10µM BA and at 15-20µM BA stunted shoots developed indicating that this level of cytokinin was toxic to the plant. Along with these shoot buds and shoots, compact whitish green callus was induced at high concentration of BA and Kin (15-20µM).

4.1.1.4 Synergistic effect of cytokinin and auxin

Individual concentrations of BA/ Kin and a synergism between the two, lead to multiple shoot formation. Similarly individual auxins (IAA and NAA) at low concentrations also induced shoots. Thus further experiments were designed where the synergism between the cytokinin and auxin was studied to evaluate their potential for enhancement of number of shoots.

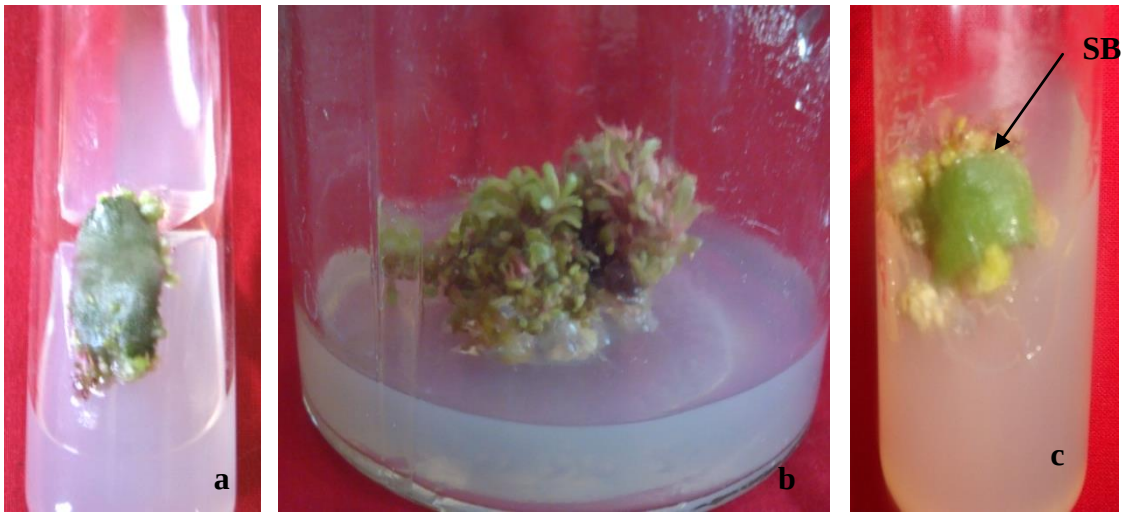


Figure8: Effect of 2.5 µM BA with different concentrations of Kin on leaf explants of *P oleracea* (a) Shoot bud induction at 2.5 µM BA and 5 µM Kin (b) Shoot buds and cluster of incipient shoot at 2.5 µM BA and 10 µM Kin (c) Green nodular callus and differentiating shoot buds (SB) at 2.5 µM BA and 15 µM Kin.

Figure 9: Effect of 5 µM BA with different concentrations of Kin on leaf explant of *P oleracea*(a) Shoot formation on 5 µM BA and 2.5 µM Kin after 8 weeks (b) shoot bud formation in presence of 5 µM BA and 10 µM Kin after 2 weeks (c) Initiation of shoot on 5 µM BA and 10 µM Kin after 3 weeks (d) Optimum number of in vitro shoot on 5 µM BA and 10 µM Kin after 8 weeks (e) Green nodular callus and shoot buds in presence of 5 µM BA and 20 µM Kin.

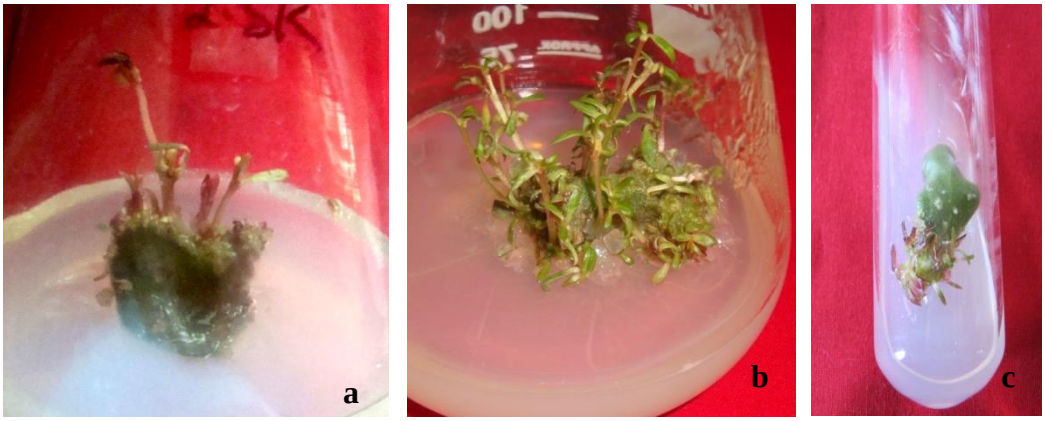


Figure10: Effect of 10 µM BA with different concentration of Kin on leaf explants of *P oleracea* after 8 weeks (a) Shoot buds and incipient shoots at 10 µM BA and 2.5 µM Kin (b) Few in vitro shoot at 10 µM BA and 5 µM Kin (c) Green compact callus and shoot bud formation at 10 µM BA and 20 µM Kin.

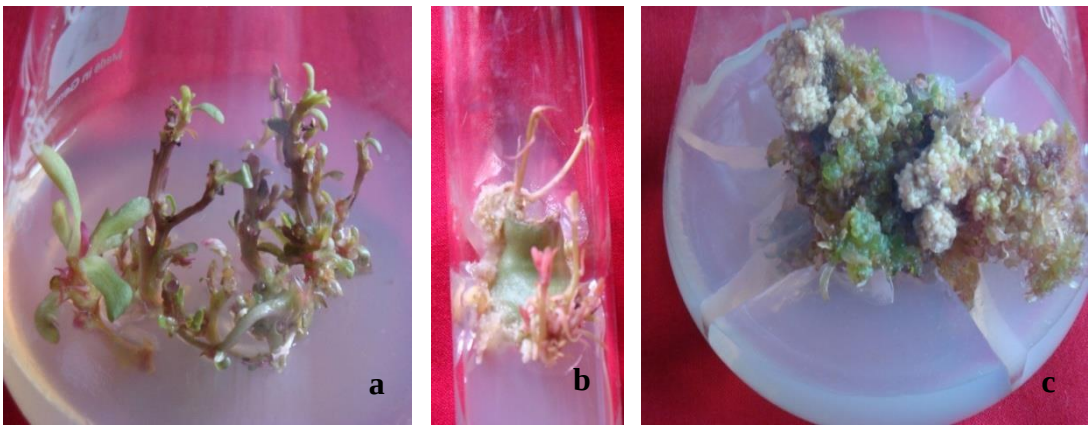


Figure11: Effect of 15/20 µM BA with Kin on leaf explants of *P oleracea* after 8 weeks (a)Stunted in vitro shoot at 15 µM BA and 2.5 µM Kin (b) Callus and fragile red shoot at 20 µM BA and 0.5 µM Kin (c) Whitish green compact nodular callus at 20 µM BA and 5 µM Kin.

Table 5: Effect of BA and Kin on in vitro shoot formation from leaf explants of *P oleracea*

Cytokinin (µM)		% Explants forming shoots	Number of shoots/explant (Mean ± S.E.)*
BA	KIN		
0	0	10	0.9 ± 0.34^{a}
0.5	0.5-15	-	-
0.5	20	-	Shoot buds
2.5	0.5	-	-
2.5	2.5-20	-	Shoot buds
5	0.5	-	-
5	2.5	40	5.4 ± 0.70^{cde}
5	5	80	6.1 ± 0.80^{de}
5	10	80	8.3 ± 1.01^{e}
5	15	20	3.8 ± 0.91^{abcd}
5	20	-	-
10	0.5	-	-
10	2.5	50	3.5 ± 0.65^{abcd}
10	5	70	4.9 ± 0.52^{bcd}
10	10	70	2 ± 0.61^{ab}
10	15	30	1.8 ± 0.41^{ab}
10	20	-	-
15	0.5-20	-	Stunted shoots
20	0.5-20	-	Stunted shoots

*Means followed by the same letters are not statistically significant according to Tukey's HSD test where the value of $p\leq 0.05$ and n=10. S.E. = Standard error, - = No response.

(i) BA and IAA

A combination of BA with IAA was evaluated where a low concentration of 0.5µM BA with IAA (0.5-10µM) evoked a late response as callus and roots were formed simultaneously within 4-6 weeks (Fig. 12a).The growth of callus and number of roots increased at 2.5µM BA and IAA (0.5-10) and highest root number (6.90±0.56) was obtained at 2.5µM BA and 2.5 µM IAA (Table 6, Fig. 12b). At 5 µM BA and 0.5 µM IAA, diversity in response was observed as here white green compact callus, roots and shoot buds were initiated. Further increase in IAA concentration (2.5-10µM) with this level of BA gave root with multiple branching and moderate whitish green callus. At a high concentration of 10 µM BA and IAA (0.5-10 µM) the morphogenic response shifted to profuse greenish white compact callus was formation (Fig. 12 c).

Table 6: Effect of BA and IAA on root and callus formation from leaf explant of *P oleracea*

PGR's (µM)		Callus	Number of roots/explant (Mean± S.E.)*	% Explants forming roots
BA	IAA			
0.5	0.5	+	2.41 ±0.62^{abc}	66.66
0.5	2.5	+	2.91±0.89^{abc}	66.66
0.5	5	++	2.66±0.63^{abc}	80
0.5	10	+	1.33±0.39^{a}	57.14
2.5	0.5	+	1.58±0.59^{a}	55.55
2.5	2.5	++	6.90 ±0.56^{d}	100
2.5	5	++	5.16 ±1.16^{cd}	85.71
5	0.5	+	0.5 ±0.19^{a}	30
5	2.5	++	1.25±0.39^{a}	42.85
5	5	++	4.91± 1.02^{bcd}	87.5
5	10	+++	4.25 ± 0.97^{abc}	75
10	0.5	+	0.91±0.31^{a}	50
10	2.5	+++	1.75 ± 0.52^{ab}	50
10	5	+++	0.66 ± 0.28^{a}	22.22
10	10	++++	1.08 ± 0.25a	70

*Means followed by the same letters are statistically not significant according to Tukey's HSD test where n=12 and $p \leq 0.05$, S.E. = Standard Error + = Poor callus, ++ = moderate callus, +++ = Good callus, ++++= Profuse callus.

Thus a combination of BA and IAA evoked callus and root formation in leaf explants of *P oleracea*. There was low callus induction at 0.5 µMBA and IAA (0.5-10µM).With increase in concentration of BA (2.5-10µM) and IAA (0.5-10µM) moderate to profuse callusing was observed and the ability to induce roots decreased.

(ii) BA and NAA

Experiments with BA and IAA induced callus and root formation therefore the auxin IAA was replaced with NAA to determine the difference in response with respect to auxins.

A very low concentration of BA (0.5 µM) and 0.5 µM NAA formed only roots and an increase in NAA concentration (2.5-10 µM) resulted in moderate callus differentiating a large number of roots (11.75±0.60) at 0.5 µM BA and 5 µM NAA (Fig. 13a). A combination of 5µM BA and 2.5 and 5µM NAA induced shoot (Fig. 13b) indicating that NAA plays a stimulatory role in differentiating shoots at high level of BA (5 µM). This morphogenic response of inducing in vitro shoots shifted to caulogenesis as the level of NAA increased to 10 µM. High concentration of BA at 10 µM coupled with 0.5 µM NAA depicted a similar response of shoot buds and callus formation. Rise in NAA concentration to 5-10µM with

10µM BA shifted the mode of organogenesis completely to caulogenesis as whitish green friable callus with few roots was induced in the cultures (Fig. 13c, Table 7).

Table 7: Effect of BAP and NAA on callus, root and shoot inductionfrom leaf explants of *P oleracea*

PGR's (µM)		Callus	Number of roots/explant(Mean ± S.E.)*	% Explants forming roots	Number of shoots/explant(Mean ± S.E.)*	% Explants forming shoots
BA	NAA					
0.5	0.5	-	2.33 ± 0.41^{ab}	100	-	-
0.5	2.5	+	6.83 ± 1.19^{c}	100	-	-
0.5	5	++	11.75 ± 0.60^{d}	100	-	-
0.5	10	+	11.33 ± 1.13^{d}	100	-	-
2.5	0.5	+	1.16 ± 0.38^{a}	100	-	-
2.5	2.5	+	4.66 ± 0.94^{abc}	100	-	-
2.5	5	+	6.08 ± 1.46^{abc}	100	-	-
2.5	10	+	6.41 ± 1.80^{bc}	100	-	-
5	0.5	+	-	-	-	-
5	2.5	+++	1.91 ± 0.72^{a}	100	0.84 ± 0.23^{a}	16.66
5	5	+++	5.83 ± 0.94^{abc}	100	1.12 ± 0.34^{a}	25
5	10	++++	3.08 ± 0.87^{abc}	100	-	-
10	0.5	+	-	100	-	-
10	2.5	+++	0.75 ± 0.30^{a}	100	-	-
10	5	++++	4.16 ± 0.77^{abc}	100	-	-
10	10	++++	1.33 ± 0.39^{a}	100	-	-

*Means followed by the same letters are statistically not significant according to Tukey's HSD test where n=12 and $p \leq 0.05$, S.E. = Standard error + = Poor callus, ++ = moderate callus, +++= Good callus, ++++= Profuse callus.

Thus the combinations of BA and NAA revealed that at low concentrations of BA (0.5 µM) with NAA (0.5-10 µM) there was poor callus induction and increase number of roots formation. On increasing the concentration of BA to 5-10µM and NAA (0.5-10 µM) the morphogenic response just changed as there was good to profuse callusing, differentiating few roots. It is further concluded that both IAA and NAA with BA had a similar morphogenic response of inducing root and callus.

(iii) BA and 2,4D

The third auxin tried with BA was 2,4D, because previous experiments individually with 2,4D induced organogenesis in terms of roots at low concentrations while cytokinins initiated shoot buds and shoots.

BA at 0.5 µM coupled with 0.5 µM 2,4D induced roots in 3rd week (Fig. 14a) while with 2.5-10 µM induced white compact callus. Increase in the concentration of BA to 2.5 µM with 0.5 µM 2,4D formed shoot buds and negligible green compact callus but when the concentration of 2,4D was increased to 2.5 and 5 µM there was a rapid growth of callus. By the end of 4 weeks for both the concentration of 2,4D the entire explant was covered with callus. Profuse callus induction was achieved at 2.5 µM BA and 2.5 µM 2,4D (Fig. 14b). A further increase of BA to 5µM induced shoot buds and callus along with 0.5 µM 2,4D. When 10 µM BA was coupled with 0.5 µM 2,4D green compact callus and shoots (2.34±0.57) were formed (Fig. 14c) and further increase in 2,4 D (2.5-10 µM) formed white compact callus which decreased with increase in 2,4D concentration. Thus 2,4D consistently had the capacity of inducing white compact callus and only at a combination of high BA (5-10 µM) and low 2,4D (0.5 µM) the explant was able to form roots or shoots.

Since profuse callusing was observed with BA and 2,4D, this combination was optimised for callus induction (in terms of fresh weight and dry weight) which was utilized in metabolite estimation. The time period when an optimized callus induces maximum callus weight was also determined by callus growth index.

The coupled interaction of PGR's BA and 2,4D as discussed produced white friable callus. At0.5µM BA and 0.5 µM 2,4D least callus (1.61±0.13 gm) was produced after 8 weeks which increased to 5.57±0.45 gm at 0.5 µM BA and 10 µM 2,4D implying that 2,4D is the determining factor in callus induction. Increase in BA concentration to 2.5 µM and 2,4D (0.5-10 µM) revealed that the callus weight increased and optimum callus amount was produced at 2.5 µM BA and 2.5 µM 2,4D (20.2±0.70 gm) after which there was a gradual decline (5-10 µM 2,4D). A similar pattern of callus growth was noted in 5-10 µM BA and 2,4D (0.5-10 µM, Fig. 15).

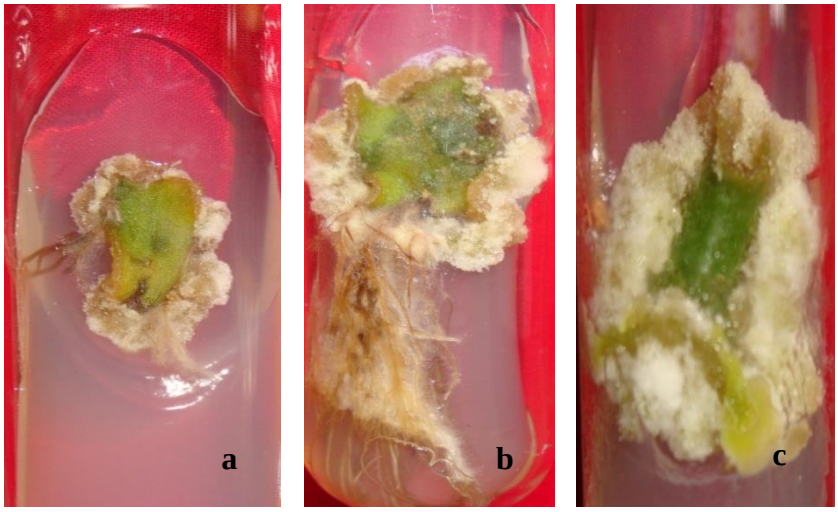


Figure12: Effect of BA and IAA on leaf explants of *P oleracea* after 8 weeks. (a) Differentiation of few roots from callus at 0.5µM BA and 2.5µM IAA. (b) Formation of highest number of roots from callus at 2.5µM BA and 2.5µM IAA, (c) Profuse greenish white callus at 10µM BA and 2.5µM IAA.

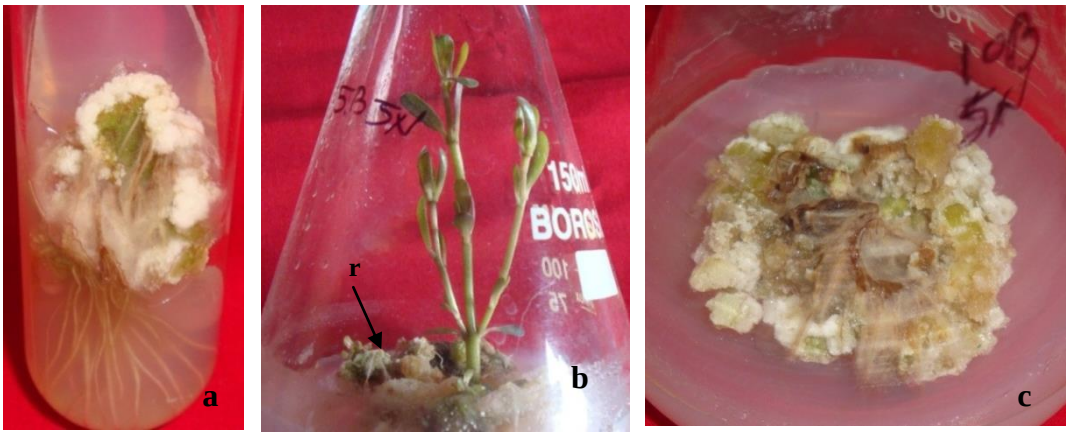
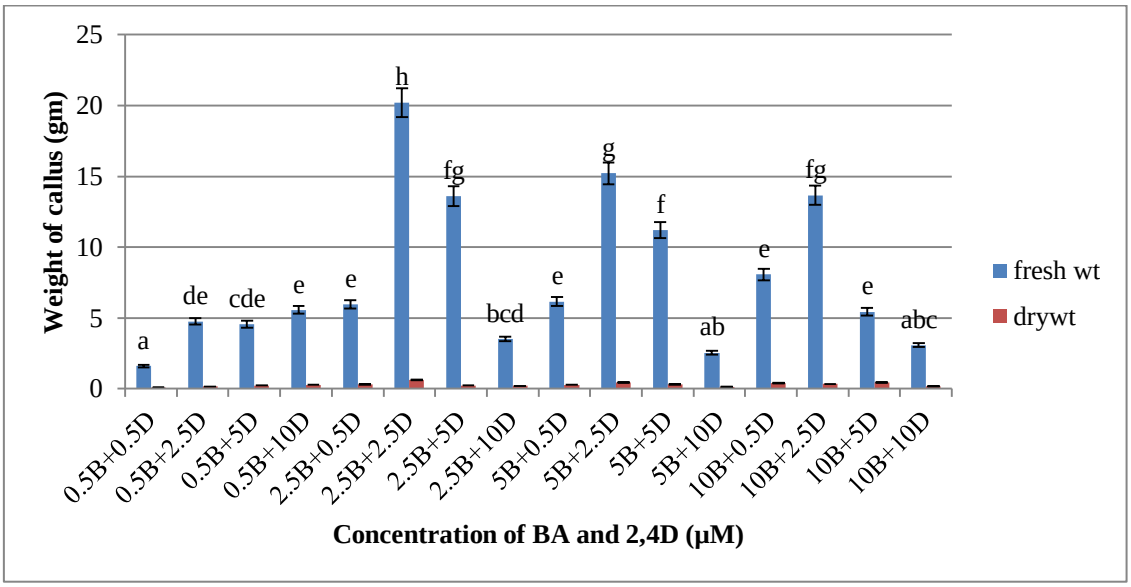


Figure13: Effect of BA and NAA on leaf explant of *P oleracea* after 8 weeks. (a) Moderate callus regenerating roots at 0.5 µM BA and 5µM NAA (b)Roots (r), and in vitro shoot induction at 5 µM BA and 5 µM NAA (c) white green friable callus at 10 µM BA and 5 µM NAA

Figure 15: Effect of BA and 2,4D on biomass of callus (gm) from leaf explant of *P oleracea*



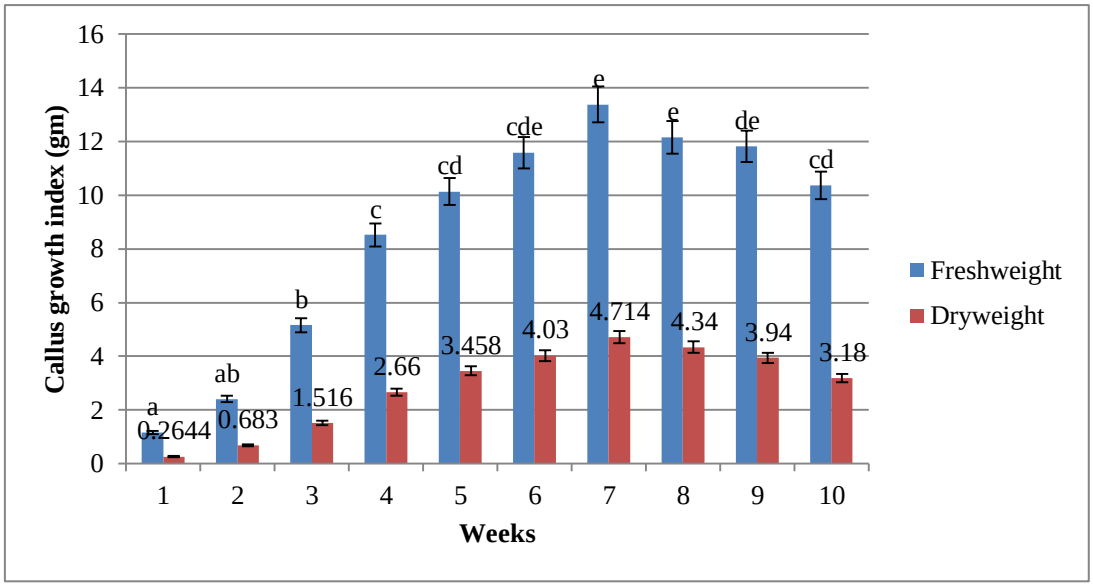
Means followed by the same letters are not statistically significant according to Tukey's HSD test (n=12) and value of $p \leq 0.05$.

Thus concentration of 2.5 µM 2,4D and BA is an optimum concentration for callus induction as when it is coupled with BA (2.5/5/10 µM) it tends to produce maximum callus weight. The dry weight of the callus also depicted a similar pattern of growth (Fig. 11).

(a) Callus Growth Index

Callus growth index in terms of fresh weight and dry weight was determined to ascertain the time period when maximum callus biomass could be achieved. It was observed that there was a steady rise in callus fresh weight until 7thweek (13.38 gm) after which there was a decline in callus fresh weight up to 10 weeks. A similar pattern of growth index was revealed with dry weight of the callus where maximum growth index was 4.714 at 7th week. This implied that 7th week is the time period where maximum callus biomass will be obtained which could be utilized for different biochemical interventions (Fig. 16).

Figure 16: Callus growth index (gm) on an optimized media (2.5µM BA and 2.5 µM 2,4D)



Means followed by the same letters are not statistically significant according to Tukey's HSD test (n=12) and value of p≤ 0.05.

It is concluded from the experiments with BA and auxin IAA, NAA and 2,4D that the activity of IAA and NAA with BA was almost similar as both of these auxins induced root and callus of varying degree. The third auxin 2,4D with BA predominantly induced callus.

Since morphogenic response of all these combinations was similar therefore another set of experiments was devised where BA was replaced with Kin and its response with auxins was evaluated.

(iv) Kin and IAA

BA and IAA induced root and callus with shoot buds in lower concentrations; hence in further experiments the effect of Kin and IAA was assessed.

A very low concentration of 0.5µM Kin with IAA (0.5-10 µM) induced root formation which grew long within 2 weeks (Fig. 17a). At 2.5-5 µM Kin and IAA (0.5-10 µM) moderate yellow callus induction and roots were initiated from the leaf explant (Fig. 17b, Table 8). A high Kin concentration of 10 µM with IAA formed greenish yellow compact callus without root or shoot. (Fig. 17c)

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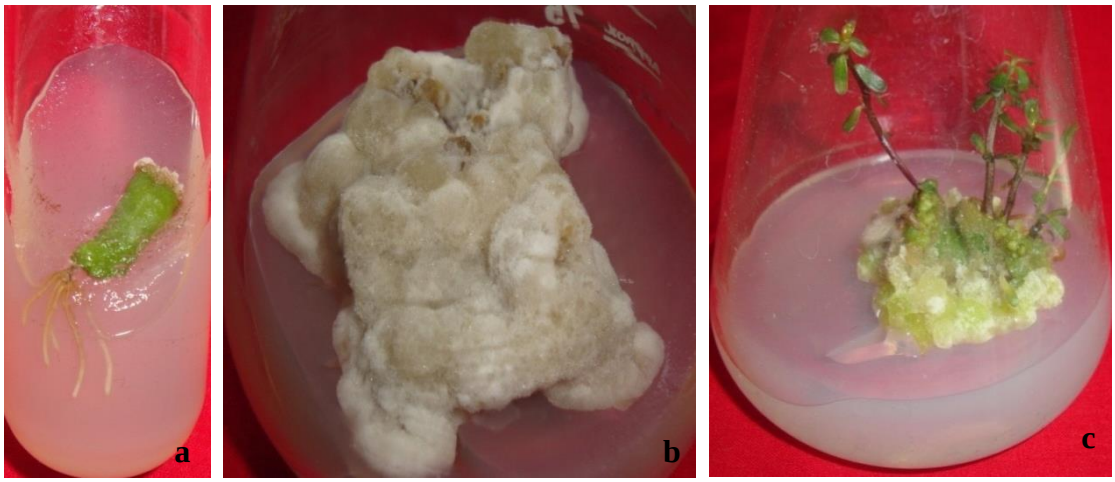


Figure14: Effect of BA and 2,4 D on leaf explant of *P oleracea* after 8 weeks. (a) Induction of few roots in presence of 0.5 µM BA and 0.5 µM 2,4D, (b) Profuse white compact callus formation on 2.5 µM BA and 2.5 µM 2,4D, (c) Induction of invitro shoots from callus on 10µM BA and 0.5 µM 2,4D

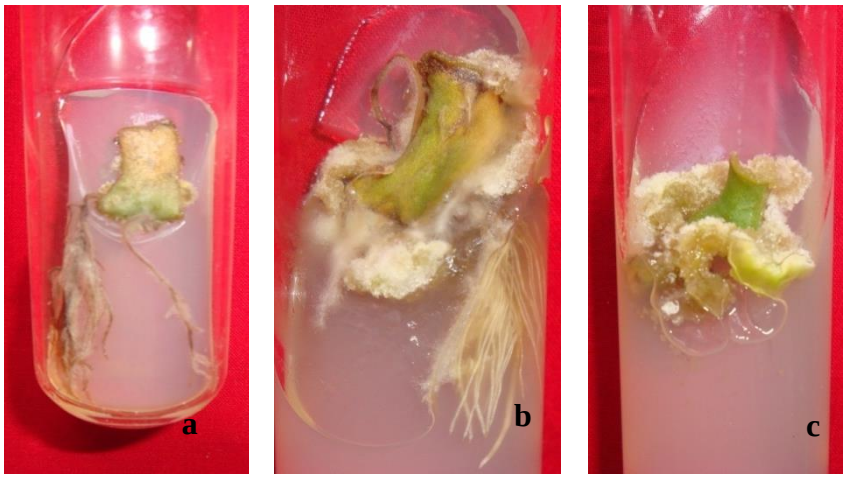


Figure 17: Effect of Kin and IAA on leaf explant of *P oleracea* after 8 weeks. (a) Roots induction on 0.5 µM Kin and 0.5 µM IAA (b) moderate callus and optimum root induction on 5 µM Kin and 2.5 µM IAA (c) Greenish white callus on 10 µM Kin and 2.5 µM IAA

Table 8: Synergistic effect of Kin and IAA on leaf explant *of P oleracea*

PGR's (µM)		Callus	Number of roots/explant (Mean± S.E.)*	% Explants forming roots
Kin	IAA			
0.5	0.5	-	6.08 ± 1.05^{cd}	85.71
0.5	2.5	-	5.58 ± 0.98^{bcd}	88.88
0.5	5	-	5.16 ± 0.82^{bcd}	87.50
0.5	10	+	4.50 ± 0.92^{bc}	100
2.5	0.5	++	3.33 ± 0.68^{ab}	66.66
2.5	2.5	+	3.50 ± 0.63^{ab}	83.33
2.5	5	++	4.50 ± 0.94^{bc}	100
2.5	10	++	3.83 ± 0.44^{ab}	100
5	0.5	++	2.50 ± 0.55^{a}	87.50
5	2.5	++	7.50 ± 1.37^{e}	100
5	5	++	2.91 ± 0.78^{a}	28.75
5	10	++	2.16 ± 0.68^{a}	42.85
10	0.5	+	-	-
10	2.5	+	-	-
10	5	+	-	-
10	10	+	-	-

*Means followed by the same letters are statistically not significant according to Tukey's HSD test where n=12 and $p \leq 0.05$, S.E.= Standard Error, + = poor callus, ++ = moderate callus, - = no response.

The results obtained with these experiments showed that combining Kin and IAA induced low to moderate callus which differentiated lesser number of roots while BA and IAA induced profuse callus and high number of roots.

BA and Kin with IAA had a similar response as both induced callus and roots therefore the auxin IAA was replaced with NAA to assess its regenerative potentialwith the cytokinin.

(v) Kin and NAA

The determination of synergistic influence of Kin and NAA was done to evaluate the difference in expression of the two cytokinin BA or Kin with NAA. The response of Kin at 0.5 µM and NAA at 0.5-2.5 µM was insignificant as there was negligible callus was formed in few cultures while at 5-10 µM NAA 1-2 roots with white callus initiated in the cultures. A similar trend of root and callus formation was noted in 2.5 µM Kin and increasing concentrations of NAA (Table 9). Optimum root and callus induction was observed at 5-10 µM NAA (Fig. 18a) while the pattern of growth remained the same for rest of the synergistic concentrations. Increase in concentration of Kin to 10 µM with NAA at 0.5 µM induced shoot buds and in vitro shoots (Fig. 18b) while the rest of the NAA concentrations (2.5-10 µM) formed 1-2 roots and white yellow friable callus. (Table 9, Fig. 18c).

Table 9: Synergistic effect of Kin and NAA on leaf explants of *P oleracea*

PGR's (µM)		Callus	Number of roots/explant (Mean ±S.E.)*	% Explants forming roots	Number of shoots/explant (Mean ±S.E.)
Kin	NAA				
0.5	0.5	-	-	-	-
0.5	2.5	+	-	77.77	-
0.5	5	++	0.66±0.24[a]	90	-
0.5	10	++	2.08± 0.16[ab]	100	-
2.5	0.5	-	-	-	-
2.5	2.5	+	1.00±0.36[ab]	91.66	-
2.5	5	++	1.16±0.27[ab]	100	-
2.5	10	++	0.66±0.33[a]	83.33	-
5	0.5	+	-	-	-
5	2.5	+	3.41±0.89[b]	100	-
5	5	++	3.83±0.83[b]	100	-
5	10	++	3.16±0.75[ab]	100	-
10	0.5	--	-	-	1.31±0.34
10	2.5	+	0.58±0.28[ab]	100	-
10	5	++	1.33±0.41[ab]	100	-
10	10	++	1.25±0.37[ab]	100	-

*Means followed by the same letters are statistically not significant according to Tukey's HSD test where n=12 and $p \leq 0.05$, S.E= Standard Error + = Poor callus, ++ = moderate callus, +++= Good callus, ++++ = Profuse callus.

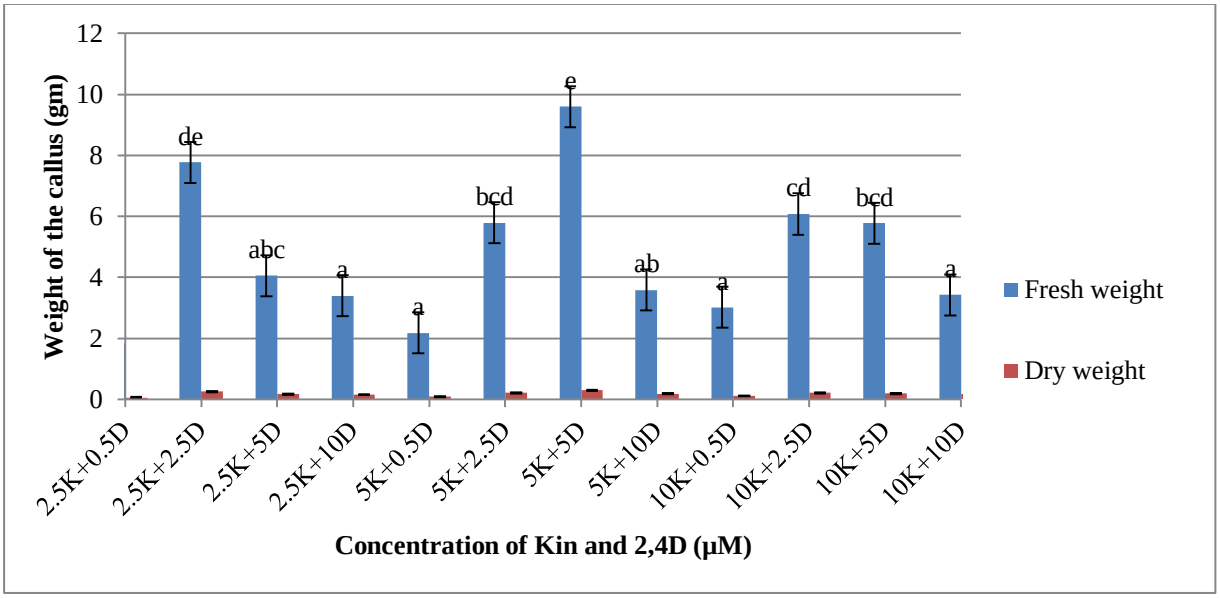
Thus the combinations of Kin and NAA revealed that their synergism failed to induce significant response in the cultures as less number of roots and moderate callus was obtained.

(vi) Kin and 2,4D

The response of Kin with IAA and NAA was similar to BA and IAA/NAA as all these combinations induced callus and roots of varying degree therefore 2,4D was tried with Kin to assess a change in response. The leaf explants were unable to induce a morphogenic response at low level of Kin (0.5 µM) and 2,4D (0.5-10 µM) even after 8 weeks. When the level of Kin increased to 2.5 µM with 0.5 µM 2,4D a responses in terms of callus, roots and in vitro shoot was obtained while further increase in 2,4D (2.5-10 µM) induced white to yellow friable callus. Kin at 5 µM coupled with 0.5 µM 2,4D induced root, callus and shoot which had short internodes and large leaves (Fig. 19a) while at higher concentrations (2.5-10 µM) white friable callus was induced. With high concentration of 10 µM Kin and 2,4D a change in morphogenic response was noted as callus was formed with 0.5 µM 2,4D and the colour of the callus changed from white to brown at higher levels (Fig. 19b).

The fresh weight and dry weight of the callus was optimised to determine whether the amount of callus produced was better than the combinations of BA and 2,4D. It was revealed in the experiment that 5 µM Kin and 5µM 2,4D produced optimum fresh and dry weight of the callus which was less than the optimum callus weight generated at 2.5µM BA and 2.5µM 2,4D (Fig. 20).

Figure 20: Effect of Kin and 2,4D on biomass of callus (gm) from leaf explant of *P oleracea*



Means followed by the same letters are not statistically significant according to Tukey's HSD test (n=12) and value of $p \leq 0.05$

Recapitulating the results obtained from the synergistic interaction of cytokinin (BA/Kin) and auxin (IAA, NAAand 2,4D) it was revealed that high level of cytokinin (5-10 µM) and low level of auxin (0.5 µM) tend to form callus and in vitro shoots. In general BA/Kin and IAA/NAA formed callus and roots while BA/Kin and 2,4D formed compact callus. It was thus concluded that for the regeneration of in vitro shoots synergistic combination of cytokinin was favourable where optimum number of shoots with shoot clusters was obtained at 5 µM BA and 10µM Kin. These clusters of shoots were elongated in further set of experiments for a highly efficient regeneration protocol. It was also concluded that combination of 2.5µM BA and 2.5µM 2,4D was an optimum concentration for callus induction.

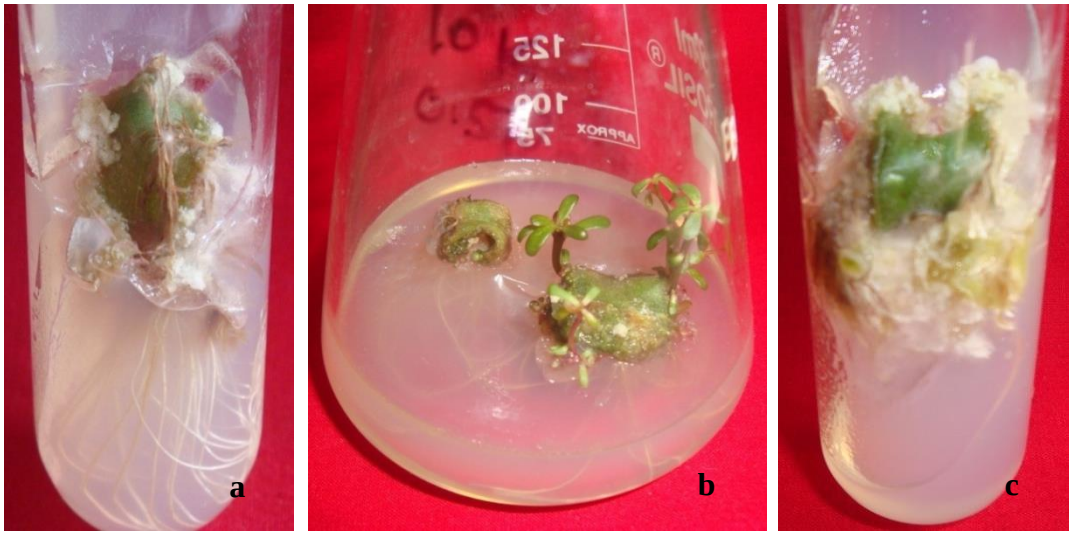


Figure18: Effect of Kin and NAA on leaf explant of *P oleracea* after 8weeks: (a) Rooting at 5 µM Kin and 10 µM NAA (b) Roots, shoot buds and in vitro shoot formation at 10 µM Kin and 0.5 µM NAA(c) Moderate callus formation with 1-2 roots at 10 µM Kin and 10 µM NAA

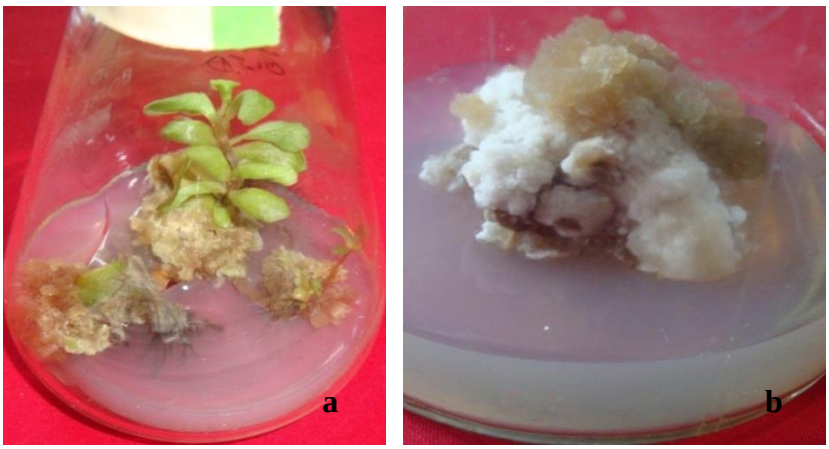


Figure 19: Effect of (Kin) and 2,4 D on leaf explant of *P oleracea* after 8 weeks. (a) Root, callus and in vitro shoot formation at 5µM Kin and 0.5 µM2,4D, (b)White to brownish callus induction at 10 µM Kin and 2.5 µM2,4D

4.1.1.5 Multiplication and elongation of in vitro shoots

During the regeneration of in vitro shoot on BA and Kin it was observed that there were incipient shoots within the cluster which failed to elongate. Therefore for elongation of these shoots they were transferred to the following medium-

(i) Basal medium

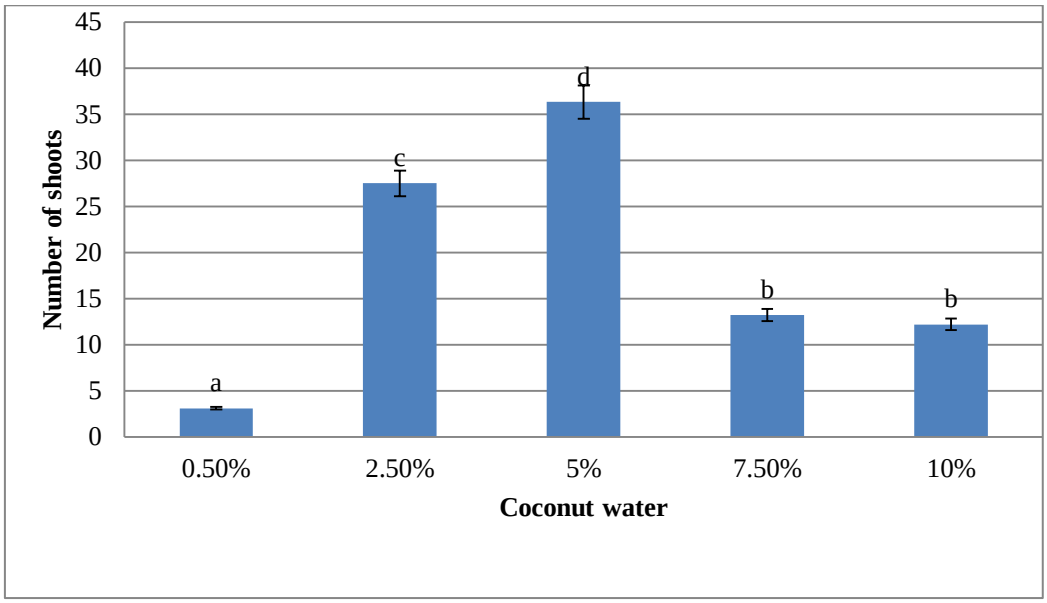
The in vitro shoots obtained on an optimized media of 5 µMBA and 10 µMKin were transferred to basal media for elongation. The shoot clusters were unable to multiply or elongate in the basal medium (Fig. 21a)

(ii) Coconut water

The six weeks old cluster of shoots obtained on the optimized media was transferred to basal media supplemented with 3% sucrose and coconut water. A concentration of 0.5% coconut water was able to elongate only 2-5 shoots in the cluster which had long internodes with 3-4 leaves. With increase in concentration of coconut water to 2.5-5% a rapid rate of multiplication and simultaneous elongation of shoots was achieved (Fig. 21b). Optimum number of shoots was obtained at 5% coconut water (36.2±3.70) after two weeks (Fig. 21c, Fig. 19). With increase in concentration to 7.5% the number of shoots declined (13.2 shoots per explant) along with the formation of green compact callus. At 10% coconut water again the number of shoots decreased and white friable callus formation was observed (Fig. 21d, Fig. 22).

Figure21: Effect of coconut water on in vitro shoot elongation of *P oleracea*(a)In vitro shoots transferred to basal media (b) Cluster of in vitro shoots obtained on 2.5% coconut water and 3% sucrose,(c) Optimum in vitro shoot elongation on 5% coconut water and 3% sucrose (d)Less shoot elongation and white callus formation at 10% coconut water and 3% sucrose.

Figure 22: Effect of coconut water on shoot number in *P oleracea*



Means followed by the same letters are not significantly different according to Tukey'sHSD test where p≤ 0.01 and n=10

Thus coconut water was beneficial in multiplication and elongation of the in vitro shoots obtained on an optimized media.

Thus regeneration from in vivo leaf explants was obtained in presence of cytokinin and shoots elongated in presence of coconut water.

4.1.1.6 Histological study of differentiating leaf explants in *P oleracea*

The regeneration of in vitro shoots from leaf explants depicted direct organogenesis which could be confirmed by studying the histological pattern of mode of development. The leaf explants were inoculated on a medium fortified with sucrose 3%, 5 µM BA and 10 µM Kin and the developmental pattern was studied weekly. On day 0 the transverse section of the leaf which served as control showed that it consist of typical kranz anatomy. The outer most layer is uniserate epidermis which is interrupted at places by sunken stomata devoid of any hairs. The vascular bundles are arranged in a wavy pattern having a bundle sheath around it and the mesophyll cells were columnar and compactly arranged. Below the vascular bundle parenchymatous cells and large intercellular spaces are present (Fig. 23a).After one week thickness of the leaf explants increased and the mesophyll cells became meristematic rapidly and started dividing (Fig. 23b) and formed some localized regions of mitotic centres which developed on the margins of the explants. The cells were isodiametric arranged in regular files with dense cytoplasm and condensed nuclei in the centre called as meristemoids (Fig. 23c). The meristemoids differentiated into shoot buds (Fig. 23d) by the end of 4^{th}-5^{th} weeks

which grew into in vitro shoots. The shoot bud formation in this study did not involve callus formation confirming direct organogenesis in the plant.

The leaves for the in vitro shoots obtained by direct organogenesis were further tested for their regenerative capacity.

4.1.1.7 Regeneration from in vitro leaf explant

The protocol which was developed for in vivo leaf explant was applied to the in vitro leaf explant and a difference in mode of regeneration pattern was observed. Direct regeneration was obtained from in vivo leaf explant while in vitro leaf explants changed to indirect mode of regeneration. It was noticed that the in vitro leaf explant swelled to a considerable degree in first two weeks of inoculation followed by development of compact green nodular callus and in vitro shoot buds differentiated within 3 weeks (Fig. 24a). By 5^{th} week induction of green nodular callus and differentiation of in vitro shoots was observed from the callus. The shoots originated from the abaxial proximal end of the leaf explant and the number reached to 5.72±0.71 by the end of 8 weeks (Fig. 24b). The leaf explant with shoot buds, callus and in vitro shoots were then transferred to 5% coconut water during 9^{th} week and was repeatedly sub cultured. The in vitro shoots multiplied and elongated in presence 5% coconut water and the number of shoots/explants increased to 19.18±0.86 after 12 weeks which further enhanced to 26.18±2.20 after 16 weeks (Fig. 24c). This suggested that the number of shoots enhanced with each subculture (Fig. 25). The regeneration from in vitro leaf explants confirmed its regenerative capacity and the information were utilized in mutation experiments.

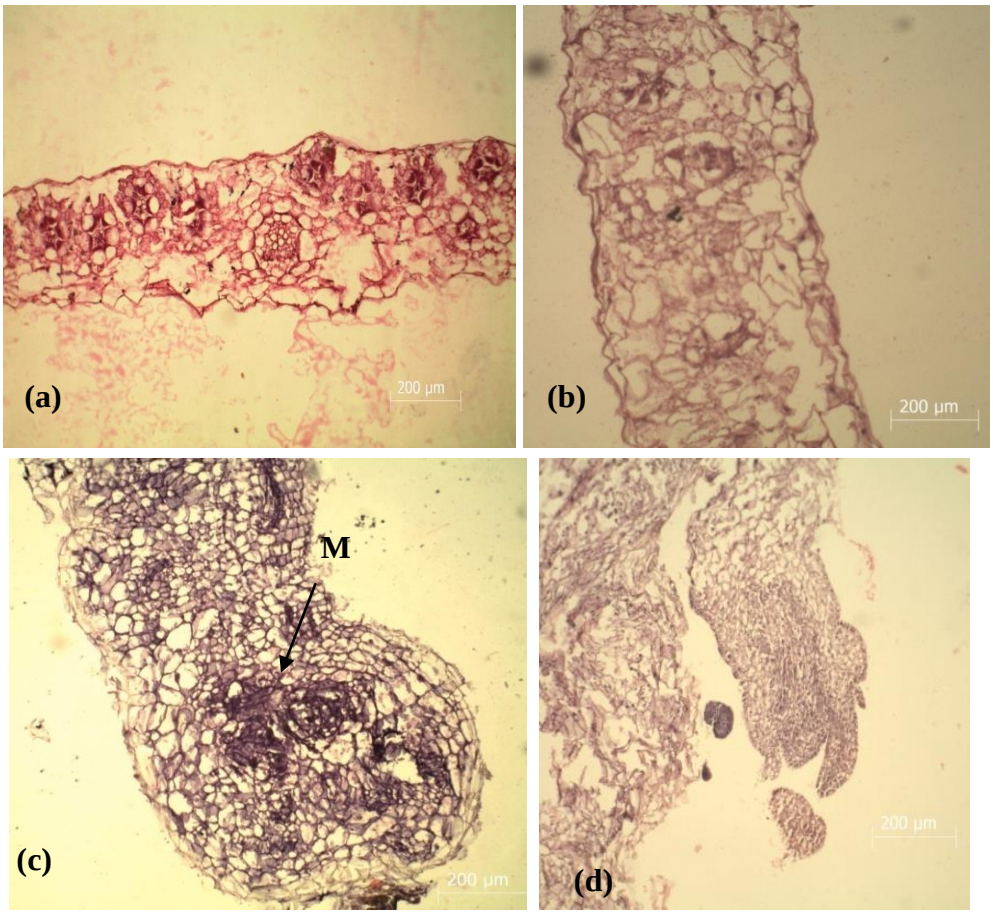
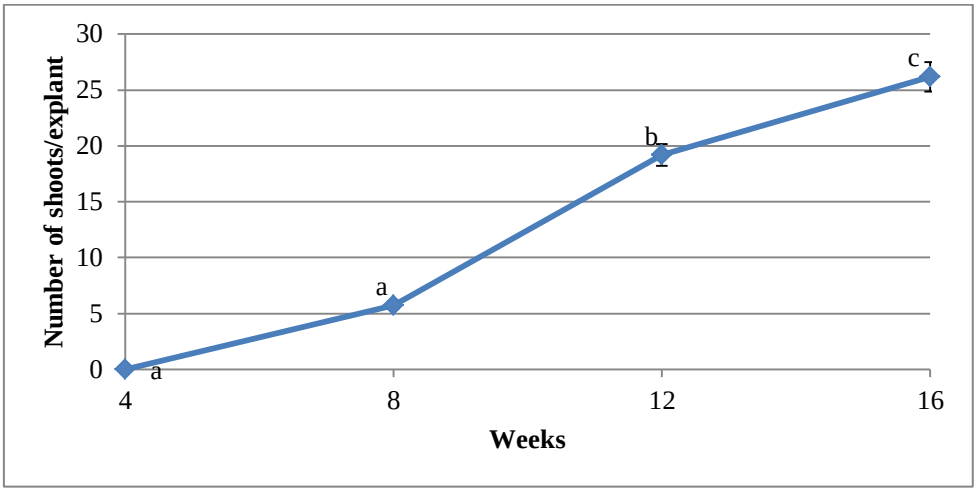


Figure23:Histological changes during in vitro regeneration of *P oleracea* from leaf explant (a) Transverse section of leaf (Control) on day 0 (b) Dividing mesophyll cells during 1st week (c) Formation of meristemoids (M) at the end of 3rd week (d) Shoot bud formation during 4th week

Figure 25: Number of shoots from in vitro leaf explant of *P oleracea* after each subculture



Means followed by the same letters are statistically not significant at Tukey's HSD test, $p \leq 0.05$ and n=10

4.1.1.8 Rooting of micro shoots

The micro shoots were placed in liquid MS medium (Half and Full strength) supplemented with 1% sucrose and different concentrations of IBA (1-4 µM).

(i) Half strength MS medium

An experiment was devised for rooting of in vitro shoots in half strength medium as it was reported to be effective in a number of plants. A control devoid of PGR's was set which gave slow response as induction of 1.6±0.56 roots were formed after 8 weeks (Fig. 26a(i)). The shoots formed roots in presence of 1-2 µM IBA during 2th week and further formed lateral roots in 4thweek. The number of roots and percent response increased up to 3µM (Table 10) and at this concentration a fast initiation of rooting in all the micro shoots was observed by the end of 4 weeks. The roots were long and stout and emerged from the cut ends of the micro shoot as well as from the nodes exposed to the rooting medium. Optimum number of roots (8.2±0.64) was observed in 100% cultures at this concentration after 8 weeks (Fig. 26a(ii), Table 10). A similar pattern of growth was observed at 4 µM with a decline in root number (Table 10) while at 5 µM short root were formed during 4th week which failed to survive further.

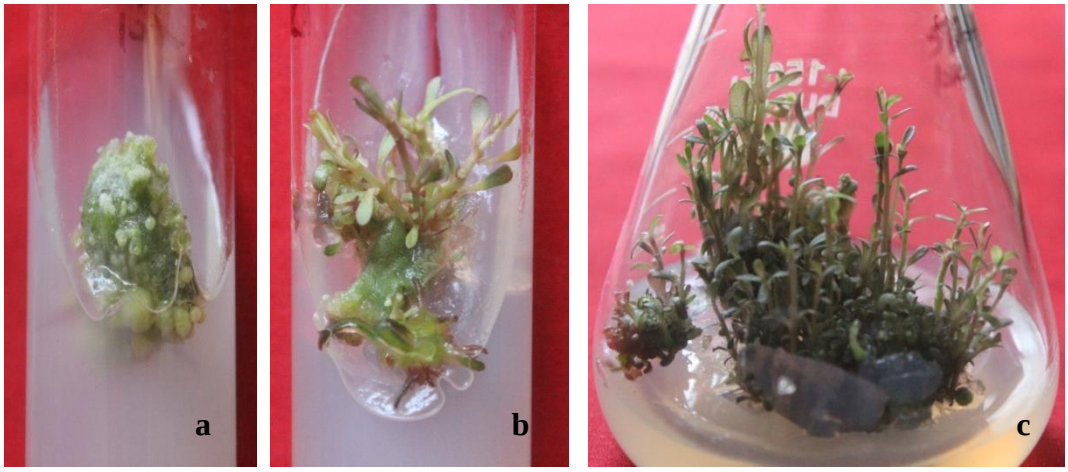


Figure 24: Regeneration of *P oleracea* from in vitro leaf explant on optimized medium of 5 µM BA and 10 µM Kin (a) Initiation of shoot buds from compact green callus after 3 weeks (b) Proliferation of in vitro shoots after 8 weeks (c) Cluster of in vitro shoot after being transferred to 5% coconut water after 16 weeks

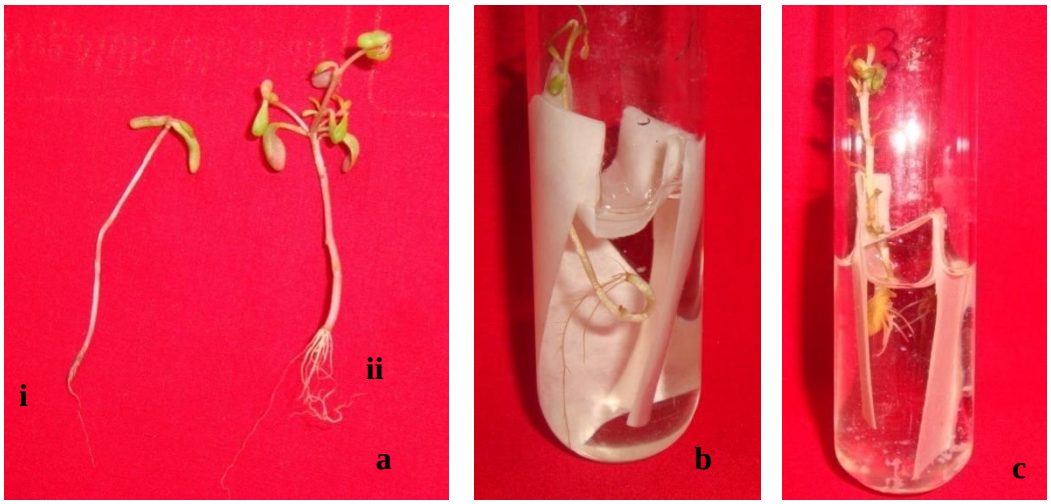


Figure26: Effect of media strength and IBA on rooting potential of *P oleracea* (a (i)) Rooting at half strength basal media (Control),(a(ii)) Rooting at half strength media supplemented with 3 µM IBA, (b) Rooting at full strength basal medium (Control), (c) Rooting at full strength medium supplemented with 3 µM IBA.

(ii) Full strength MS medium

Half strength medium was able to induce roots in presence of IBA. Full strength medium was also utilized to evaluate its role in formation of roots. A control without PGR's was maintained which depicted slow initiation of rooting in 5th week with 70% response (Fig. 26b). It was noted that 1 and 2 µM IBA was able to induce only 50% rooting response after 4th weeks (Table 10). With increase in IBA concentration to 3 µM there was a positive shift in root development and optimum number of roots (4.2±0.75) were initiated (Fig. 26c, Table 10). The number of root decreased at 4 µM with 80% response and the roots formed failed to elongate.

Table 10: Effect of IBA and MS medium strength on number of roots of *P oleracea*

IBA(µM)	% Shoots forming roots	Number of roots/shoot (Mean ±S.E.)*
Half Strength		
0	80	1.6 ± 0.56^{ab}
1	70	3.9 ± 0.79^{ab}
2	90	4.4 ± 0.65^{bc}
3	100	8.2 ± 0.64^{d}
4	100	6.8 ± 0.57^{cd}
Full Strength		
0	70	2 ± 0.42^{ab}
1	50	1.5 ± 0.45^{a}
2	50	1.5 ± 0.63^{a}
3	90	4.2 ± 0.75^{abc}
4	80	3.2 ± 0.64^{ab}

*Means followed by the same letters are statistically not significant with Tukey'sHSD test (n=10) and the value of $p\leq0.05$.

Thus in the present experiment it was revealed that half strength media is better suited to rooting of shoots as compared to full strength media. It was also noted that 3 µM IBA is a suitable concentration as optimum number of root/shoot are formed both in half and full strength media.

Thus the regeneration protocol of P oleracea from leaf explant was obtained at a synergistic combination of 5 µM BA and 10 µM Kin. Multiplication and elongation of the micro shoot was effective at 5% coconut water. Half strength MS medium fortified with 3 µM IBA was optimum for rooting. Anatomical studies of cultured leaf explants confirmed direct organogenesis. Optimum callus was achieved at 2.5 µM BA and 2.5 µM 2,4D.

4.1.2 *P grandiflora*

In the present studies another species of genus *Portulaca* i.e. *P grandiflora* was evaluated for regeneration from leaf explant. The different PGR's employed for the study were individual cytokinin (BA/Kin), auxin (IAA/NAA/2,4D) and a synergistic combination of both. A control devoid of PGR's was set for the experiment which depicted root formation by the end of 8 weeks (Fig. 27a)

4.1.2.1 Effect of cytokinin

The effect of individual cytokinin BA/Kin on leaf explant was evaluated for its potential for regeneration. It was observed that all concentration of BA (0.5-20 µM) failed to evoke morphogenic response and the explants turned brown after 8 weeks. Replacing BA with Kin also failed to evoke any response as only white friable callus was induced (5-10 µM) after six weeks (Table 11).

4.1.2.2Effect of individual auxins

Individual concentration of all the three auxins (IAA/NAA/2,4D) failed to evoke appreciable morphogenetic response however only 0.5 µM IAA induced roots (Table 11, Fig. 27b).Thus it was concluded that individual concentrations of both cytokinin and auxins were unable to induce a morphogenic response and therefore synergistic combinations with two cytokinins and cytokinin and auxin were tried for regeneration of in vitro shoots.

4.1.2.3 Synergistic effect of cytokinins

(i) BA and Kin

Individually cytokinins were inadequate to induce an appreciable morphogenic response hence a synergistic concentration of the two cytokinins (BA/Kin) were employed to evaluate their effect on regeneration from leaf explant. A very low concentration of BA (0.5 µM) and Kin (0.5 µM) induced 1.82±0.23 roots per explant in 3rd week with 75% response. Further increase in BA to 2.5µM and Kin (2.5-10 µM) ceased rhizogenesis completely and differentiated shoot bud at 5-10 µM BA and 0.5-10 µM Kin (Table 12). The shoot buds arose from the abaxial surface, on the margins of the explant during 3rd week which were initially light green and shiny and became bright red and large by the end of 8 weeks with no further development (Fig.28a,b). Thus the synergistic combination of BA and Kin revealed that

moderate concentration of BA and Kin was able to induce shoot buds, which did not form in vitro shoots even after 8 weeks.

Table 11: Effect of individual cytokinins and auxins on leaf explant of *P grandiflora*

PGR's (µM)					Number of roots/explants (Mean±S.E.)	% Explants forming root	Callus	% Explants forming callus
BA	Kin	IAA	NAA	2,4D				
-	-	0.5	-	-	2.66	66.66	-	-
-	-	2.5-10	-	-	-	-	-	-
-	-	-	0.5-10	-	-	-	-	-
-	-	-	-	0.5-10	-	-	-	-
0.5-20	-	-	-	-	-	-	-	-
-	0.5-2.5	-	-	-	-	-	-	-
-	5	-	-	-	-	-	+	33.33
-	10	-	-	-	-	-	+	16.66
-	15-20	-	-	-	-	-	-	-

- = No Response, S.E. = Standard error

Table 12: Effect of synergistic concentrations of cytokinin (BA and Kin) in leaf explant of *P grandiflora*

Cytokinin (µM)		Number of roots/explant	% Explants forming roots	% Explants forming shoot buds
BA	Kin			
0.5	0.5	1.82±0.23	75	-
0.5	2.5-10	-	-	-
2.5	0.5- 10	-	-	-
5	0.5-10	-	-	50
10	0.5	-	-	50
10	2.5-10	-	-	20

- = No Response, + = poor callus

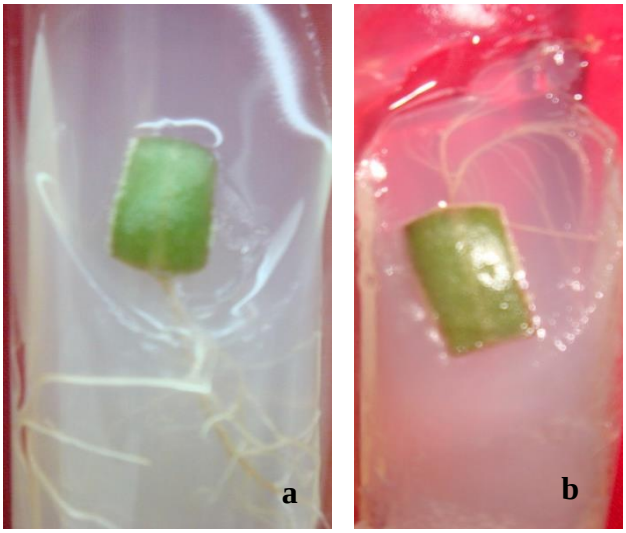


Figure27: Effect of individual auxin on leaf explant of *P grandiflora* after 8 weeks: (a) Control showing root induction (b) Root induction at 0.5 µM IAA

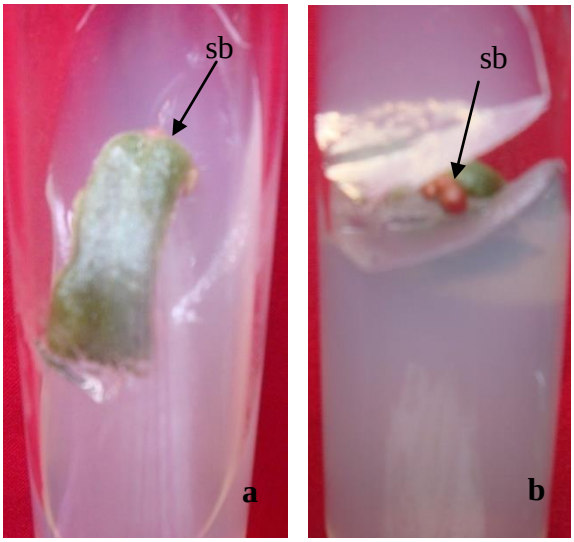


Figure 28: Synergistic effect of BA and Kin on leaf explant of *P grandiflora* after 8 weeks (a)Shoot bud (sb) on the edge of abaxial surface of leaf explant at 5 µM BA and 0.5 µM Kin (b) Red shoot bud at 5 µM BA and 2.5 µM Kin

4.1.2.4 Synergistic effect of cytokinins and auxin

Synergism between the two cytokinins evoked 2-3 shoot bud in different combinations with low percent response. Auxins have been reported to be stimulatory in shoot bud induction with cytokinin therefore synergistic interaction between cytokinins and auxins was studied to determine their role for in vitro regeneration.

(i) BA and IAA

It was observed that synergistic interaction of BA and IAA initiated an indirect mode of regeneration as callus differentiated shoot buds. A very low concentration of BA (0.5 µM) with IAA (2.5-10 µM) formed poor to moderate compact whitish green callus cultures during 4^{th} week. An increase in concentration of BA to 2.5-5 µM with 2.5-10µM IAA induced vitrified green shoot from the callus during 6^{th} week with 40-100% response (Table 13,Fig. 29a).

A high concentration of BA (10 µM) with IAA depicted a faster response and with 0.5-2.5 µM IAA induced callus and shoot buds in the 3^{rd} week with 50 and 40% response respectively (Table 13). The shoot buds differentiated on the abaxial surface along with white callus. Combination of 10µM BA and 5 µM IAA enhanced the response as along with callus, shoot buds were differentiated which emerged into shoots after 4 weeks (Fig. 29b). Another significant observation was that the shoot buds were light green and vitrified at the time of initiation which later became dark pink within 4-5 weeks. In 30% of the explants shoot emerged during 6^{th} week (Table 13, Fig. 29c). A high concentration of BA (15-20 µM) and IAA (15-20 µM) formed green white callus with decreased percent response (60%).

Therefore it was concluded that synergistic combination of BA and IAA had the potential to form in vitro shoots as shoot buds were induced in all the combinations and 10 µM BA and 5 µM IAA formed shoots.

(a) Effect of age of the explant for regeneration

At 10 µM BA and 5 µM IAA 30% of the explants formed in vitro shoots. The leaf explant used for the study was mature and hence regeneration was tried utilizing young leaves which were placed on the same optimized medium. A much faster response was depicted from young leaf as compared to mature leaf, as clusters of shoot buds initiated in the 2^{nd} week without callus induction (Fig. 30a) which formed in vitro shoots (2-3) after 6 weeks but the formation of

Figure 29: Effect of BA and IAA on leaf explants of *P grandiflora* after 8 weeks. (a) Shoot bud (SB) induction at 2.5 µM BA and 5 µM IAA after 3 weeks (b) In vitro shoot initiation after 4 weeks at 5 µM BA and 10 µM IAA (c) Shoot buds and incipient shoots after 6 weeks at 5 µM BA and 10 µM IAA

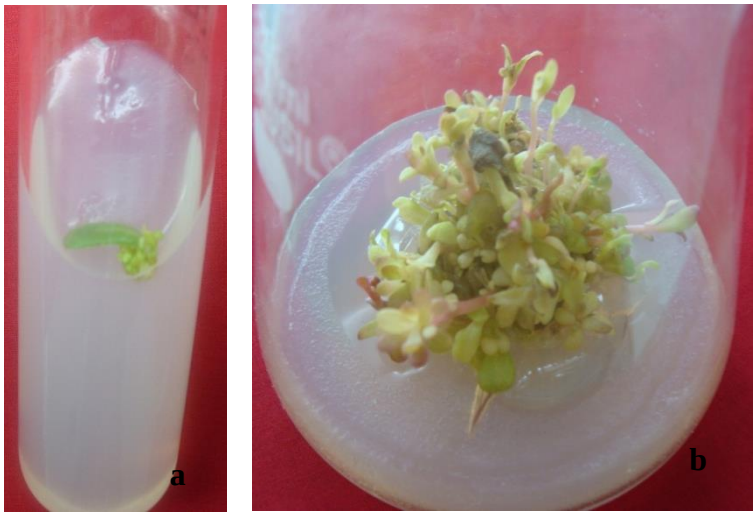


Figure 30: In vitro shoot regeneration from young leaf explant in *P grandiflora* (a) Induction of shoot buds after 2 weeks in 10 µM BA and 5 µM IAA. (b) Shoot formation at 20 µM BA after 10 weeks.

appreciable number of in vitro shoots was still not achieved. Therefore the next series of experiments were aimed to improve on the regeneration protocol by the intervention of other PGRs.

The 4 week cultured young leaf explant growing on 10 µM BA and 5 µM IAA was transferred to to individual higher concentrations of BA (15-30 µM). It was observed that at

15 µM 1-2 in vitro shoots were formed while at 20 µM 6.25± 0.85 shoots per explant and cluster of incipient shoots were initiated after 2 weeks (Fig. 30b). Further increase in BA concentration failed to develop shoots and the explant turned brown after 3 weeks. Higher concentrations of Kin (10-20 µM) was also ineffective as the shoot bud cluster failed to depict increase in number of shoots and the explants gradually became brown.

(ii) BA and NAA

It was noted that BA with IAA induced predominantly callus and shoot buds with few shoots in one combination. Therefore to increase the morphogenic response IAA was replaced by NAA to evaluate its role for formation of shoots. A low concentration of BA (0.5 µM) and NAA (0.5-10 µM) failed to induce any morphogenic response and the explant turned brown after 6 weeks. Concentration of 2.5-5 µM BA and 0.5-10 µM NAA formed reddish green callus with shoot buds (Fig. 31a, b, Table 13). Similarly a high concentration of BA at 10 µM and NAA (0.5-10 µM) induced green compact profuse - moderate callus with 100 percent response (Fig. 31c).

(iii) BA and 2,4D

Along with IAA and NAA, experiments were also set with another auxin 2,4D in synergism with cytokinins. 2,4D mostly induces callus formation within the species and a similar effect was observed when 2,4D was coupled with BA. A very low concentration of 0.5 µM BA coupled with increasing concentrations of 2,4D (0.5-10 µM) failed to induce a morphogenic response within the species. At 2.5 µM BA and 2.5 µM 2,4D there was a poor callus induction with 50% response which increased to 100% at higher concentrations (5-10 µM) of 2,4D (Fig. 32a). A similar response of moderate-profuse callus induction was noted at 5-10 µM BA and 0.5-10 µM 2,4D (Table 13, Fig. 32b, c).

From the experiments with BA and IAA/NAA it was concluded that predominantly both formed callus and few in vitro shoots while with 2,4D there was only callus induction.

BA with IAA and NAA induced callus and shoot buds which failed to form in vitro shoots except at 10 µM BA and 5µM IAA while 2,4D with BA induced callus. It was thus evident that auxins with BA were insufficient to induce shoots therefore BA was replaced with Kin and its effect with different auxins was evaluated.

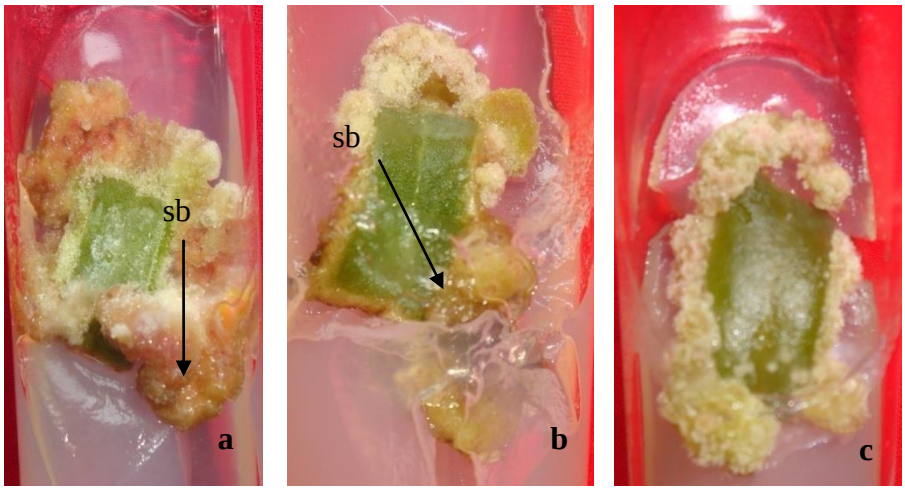


Figure 31: Effect of BA and NAA on leaf explants of *P grandiflora* after 8 weeks.: (a) Profuse reddish green callus and shoot bud (sb) formation at 2.5 µM BA and 2.5 µM NAA (b) whitish green compact callus at 5 µM BA and 5 µM NAA(c) Profuse whitish green compact callus at 10 µM BA and 5 µM NAA

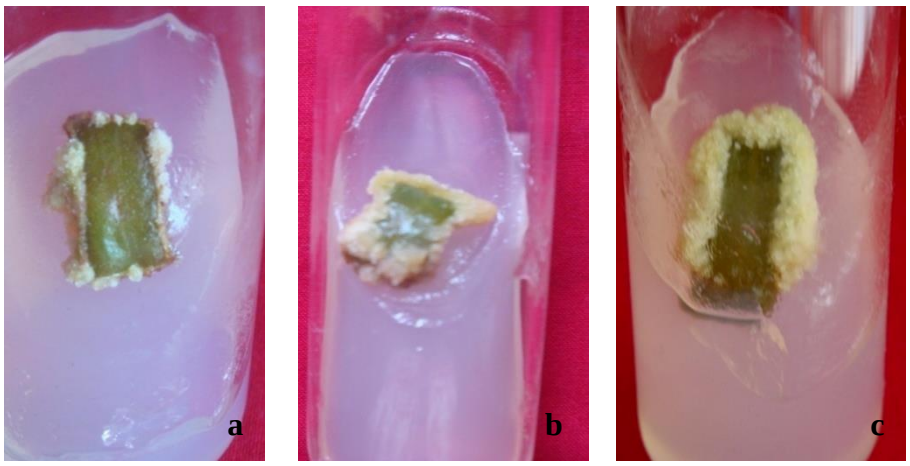


Figure 32: Effect of BA and 2,4D on leaf explant of *P grandiflora* after 8 weeks: (a) Poor callus induction at 2.5 µM BA and 2.5 µM 2,4 D (b) moderate green callus at 5 µM BA and 5 µM 2,4D (c) moderate green compact callus at 10 µM BA and 5 µM 2,4D

Table 13: Effect of synergistic concentrations of BA and auxins on leaf explant of *P grandiflora*

PGR's (µM)				Callus	% Explants forming callus	% Explants forming shoot buds
BA	IAA	NAA	2,4D			
0.5	0.5	-	-	-	-	-
0.5	2.5-10	-	-	+	100	-
2.5	0.5	-	-	-	-	-
2.5	2.5	-	-	+	100	40
2.5	5	-	-	++	100	50
2.5	10	-	-	++	100	70
5	0.5	-	-	+	30	30
5	2.5	-	-	+	100	60
5	5	-	-	+	100	100
5	10	-	-	+	100	100
10	0.5	-	-	+	50	40
10	2.5	-	-	+	100	50
10	5	-	-	++	100	100
10	10	-	-	++	100	100
0.5	-	0.5-10	-	-	-	-
2.5	-	0.5	-	+	100	-
2.5	-	2.5	-	++	100	72.72
2.5	-	5	-	++	100	66.66
2.5	-	10	-	++	100	50
5	-	0.5	-	+	41.66	-
5	-	2.5	-	+	83.33	100
5	-	5	-	++	100	33.33
5	-	10	-	+++	72.72	-
10	-	0.5	-	+	90.90	72.72
10	-	2.5	-	+	100	50
10	-	5	-	++	100	50
10	-	10	-	++	100	-
0.5	-	-	0.5-10	-	-	-
2.5	-	-	0.5	-	-	-
2.5	-	-	2.5	+	50	-
2.5	-	-	5	+	100	-
2.5	-	-	10	++	100	-
5	-	-	0.5	-	-	-
5	-	-	2.5	+	60	-
5	-	-	5	++	100	-
5	-	-	10	++	100	-
10	-	-	0.5	-	-	-
10	-	-	2.5	+	100	-
10	-	-	5	++	100	-
10	-	-	10	+	70	-

- = No Response, + = little callus, ++ = moderate callus, +++ = profuse callus.

(iv) Kin and IAA

A low concentration of Kin at 0.5µM and IAA (0.5-10µM) failed to evoke morphogenic response after 8 weeks. A delayed response which resulted in formation of poor white friable callus from abaxial surface in 6th week when the concentration of Kin was increased to 2.5µM with IAA (2.5-10 µM).Kin at5- 10 µM with 0.5- 10 µM IAA again formed callus and shoot buds which failed to elongate into shoots (Table 14, Fig. 33a, b).

(v) Kin and NAA

When IAA was replaced with NAA and combined with Kin it was capable of inducing shoots. A low concentration of Kin (0.5µM) and increasing concentration of NAA (0.5-10µM) failed to initiate a response. When the concentration of Kin increased to 2.5µM and coupled with NAA (0.5-10 µM) a moderate reddish green callus was obtained which was compact with varying percent response. There was induction of callus in 5-10 µM Kin and NAA (0.5-10 µM) with different colour and texture. With 5µM Kin and NAA moderate to profuse whitish green compact callus was initiated while with 10µM Kin and NAA reddish green to whitish green nodular to friable callus was induced (Table 14, Fig. 34a, b).

(vi) Kin and 2,4D

Another cytokinin Kin was coupled with 2,4D to determine its effect on morphogenesis from leaf explant of *P grandiflora*. When the explants was inoculated on 0.5 µM Kin coupled with increasing concentrations of 2,4D (0.5-10 µM) it failed to evoke a morphogenic response. There was a late response in the concentrations of 2.5 µM BA and 2,4D (2.5-10 µM) as the callus initiated in the 6th week from the margins of the explants (Fig. 35a). A slight increase in callus was observed as the concentration of Kin increased to 5-10 µM along with 2,4D (2.5-10 µM, Fig 35b, c). The callus in these combinations was white at the time of initiation and turned whitish green and compact at the end of 8 weeks. It was noted that both BA and Kin along with 2,4D induce callus which was white to green and poor to moderate in growth (Table 14).

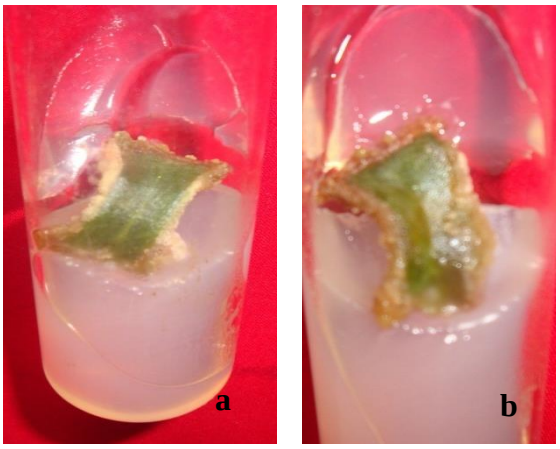


Figure 33: Effect of Kin and IAA on leaf explants of *P grandiflora* after 8 weeks. (a) White friable callus induction at 5 µM Kin and 10 µM IAA (b) Shoot bud induction at 10 µM Kin and 5 µM IAA

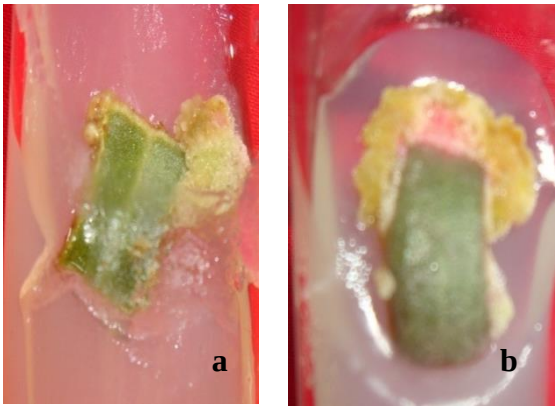


Figure 34 : Effect of Kin and NAA on leaf explants of *P grandiflora* after 8 weeks.: (a)Moderate whitish green callus induction at 5 µM Kin and 0.5 µM NAA (b) Reddish green callus at 10 µM Kin and 0.5 µM NAA.

Table 14: Effect of synergistic concentrations of Kin and auxins in leaf explant of *P grandiflora*

PGR's (µM)				Callus	% Explants forming callus	% Explants forming shoot buds
Kin	IAA	NAA	2,4D			
0.5	0.5-10	-	-	-	-	-
2.5	0.5	-	-	-	-	-
2.5	2.5	-	-	+	70	-
2.5	5	-	-	+	100	20
2.5	10	-	-	+	100	20
5	0.5	-	-	-	-	-
5	2.5	-	-	+	60	60
5	5	-	-	+	100	100
5	10	-	-	+	100	100
10	0.5	-	-	+	40	40
10	2.5	-	-	+	100	100
10	5	-	-	+	100	100
10	10	-	-	+	100	70
0.5	-	0.5-10	-	-	-	-
2.5	-	0.5	-	+	50	-
2.5	-	2.5	-	+	50	-
2.5	-	5	-	++	70	-
2.5	-	10	-	++	100	-
5	-	0.5	-	+	50	-
5	-	2.5	-	++	75	-
5	-	5	-	++	70	-
5	-	10	-	+++	70	-
10	-	0.5	-	+	70	-
10	-	2.5	-	++	66.66	-
10	-	5	-	+++	100	-
10	-	10	-	+++	100	-
0.5	-	-	0.5-10	-	-	-
2.5	-	-	0.5	-	-	-
2.5	-	-	2.5	+	50	-
2.5	-	-	5	+	100	-
2.5	-	-	10	++	100	-
5	-	-	0.5	-	-	-
5	-	-	2.5	+	60	-
5	-	-	5	++	100	-
5	-	-	10	++	100	-
10	-	-	0.5	-	-	-
10	-	-	2.5	+	100	-
10	-	-	5	++	100	-
10	-	-	10	+	70	-

- = No Response, + = little callus, ++ = moderate callus, +++ = profuse callus.

The experiments with synergistic cytokinin and auxins revealed that the concentrations of Kin and auxins induced callus of varying texture and colour. BA and auxins IAA/NAA formed callus and shoot buds while 2,4D induced callus.

Besides the combinations of BA and IAA it was also observed that at 2.5 µM BA and 2.5 µM NAA optimum number of shoot buds (7-8) were formed which were also subjected to bud break combinations.

The auxin IAA was supplmented with 2.5 µM BA and 2.5 µM NAA at very low concentrations. It was observed that 2.5 µM BA, 2.5 µM NAA and 0.1 µM IAA formed reddish brown callus. Percent response was found to be 100% for callus and 30% for shoot buds. Similarly 2.5 µM BA, 2.5 µM NAA and 0.5-1 µM IAA formed greenish brown callus with 100% callus and shoot bud response (Fig. 36a).Thus coupling of IAA to 2.5 µM BA, 2.5 µM NAA induced callus which was insufficient to improve on the protocol of regeneration.

Supplementation of Phloroglucinol (0.5-1 µM) to this bud inducing medium evoked reddish yellow callus and was insufficient to induce shoot formation (Fig. 36b).

Another additive Silver nitrate was also supplemented with 2.5 µM BA and 2.5 µM NAA to determine its effect on bud break. At 2.5 µM BA, 2.5 µM NAA and 2 mg/l $AgNO_3$ profuse yellow red callus was obtained with the formation of suppressed shoot. At 2.5 µM BA, 2.5 µM NAA and 4 mg/l $AgNO_3$ moderate white callus was formed while with 8 mg/l the explant turned brown indicating that $AgNO_3$ is not beneficial in inducing in vitro shoots in the plant (Fig. 36c).

It was thus concluded that the combinations with BA and NAA induced shoot buds which with different intervention failed to induce shoots.Combination of 10 µM BA and 5 µM IAA formed shoot buds and shoots which multiplied at a concentration of 20 µM BA. The next experiment was devised where the shoots and shoot cluster obtained on 20 µM was subjected to elongation.

4.1.2.5Multiplication and elongation of in vitro shoots

A cluster of in vitro shoots which were mostly short in length were obtained when the young leaf explant was inoculated on 10 µM BA and 5 µM IAA followed by its transfer to 20 µM BA. The elongation of these in vitro shoot cluster was tried in further experiments.

(i) Basal media

Basal media was inadequate to elongate the shoots which turned yellow after 2 weeks.

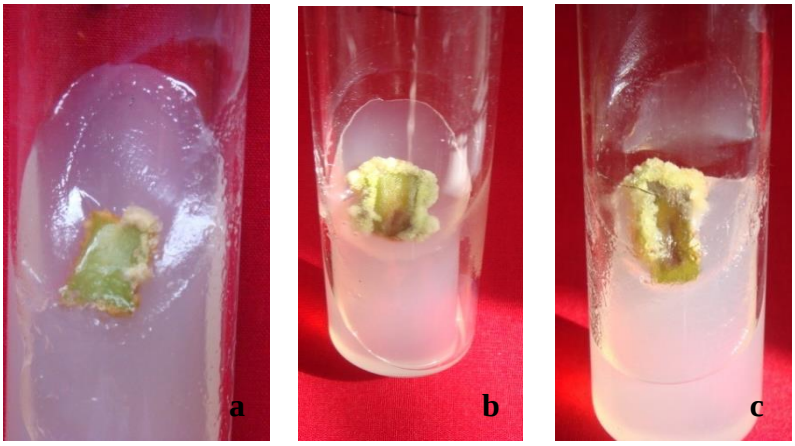


Figure 35: Effect of Kin and 2,4D on leaf explant of *P grandiflora* after 8 weeks: (a) Poor white friable callus induction 2.5 µM Kin and 5 µM 2,4D (b) white green friable callus formation at 5 µM Kin and 10 µM 2,4 D (c) green compact callus formation at 10 µM Kin and 5 µM 2,4D

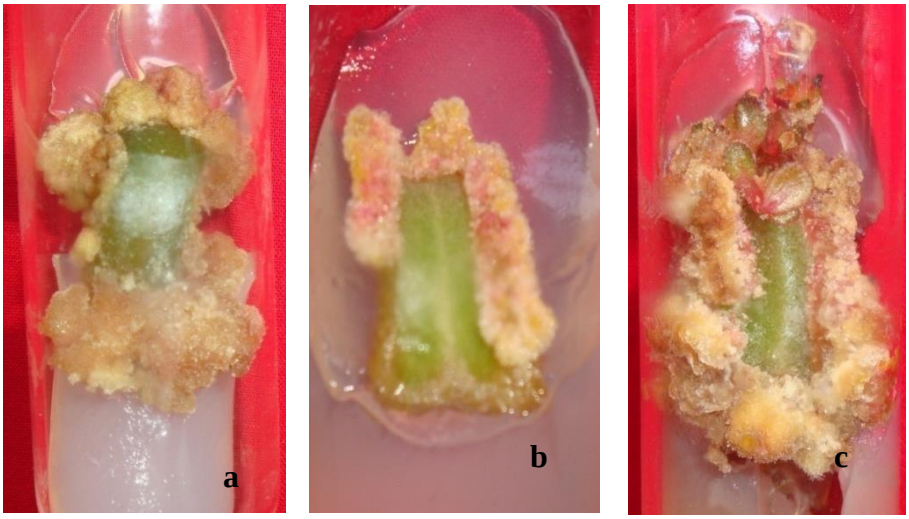
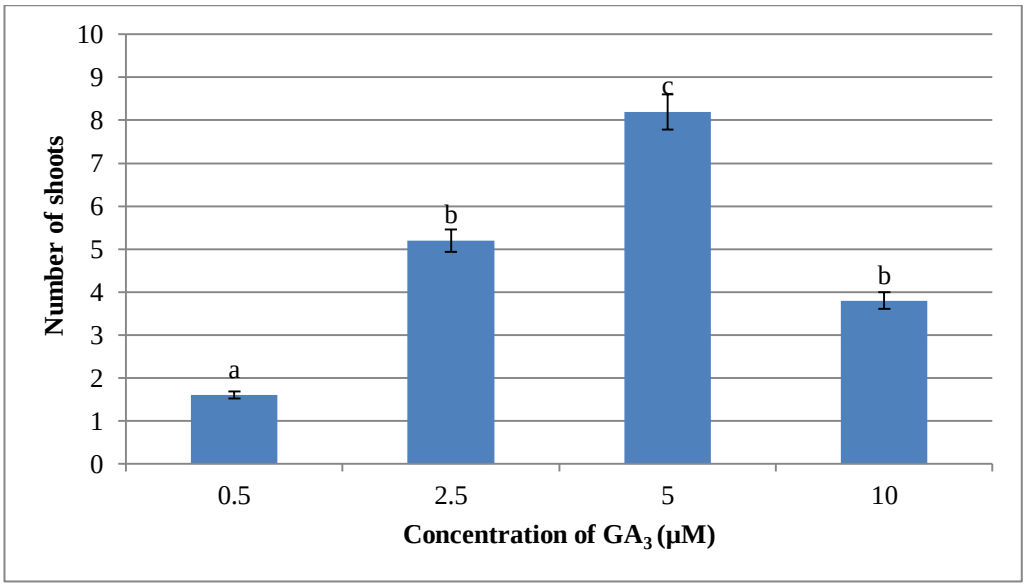


Figure 36: Shoot buds formed at 2.5 µM BA and 2.5µM NAA transferred to different media's for bud break in *P grandiflora* after 2 weeks (a) good friable callus formation at 2.5 µM BA , 2.5 µM NAA and 0.5µM IAA reddish brown callus (b) Reddish yellow callus formation at 2.5 µM BA ,2.5µM NAA and 1 µM Phloroglucinol (c) Emergence of in vitro leaves from shoot in presence of 2.5 µMBA , 2.5 µMNAA and 2 mg/l $AgNO_3$

(ii) GA_3

Gibbrellic acid is a hormone which is generally employed for the elongation of shoots and it proved to be effective in elongating the cluster of incipient shoots obtained on 10µM BA and 5µM IAA transferred on 20µM BA. In *P.grandiflora* low concentration of 0.5 µM GA_3 was insufficient in elongating the in vitro shoots as only 1-2 shoots depicted elongation. With increase in concentration to 2.5 µM there was an increase in number of shoots (5.2±0.37) and optimum response was achieved at 5 µM GA_3 where the number of shoot was 8.2±0.37 (Fig. 37, 38). In these two concentration the shoots started to elongate after 1 week but were thin and green. In the 2nd week the shoot elongated and the stem of the shoot became red indicating the presence of betalains. Hyper hydricity was also noted in the leaves of the shoots which did not elongate. A decline in shoot number was observed at a high concentration of 10 µM GA_3.

Figure 38: Effect of GA_3 on the number of shoots in *P grandiflora* after two weeks



Means followed by the same letters are statistically not significant with Tukey's HSD test, p≤ 0.05.

(iii) Coconut water

Coconut water (0.5-5%) was also ineffective in elongating the shoots and the cluster gradually turned yellow.Thus in the present experiment it was noted that GA_3 proved beneficial in elongating the shoots while basal medium and coconut water were inadequate to elongate the shoots in *P.grandiflora*.

d

Figure. 37: Shoot elongation at 5 µM GA_3in *P grandiflora* after 12 weeks

4.1.2.6 Histological study of differentiating leaf explants in *P grandiflora*

P grandiflora is a succulent plant and the anatomy of young leaf depicts typical anatomical features of the same. Transverse section of the leaf revealed kranz anatomy similar to *P oleracea*. The mesophyll cells were round present between bundle sheath cells and epidermis. Large water storage cells with intercellular spaces were present towards the margins of the leaf indicating its succulent nature (Fig. 39a). Inoculation of the explants was initiated on MS induction media supplemented with 3% sucrose and 10 µM BA and 5 µM IAA. After first week it was observed that the initiation of meristematic activity occurred from the epidermal cells. There was considerable swelling of the explants which was due the rapid rate of cell division. (Fig.39b). The mitotic activity intensified during 4^{th} week such that isodiametric cells with conspicuous nucleus and dense cytoplasm were observed distributed all over the surface which started coalescing into a more localised regions of meristemoids by 5^{th} week (Fig. 39c). As the cell proliferation and division increased these meristemoids arranged into layers organising into apical meristems. The primordias were in different stages of development ranging from protuberances to early shoot meristem formation (Fig. 39d) and by the end of sixth week in vitro shoots proliferated from the explants.

4.2.7 Regeneration from in vitro leaf explant

The regeneration protocol obtained for in vivo leaf explant was followed on in vitro leaf explant to determine its regeneration potential. The in vitro leaf explant was inoculated on 10 µM BA and 5 µM IAA where it swelled to a considerable size during 1^{st} week which was followed by callus and shoot bud induction along the margins during 3^{rd} week (Fig. 40a). In 100% cultures the callus formed was green, compact and nodular. After 4^{th} week the explant were transferred to a high concentration of 20 µM BA where it grew upto 7^{th} week. At this concentration the explant developed into cluster of incipient shoots which has green stems (Fig. 40b). The cluster of incipient shoots was transferred to medium fortified with 5 µM GA_3 where 4.6±0.50 shoot/explant were formed (Fig. 40c). A t test was performed between the number of shoots from in vivo leaf explant and in vitro leaf explant and the p value was 0.0045 which was significant at 0.01.

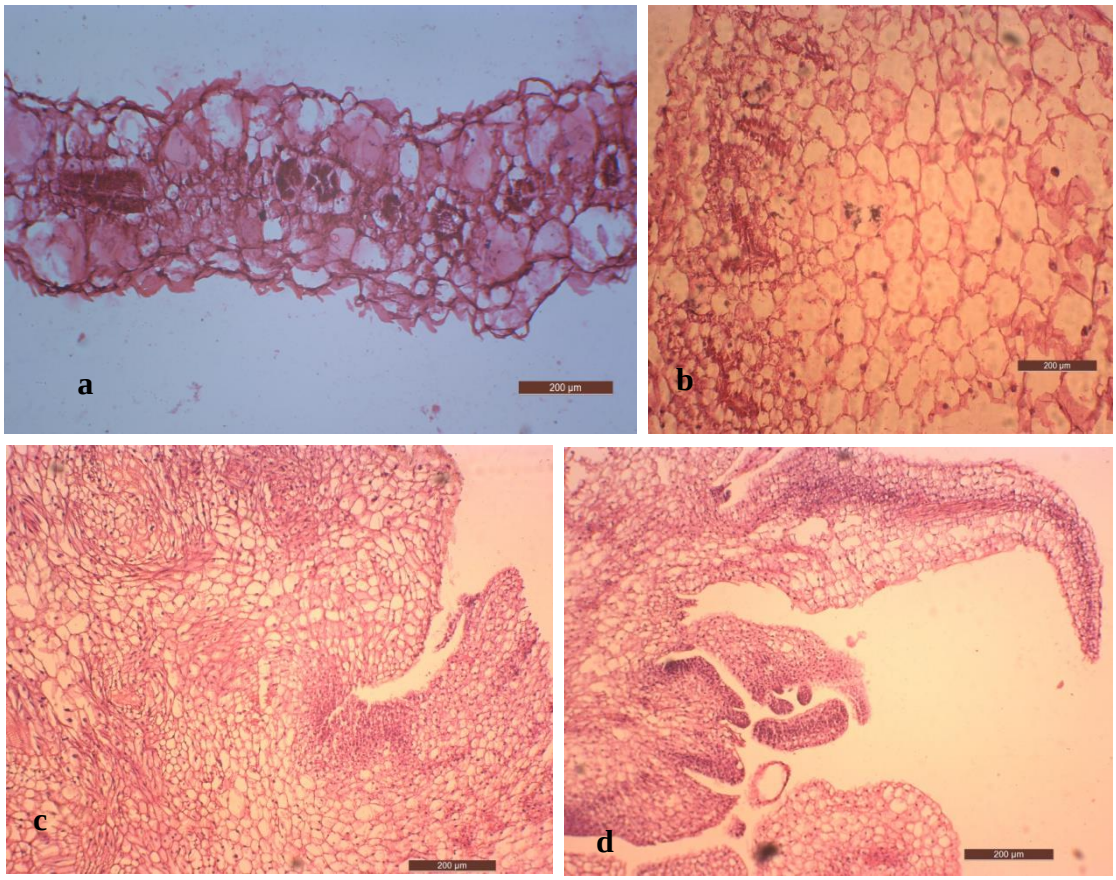


Figure 39: Histological changes during in vitro shoot regeneration of *P grandiflora* from leaf explant (a) Transverse section of leaf (Control) on day 0 (b) Water storage cells are replaced with parenchymatous cells due to massive cell division, (c) development of meristemoids at the end of 5th week, (e) Differentiation of shoot buds in different stages of development during 6th week

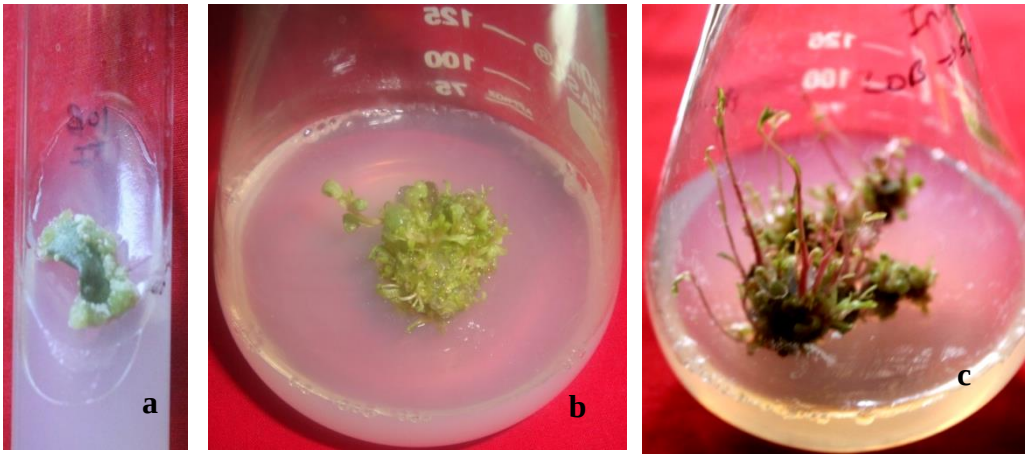


Figure 40: Regeneration from in vitro leaf explants of *P grandiflora* (a) green compact callus and shoot bud induction during 3rd week of incubation, (b) Cluster of incipient shoots on 20µM BA in 7th week, (c) Elongated in vitro shoots on 5µM GA_3

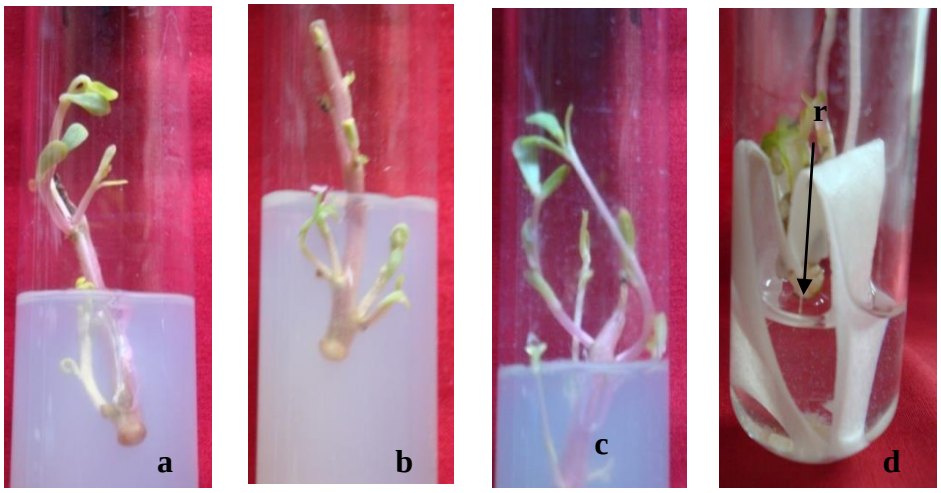


Figure41: Multiple shoot formation in *P grandiflora* from nodal explants after 8 weeks. (a) Axillary bud proliferation in 2µM Kin, (b) Induction of multiple shoots in 2µM BAP, (c) Synergistic effect of Cytokinin (8µM KIN and 2µM BAP), (d) Rooting of the regenerated shoots in presence of 2µM IBA, (r- root induction)

4.1.2.7 Regeneration from nodal explant

As the leaf explant of *P grandiflora* failed to differentiate appreciable number of shoots, regeneration utilizing nodal explants was also tried on a cytokinin based medium. It was observed that the axillary buds emerged as shoots after 2-3 days without an intervening callus phase. Individual concentration of BAP (2-10 µM) was effective as compared to individual concentration of Kin (2-10 µM) and BAP at 10 µM induced a better response as the number of shoot (3.7±0.25) and leaves (9.5±0.95) was high as compared to the rest of the concentration (Fig. 41a, b). A synergistic combination of BAP and Kin (2-10 µM) enhanced the number of shoot and leaves per explant. It was observed that a low concetration of BAP (2-4 µM) when coupled with 8 µM Kin induced optimum response where the number of shoot (4±0.63)and leaves were high while the remaining concentrations depicted almost a similar response (Fig. 41c, Table 15). These shoots obtained were rooted at half strength MS medium supplemented with 2 µM IBA where roots were initiated within 5-6 days (Fig. 38d). It was also noted that the plants which grew from nodal explants depicted necrosis of shoot tips by 4^{th} week . Initially the problem was addressed by subculturing in short time duration and a study was also conducted on this phenomenon.

4.1.2.8 Control of shoot tip necrosis (STN)

In *P grandiflora* a combination of 4 µM BA and 8 µM Kin was optimized for regeneration from nodal explants. During regeneration the in vitro plants depicted apical browning which was followed by basipetal necrosis. The multiple shoots grew to a length of 2-3 cms which was followed by the development of a necrotic region in the upper portion while the lower portion remained unaffected from which the axillary buds gave rise to in vitro shoots. Symptoms of necrosis occurred from the 1^{st} week, and by 4^{th} week the anomaly was so severe that 90% of the explants were lost (Fig. 42a). This shoot tip necrosis (STN) is generally caused by the deficiency of Boron and Calcium and so experiments were devised with different concentration of B and concentrations and source of Ca. The two source of Ca used for the experiment were $CaCl_2$ and Ca-gluconate while H_3BO_3 was used as a source of B.

Table 15: Effect of cytokinins on nodal explants of *P grandiflora*

Cytokinin (µM)		Number of shoots/explants (Mean ± S.E.)	Number of leaves/explants (Mean ± S.E.)
Kin	BAP		
2	-	2.8±0.24	9.7±0.96
4	-	2.2±0.27	7.2±0. 88
8	-	2.5±0.28	9.1±1.14
10	-	2.7±0.30	9.3±1.08
-	2	2.7±0.85	7.2±1.80
-	4	3.2±1.10	8.5±2.98
-	8	3.5±0.64	7.5±0.95
-	10	3.7±0.25	9.5± 0.95
2	2	2.8±0.43	8 ±0.63
2	4	2.5±0.34	8.6±0.42
2	8	2.4±0.57	8.5±1.19
2	10	3.1±0.60	8.5±1.40
4	2	3.3±0.21	10.5±1.02
4	4	3.1±0.43	7.0±1. 23
4	8	3.6±0.58	8.8±1.62
4	10	3.6±0.55	9.6±0.80
8	2	4 ±0.63	11.8±2.78
8	4	3.8±1.65	12 ±1.87
8	8	3.2±0.42	7.1 ± 0.64
8	10	3 ±0.81	9.5±2.21
10	2	2.7±0.47	6.5±0.95
10	4	3.7±0.47	9.5±1.25
10	8	2.7±0.47	7.5±1.70
10	10	2 ±0.41	7.3±0.76

S.E. = Standard Error

(i) $CaCl_2$ (3-30mM)

The normal concentration and source of Ca in MS medium is $CaCl_2$with a concentration of 3 mM which caused 90% loss of shoot cultures after 4 weeks. STN gradually decreased with increase in concentration and minimum necrosis (40%) was achieved at 18 mM (Fig. 42b, 43a). The micro shoots formed were healthy and stout but the number of shoot/explant was not drastically affected due to increased concentration of $CaCl_2$(Table 16). Thus 18mM concentration of $CaCl_2$ showed minimum necrosis without any loss in number of shoots/explant.

(ii) Ca-gluconate (3-30mM)

When $CaCl_2$was replaced with 3-30 mM concentration of Ca-gluconate in MS medium, minimum necrosis was achieved at 9 mM (40%) beyond which there was further increase in necrosis with 100% loss of cultures at 24 and 30mM (Fig. 43a). The number of micro shoots formed from the axillary buds was not affected but the in vitro shoots formed had long internode with pale yellow leaves.

(iii) Boron (0.1-0.4 mM)

Boron is a micro element which is used in M.S media in the concentration of 0.1 mM. Increase in concentration of B from 0.1-0.4 had no effect on % STN as 80% of the cultures were lost after 4 weeks (Fig. 43b). Increased concentration of B was detrimental to the growth of the micro shoots as their number decreased and the micro shoots were thin and yellow (Table 16).

Table 16: Effect of Boron, $CaCl_2$ and Ca-gluconate on number of shoots/explant of *P grandiflora* after 4 weeks

Concentration (mM)	Boron	$CaCl_2$	Ca-gluconate
0.1	1.83±0.24	-	-
0.2	1.68±0.21	-	-
0.3	1.50±0.56	-	-
0.4	1.40±0.87	-	-
3	-	1.83±0.24	2.08±0.09
6	-	1.63±0.38	2.47±0.51
9	-	2.5±0.32	2.23±0.10
12	-	2.12±0.39	2.35±0.15
18	-	2.83±0.30	1.90±0.43
24	-	2.50±0.18	1.75±0.36
30	-	2.07±0.12	1.40±0.37

S.E. = Standard error

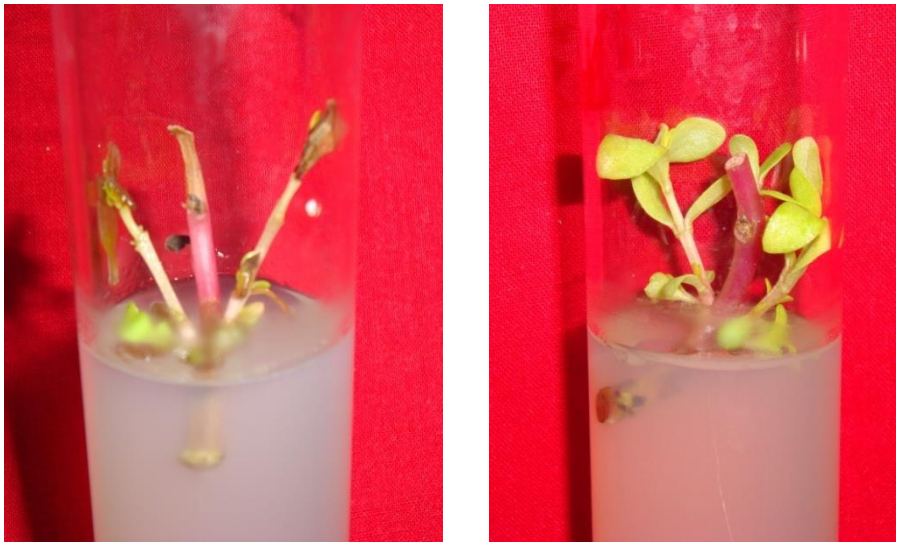


Figure 42: Shoot tip necrosis in shoot cultures of *P grandiflora*. (a) Severely affected shoot tips after 1 week, (b) STN alleviated in presence of 18 mM $CaCl_2$.

Figure 43a: Effect of $CaCl_2$ and Ca-gluconate on % Necrosis in shoot cultures of *P.grandiflora* after 4 weeks

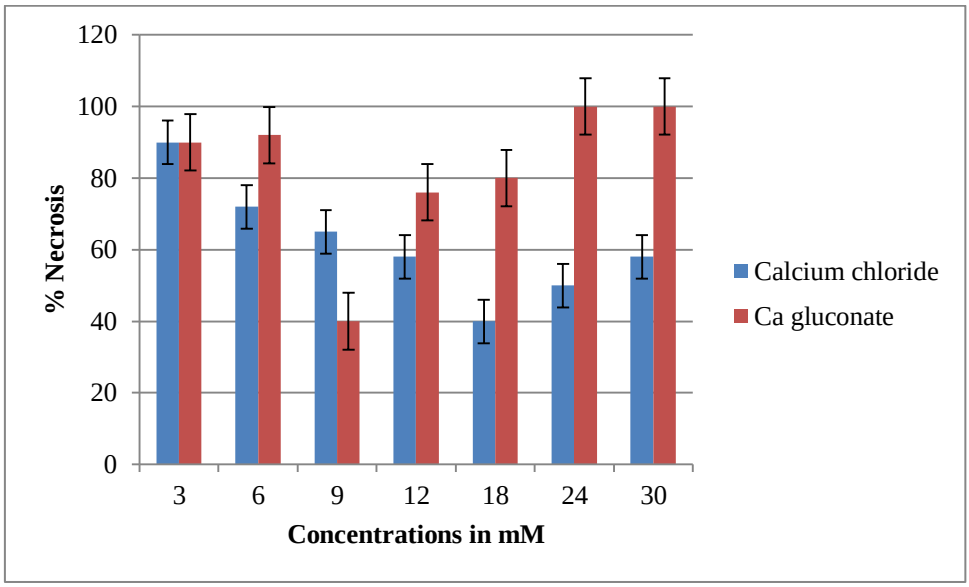
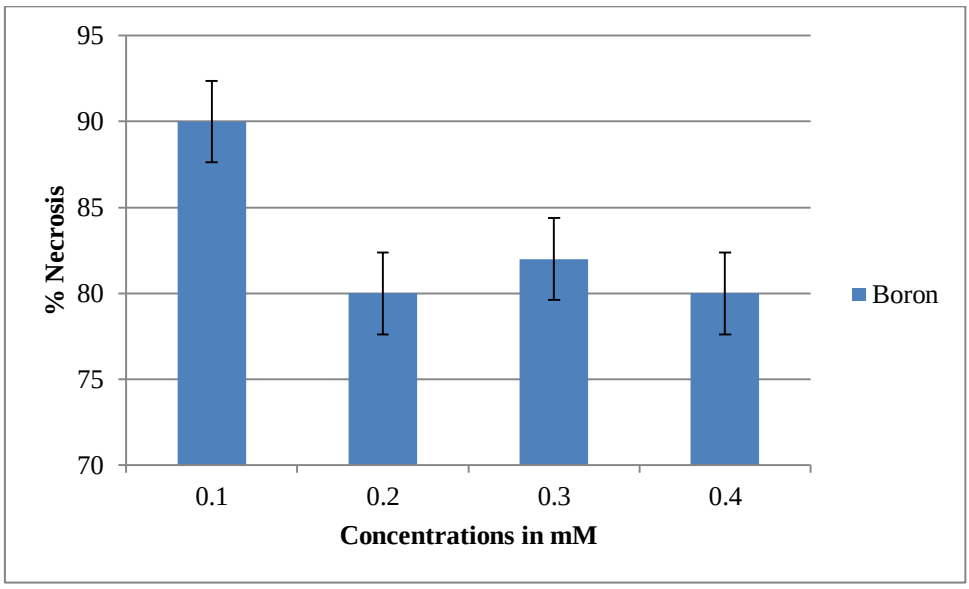


Figure 43b: Effect of Boron on % Necrosis in shoot cultures of *P grandiflora* after 4 weeks



Thus regeneration studies of P grandiflora revealed that the leaf explants under the influence of PGR 10µM BA and 5µM IAA induced shoot buds which proliferated into shoots. Multiplication of these shoots was done on 20µM BA and the shoots elongated on 5µM GA_3.Anatomy of incubating leaf explants confirmed the formation of leaf primordias which formed shoots. Since the appreciable number of shoots failed to regenerate from leaf explants therefore nodal explant was also tried for regeneration which gave optimum number of shoots at 4µM BA and 8µM Kin. The shoots formed were affected with necrosis of the shoots which was alleviated at 18mM $CaCl_2$.

Comparing the regeneration potential of the two species of Portulaca the present study proposes that the leaf explants of P oleracea has a better regenerative capacity as compared to P grandiflora

4.2 SECTION II METABOLITE STUDY

Plants are a rich reservoir of primary and secondary metabolites which are beneficial to us. The two species of *Portulaca* under study are an abundant source of the metabolites as *P oleracea* has a high content of omega fatty acids particularly Linoleic and Linolenic acid and *P grandiflora*, is a good source of betalains. Studies were undertaken in *P oleracea* to determine the relative content of the different fatty acids present in the in vivo shoot, stem, leaf, root, in vitro shoots and callus cultures. Similarly *P grandiflora* was evaluated for the content of betalains in whole plant, leaf, stem and root.

4.2.1. *P oleracea*

The content of fatty acids present in different parts of the plant as well as in vitro cultures was evaluated with respect to dry weight of the biomass.

4.2.1.1 Extraction of oil by Soxhlet apparatus

The in vivo and in vitro powdered samples were subjected to soxhlet extraction in petroleum ether to obtain its oil (Fig. 44).

4.2.1.2 Qualitative test for the presence of fat

A preliminary qualitative test to detect the presence of fatty acids was done on all the samples by performing Sudan III test and Emulsion test on oil obtained after soxhlet extraction.

(i) Sudan III Test

Oil sample when treated with Sudan III solution forms a red stained oil layer of fat globules. All six oil samples obtained after soxhlet extraction were subjected to Sudan III test (as described in Material and methods section 3.5.2.2) a red coloured layer was observed on the top of test

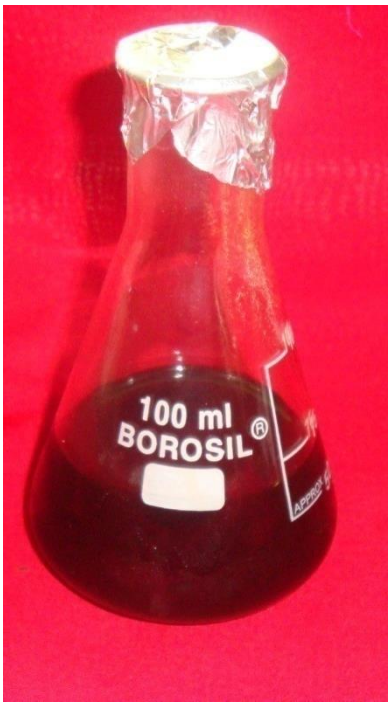


Figure44: Oil yielded from the whole plant of *P oleracea* after soxhlet extraction.

sample indicating presence of oil globules. Being a lipid soluble dye Sudan III reacts with oil globules and produces red colour while the water in the sample gets decolourised. All the treated samples showed positive results towards presence of oil except root. Result obtained in this experiment indicated presence of oil in the extracted sample which was further confirmed by emulsion test.

(ii) Emulsion Test

In this confirmatory test a white emulsion was formed because water and lipid are immiscible. This test further authenticated that except roots all the samples had the presence of fatty acids.

4.2.1.3 Estimation for the presence of total fats

The fats present in different parts of the plant, in vitro shoot and callus cultures were estimated. Results indicated that in vivo leaves had the highest content of fat (74.6 mg/g) followed by stem and whole plant (Table 16). Within the in vitro cultures, callus had a higher content of fat (58.1 mg/g) as compared to in vitro shoots (52.3 mg/g) whereas both in vivo and in vitro shoots had a similar fat content (52-53 mg/g).

Thus it was proved from the experiment that leaves are a rich source of fat followed by callus cultures. The content of fats is similar to in vivo and in vitro shoots therefore in vitro shoots can be considered as a potent source of omega fatty acids which may be enhanced by different biochemical manipulations.

Table 17: Content of total fats (mg/g) present in different samples of *P oleracea*

Sample	Total fats(mg/g)
In vivo plant	53.4
Stem	50
Leaf	74.6
Invitro shoots	52.3
Callus	58.1

4.2.1.4 Fingerprinting of the fatty acids present in the in vivo and in vitro samples

The oil obtained by soxhlet extraction was esterified with methanolic $BF_3$and subjected to gas chromatography to identify the different fatty acids and their relative quantities in the samples. The fatty acids were identified by comparing the retention time (RT) of the standard fatty acid mixture with that of the sample.

(a) Gas Chromatography of the solvents and standard

Prior to the analysis of fatty acids, gas chromatography (GC) of the solvents utilized in the experiments was done to identify their peaks and RT. In this view GC analysis of petroleum ether and hexane was done. Standard fatty acid mixture was also analyzed to determine the RT of each of the 37 fatty acids (Fig. 45).The RT of Palmitic acid (C16:0, RT=32.82), Linoleic acid (C18:2, RT=36.13), Linolenic acid (C18:3, RT=38.86), Docosanoic acid (C22:0, RT=50.35), Tricosanoic acid (C23:0, RT=58.82) and Tetraosanoic acid (C24:0, RT=62.4) was obtained in the standard mixture.

(b) Relative fatty acid content and it's fingerprinting in the in vivo and in vitro samples

The relative fatty acids in different parts of the plant and in vitro cultures were analysed. Simultaneously a control was maintained with each set of sample.

(i) In vivo shoot

Gas Chromatography analysis of in vivo shoots extract indicated the presence of Palmitic acid, Linoleic acid and Linolenic acid (RT of 30.53, 36.27, and 38.37 respectively) as major fatty acid present in this plant (Table 18). The Linolenic acid content of the plant was the highest(20.15±1.06%) followed by Palmitic acid (12.49±0.56%) and Linoleic acid (9.63±3.38%) indicating that the plant is a rich source of omega fatty acid. Long chain fatty acid such as Docosanoic acid and Tetracosanoic acid (1.40±0.33%) was also detected in the sample (Fig. 46).

(ii)In vivo leaves

Leaf analysis indicated that Linolenic acid (17.72±1.62%)is the major fatty acid followed by Palmitic acid (8.51±1.60) and a decrease in Linoleic acid content was noted as compared to invivo shoot (Table 18). Oleic acid was absent in the leaves however, Stearic acid was detected (2.98±0.34%) which is an isomer of Oleic acid. Long chain fatty acids such as Arachidic acid (C20:0), Docosanoic acid (C22:0, RT= 50.4) and Tetracosanoic acid (C24:0, RT= 62.8) were also detected in the sample indicating the potential of *P oleracea* to synthesise long chain fatty acids (Fig.47).

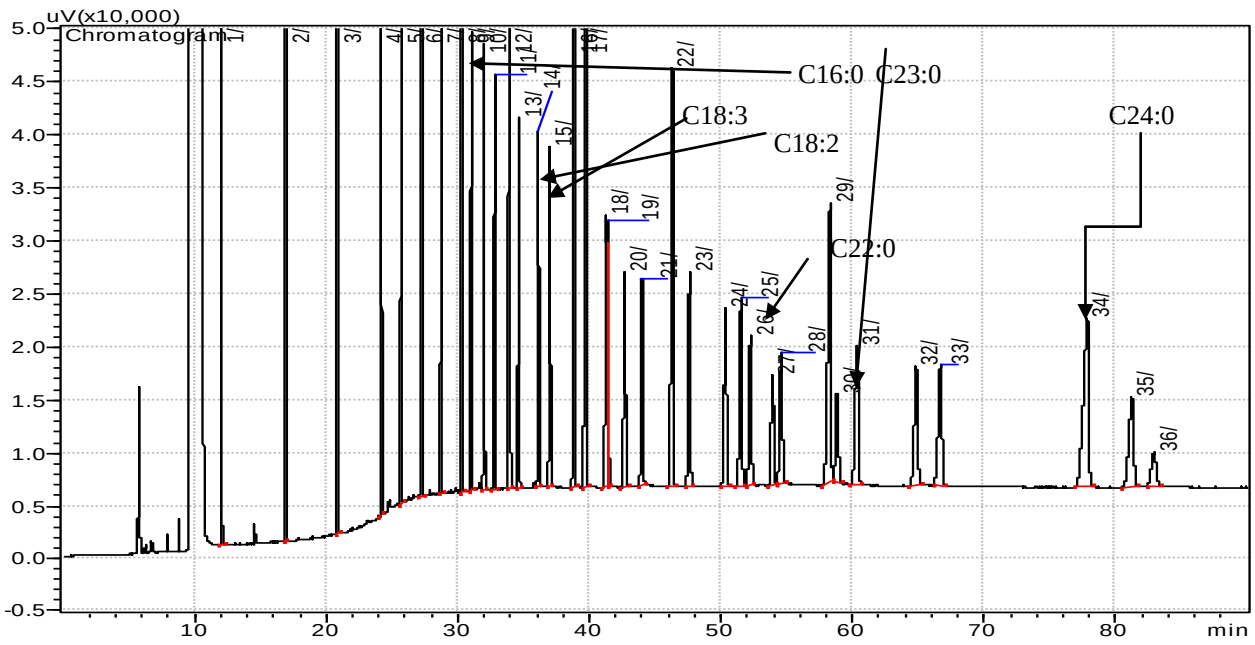


Figure45: GC analysis of Standard fatty acid mixture, 16:0= Palmitic acid, 18:2=Linoleic acid, 18:3= Linolenic acid, C22:0= Docosanoic acid, C23:0= Tricosanoic acid, C24:0= Tetracosanoic acid

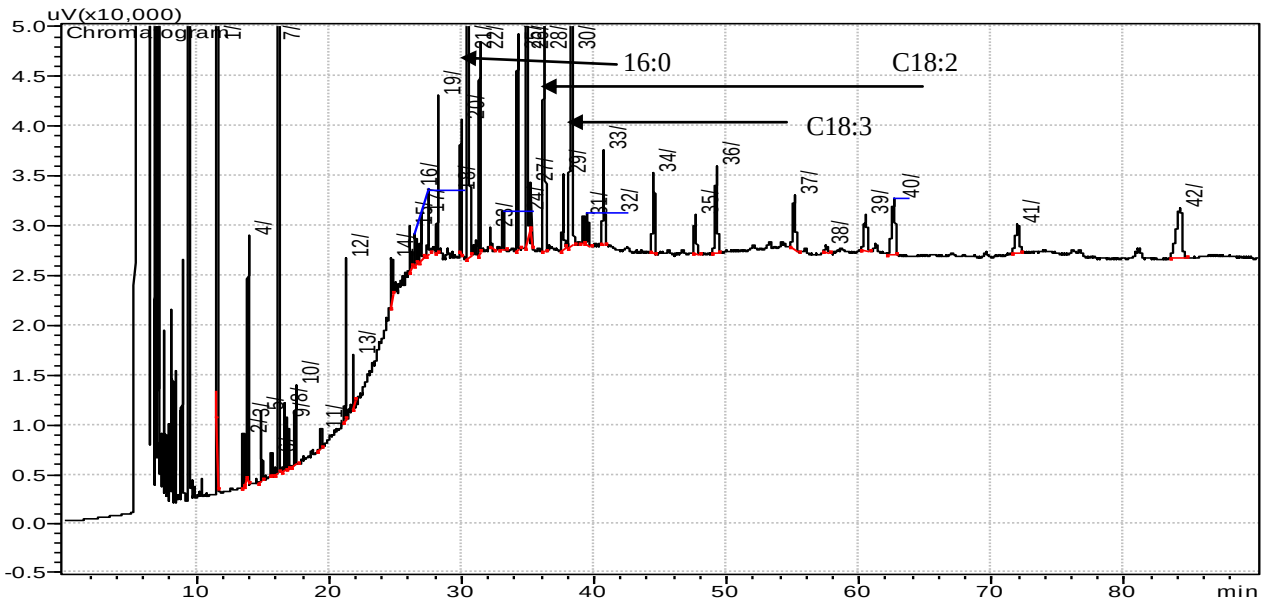


Figure46: GC analysis of fatty acids profiling of vivo shoot of *P oleracea* C16:0= Palmitic acid, C18:2= Linoleic acid, C18:3= Linolenic acid

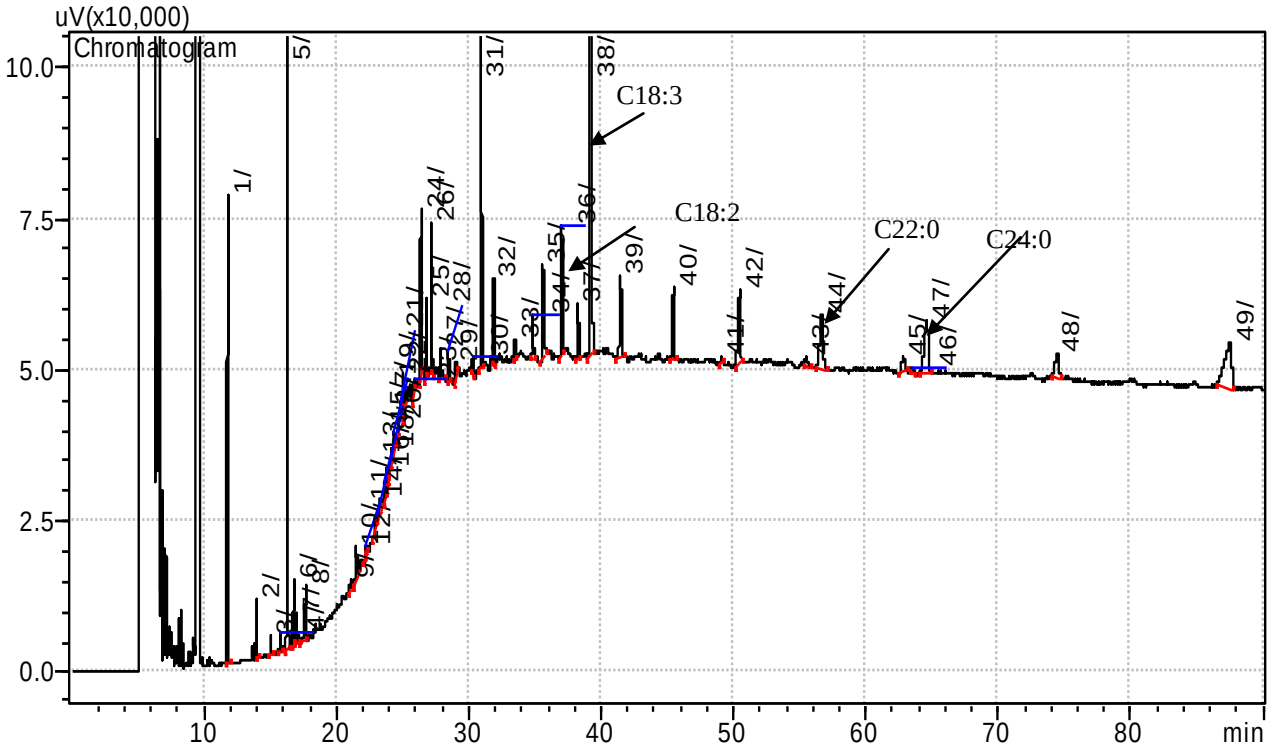


Figure 47 : GC analysis for the fatty acid profiling of leaf of *P.oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid, C22:0= Docosanoic acid, C24:0= Tetracosanoic acid.

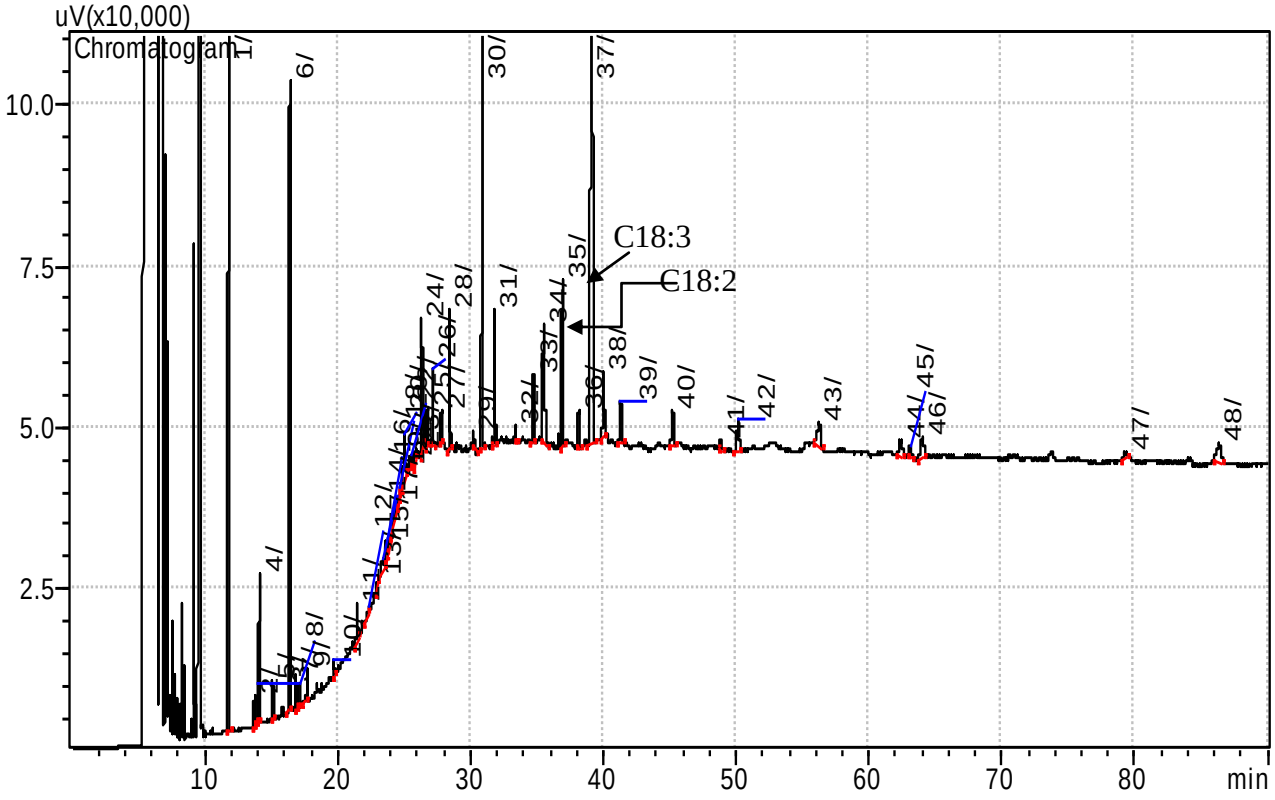


Figure48: GC analysis for the fatty acid profiling of stem of *P oleracea,* C18:2= Linoleic acid, C18:3=Linolenic acid

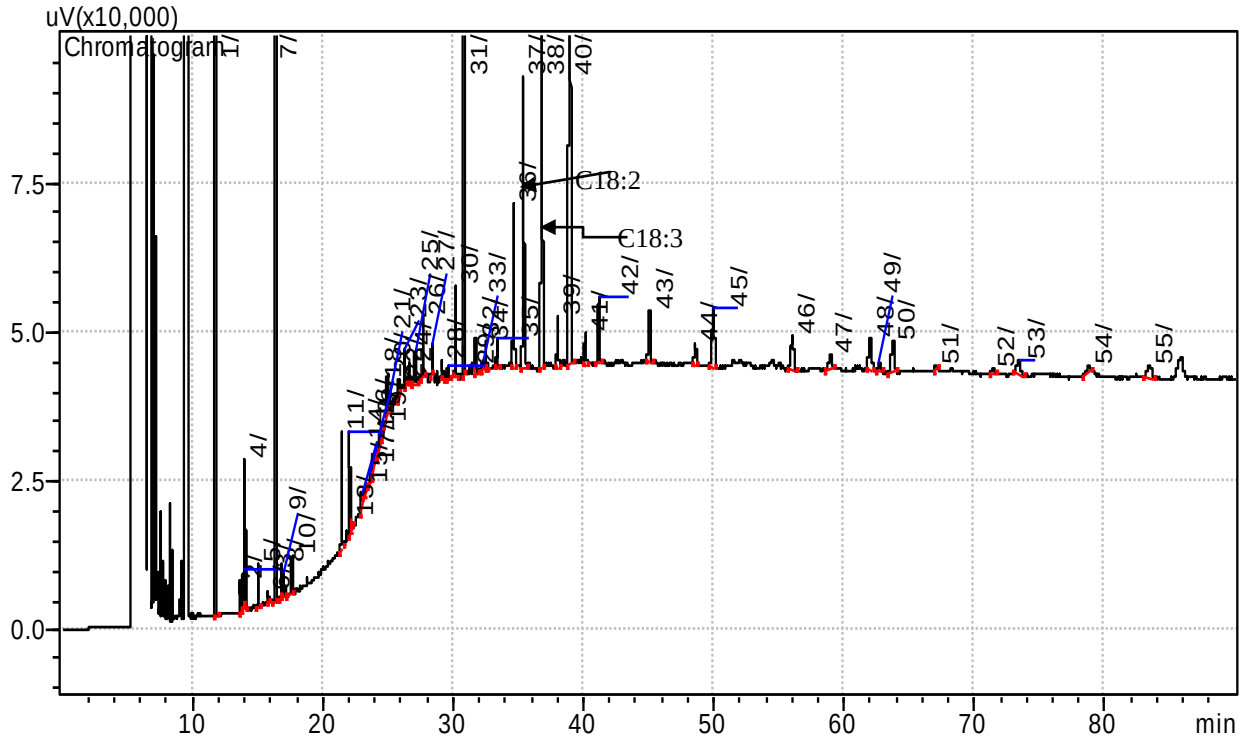


Figure49: GC analysis for the fatty acid profiling of in vitro shoots of *P oleracea*, C18:2= Linoleic acid, C18:3 = Linolenic acid

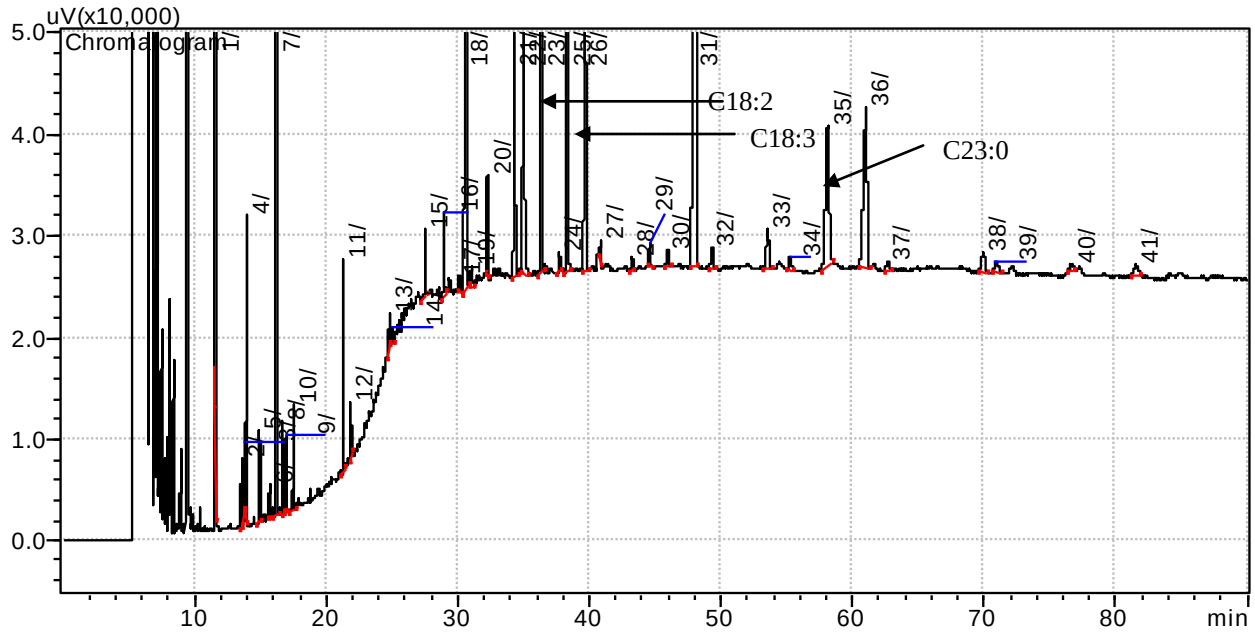


Figure 50: GC analysis for the fatty acid profiling of callus cultures of *P oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid, C23:0= Tricosanoic acid

(iii) In vivo stem

The fatty acid analysis of stem revealed that just like in vivo leaves Linolenic Acid, Palmitic acid and Linoleic acid were the major fatty acid however the content of Linoleic acid was high (10.59±1.39%) as compared to leaf indicating that stem is the main source of this acid (Table 18). Docosanoic Acid and Arachidic acid were present but long chain fatty acid Tetracosanoic acid was absent as compared to in vivo shoot and leaves (Fig. 48). This indicated that stem is rich in Linoleic acid which is an 18 carbon compound but has an inability to synthesise very long chain fatty acids.

(iv)In vitro shoots

The fatty acid profiling of in vitro shoots was similar to in vivo shoots and minor difference in the relative content of fatty acids was noted. Similar to in vivo shoots, the major fatty acids recorded within vitro shoots were Linolenic acid, Palmitic Acid and Linoleic Acid. The content of oleic acid was twofold (5.03±0.255%) in the sample and Tetracosanoic acid was absent as compared to the in vivo plant (Fig. 49, Table 18).

(v) Callus cultures

Results obtained after GC analysis of callus cultures depicted diversity in the content and variety of fatty acids. Palmitic acid was the abundant fatty acid and contrary to the results obtained in the previous samples the content of important fatty acids like Linoleic and Linolenic acid was very less (3-5%). Callus cultures also synthesized odd chain fatty acids such as Heptadecanoic acid and Tricosanoic acid (Fig. 50, Table 18).

Comparing the fatty acid content within different samples it was concluded that leaves were rich in Linolenic acid while the content of Linoleic acid was more in stem. Samples of in vivo shoot and in vitro shoot revealed that both the samples had a similar fatty acid profile with minor variations in their relative content (Table17). Within callus cultures the synthesis of Linoleic and Linolenic acid was suppressed but it had the potential to synthesise odd chain fatty acid.

Table 18: Relative fatty acids content of different samples of *P oleracea*

Fatty acids (%)	In vivo shoots	Leaf	Stem	In vitro shoots	Callus
Pentadecanoic acid	0.90±0.05	1.04±0.74	0.57±0.11	0.91±0.05	0.48±0.03
Palmitic Acid	12.49±0.56	8.51±1.60	11.90±1.25	13.11±0.37	14.94±0.36
Heptadecanoic acid	-	-	-	-	2.64±0.07
Oleic Acid	2.91±0.10	-	-	5.03±0.25	4.43±0.17
Stearic Acid	-	2.98±0.34	5.20±0.68	-	-
Linoleic Acid	9.63±3.38	3.25±0.14	10.59±1.39	7.94±0.44	5.77±0.29
Arachidic Acid	0.59±0.12	1.03±0.25	3.29±0.32	0.85±0.10	2.87±0.38
Linolenic Acid	20.15±1.06	17.72±1.62	16.46±1.79	14.16±0.88	3.08±0.16
Docosanoic Acid	1.40±0.31	2.22±1.10	4.26±0.28	1.63±0.21	7.07±0.33
Tricosanoic Acid	-	-	-	-	3.08±0.10
Tetracosanoic Acid	1.40±0.33	0.71±0.09	-	-	-

4.2.1.5 Gas chromatography Mass spectrometry of the in vivo and in vitro samples

The fatty acids identified in GC analysis were confirmed by comparing with the mass spectra of National Institute of Science and Technology (NIST) library in EI mode. NIST Library is a standard library which is frequently used for identification of different compounds. The presence of all the fatty acids identified in GC analysis was confirmed by GCMS and also which remained to be identified in GC were confirmed by this technique.

(i) In vivo shoots

The MS of in vivo shoots revealed that besides the fatty acids identified in GC it also has the presence of long chain fatty acids such as Tetracosanoic acid and Hexacosanoic acid. Linoleic acid and Linolenic acid were identified by comparing the constituent peaks with the standard mass spectra of these acid obtained from NIST library (Fig. 51). The plant is also a rich source of alcohols and alkaloids such as Phytol, Heptacosanol, Hexatricontane, Tetrapentacontane and Hexacontane indicating its high phytochemical importance.

(ii) In vivo Leaves

Besides the fatty acids identified in GC analysis MS of in vivo leaves confirmed the presence of long chain fatty acids such as methyl eicosenate, methyl hexacosanoate and methyl octacosanoate (Fig. 52). Other compounds reported from the samples were Eicosane, Tricontane, Hexatricontane, Tetratricontane, Tetrapentacontane, Hexacontane, Tetracosanol and Heptacosanol.

(iii) Invivo Stem

Stem revealed the presence of long chain fatty acid such as methyl pentacosanoate, hexacosanoate and octacosanoate and also the fatty acids detected in GC analysis (Fig. 53). Alkaloids and alcohol detected in the sample were Eicosane, Tricontane, Hexatricontane Tetratricontane, Tetrapentacontane, Hexacontane Tetracosanol and Heptacosanol.

(iv) In vitro shoots

GC MS analysis of in vitro shoot confirmed the presence of other fatty acids esters such as (2E)-2-Undecenyl pentanoate, methyl 3 propanoate, methyl eicosenate and methyl hexacosanoate (Fig. 54). Other compounds identified in the sample were Tetrapentacontane, Tetracosanol, Heptacosane, Tetrapentacontane, Hexacontane, Pentacosanol and Heptacosanol

(v) Callus cultures

Callus revealed the presence of Methyl nonadecanoate, Methyl 5,13-docosadienoate and long chain fatty acid detected were Methyl eicosanoate, Methyl pentacosanoate and Methyl cerebronate (Fig. 55). Other compounds detected were Eicosene, Diisooctyl phthalate, Tetracosanol and Octadecanamide.

Summarizing the above results it is reported that the amount of total fats is highest in leaves of in vivo plant of P oleracea. Similarly Linolenic and Linoleic acid are the major omega 3 fatty acids present in the in vivo plant. The in vitro shoots had a similar fatty acid profiling except Tetracosanoic acid. Evaluation of fatty acids contents in callus cultures depicted that Palmitic acid and Docosanoic acid were synthesized in larger content. The presence of Heptadecanoic

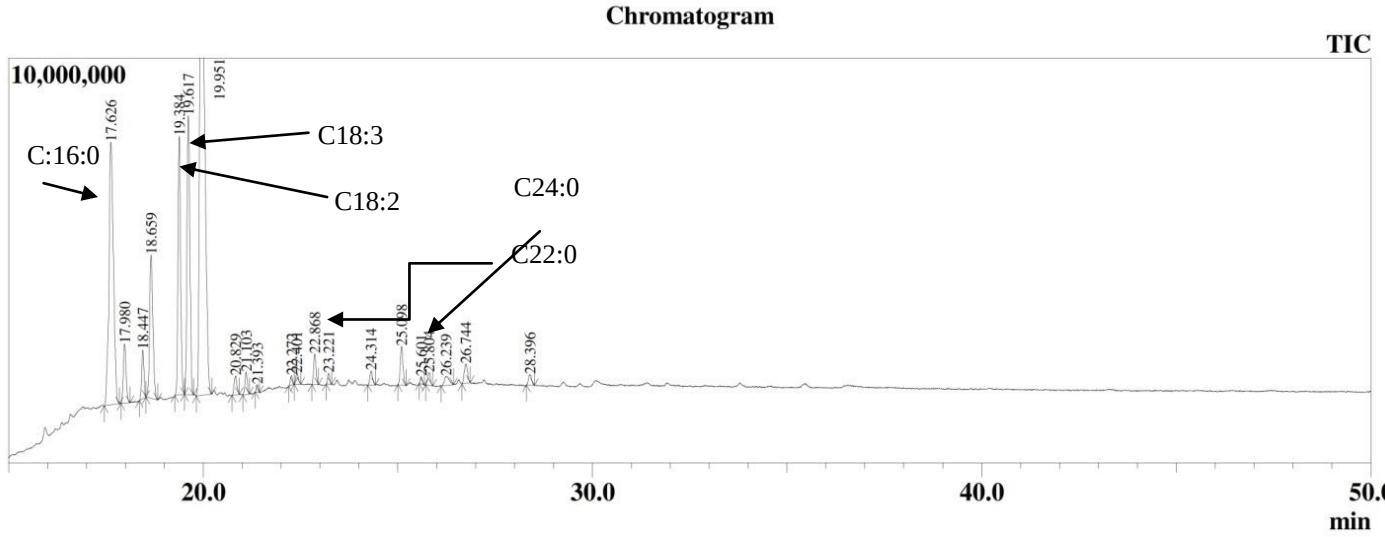


Figure51: Total Ion Chromatogram in EI mode of In vivo shoots of *P.oleracea* C16:0= Palmitic acid, C18:2= Linoleic acid, C18:3= Linolenic acid, C22:0= Docosanoic acid, C24:0= Tetracosanoic acid.

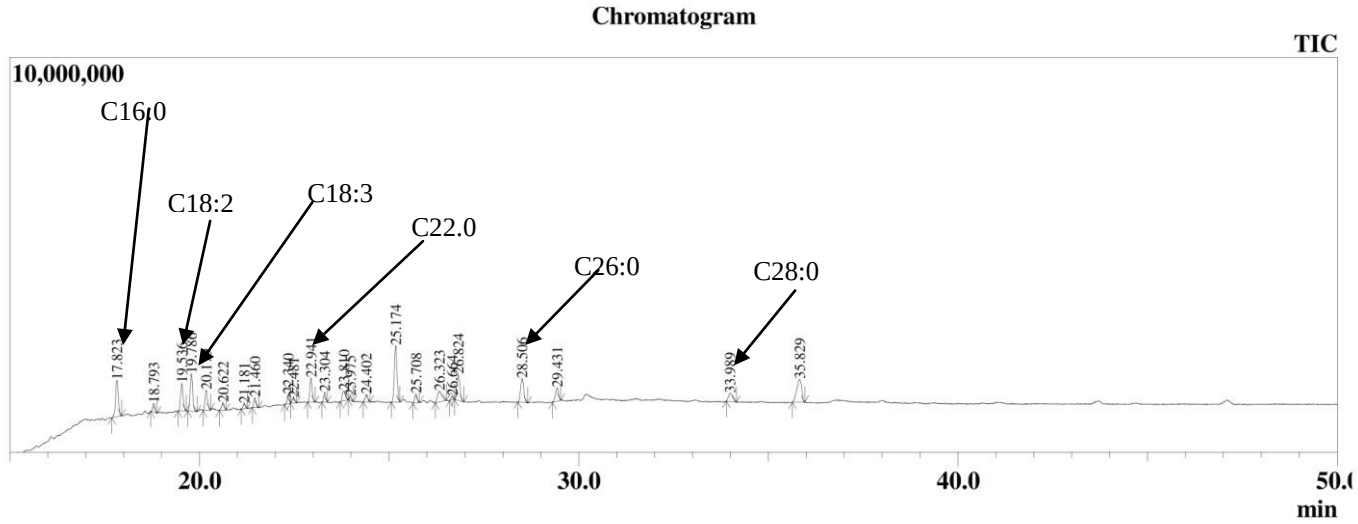


Figure 52: Total Ion Chromatogram in EI mode of In vivo leaves of *P.oleracea* C16:0= Palmitic acid, C18:2= Linoleic acid, C18:3= Linolenic acid, C22:0= Docosanoic acid, C26:0= Hexacosanoic acid, C28:0= Octacosanoic acid.

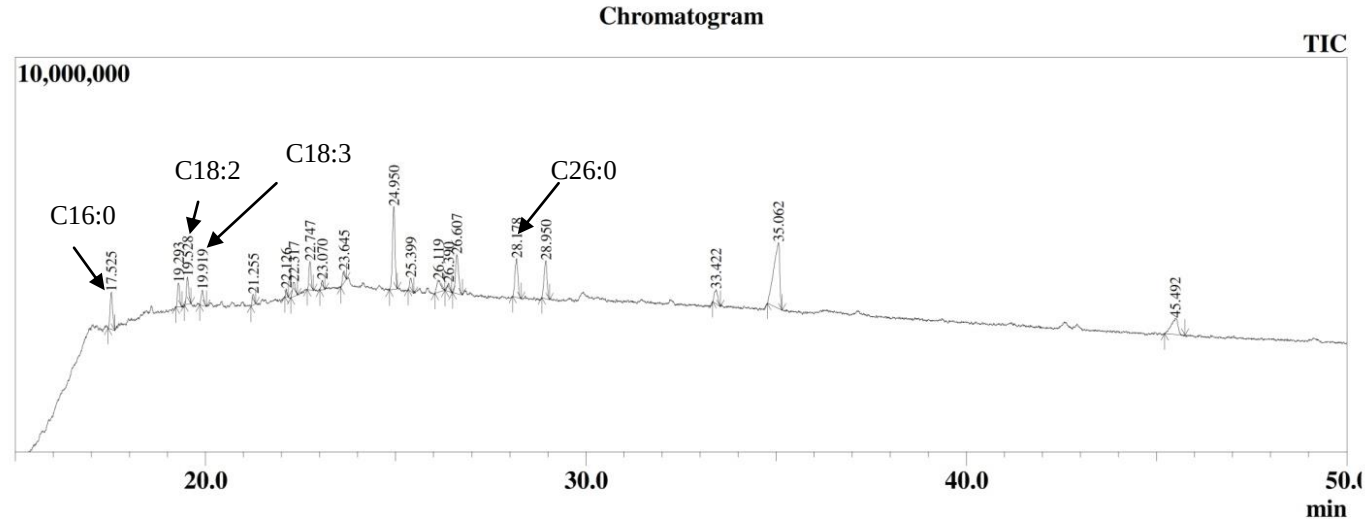


Figure 53: Total Ion Chromatogram in EI mode of stem of *P.oleracea* C16:0= Palmitic acid, C18:2= Linoleic acid, C18:3= Linolenic acid, C22:0= Docosanoic acid, C26:0= Hexacosanoic acid.

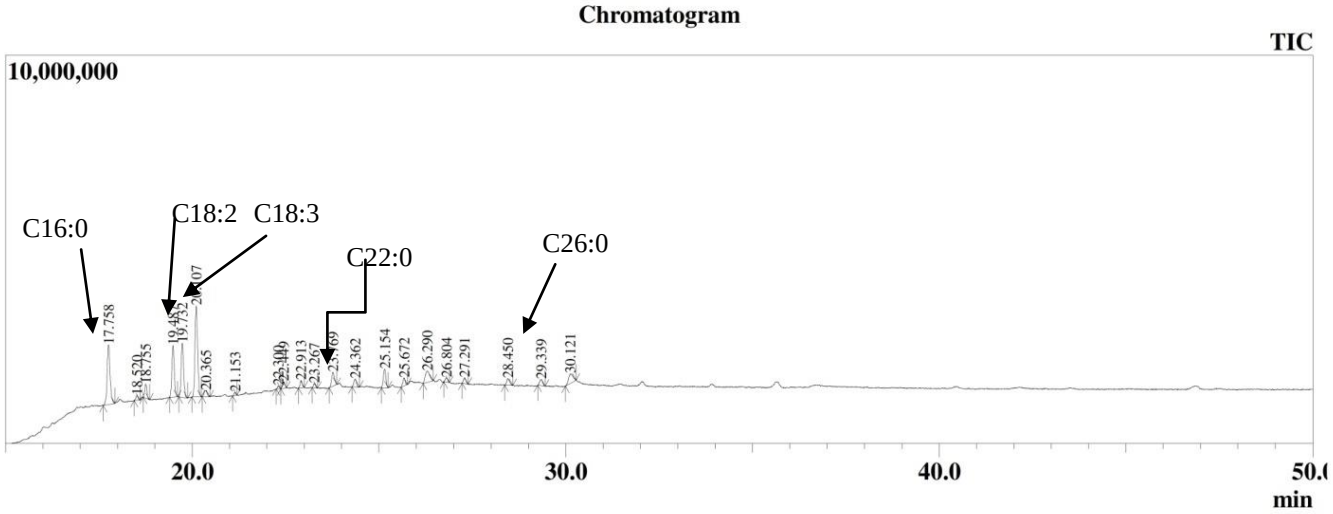


Figure 54: Total Ion Chromatogram in EI mode of In vitro shoots of *P.oleracea* C16:0= Palmitic acid, C18:2= Linoleic acid, C18:3= Linolenic acid, C22:0= Docosanoic acid, C26:0= Hexacosanoic acid.

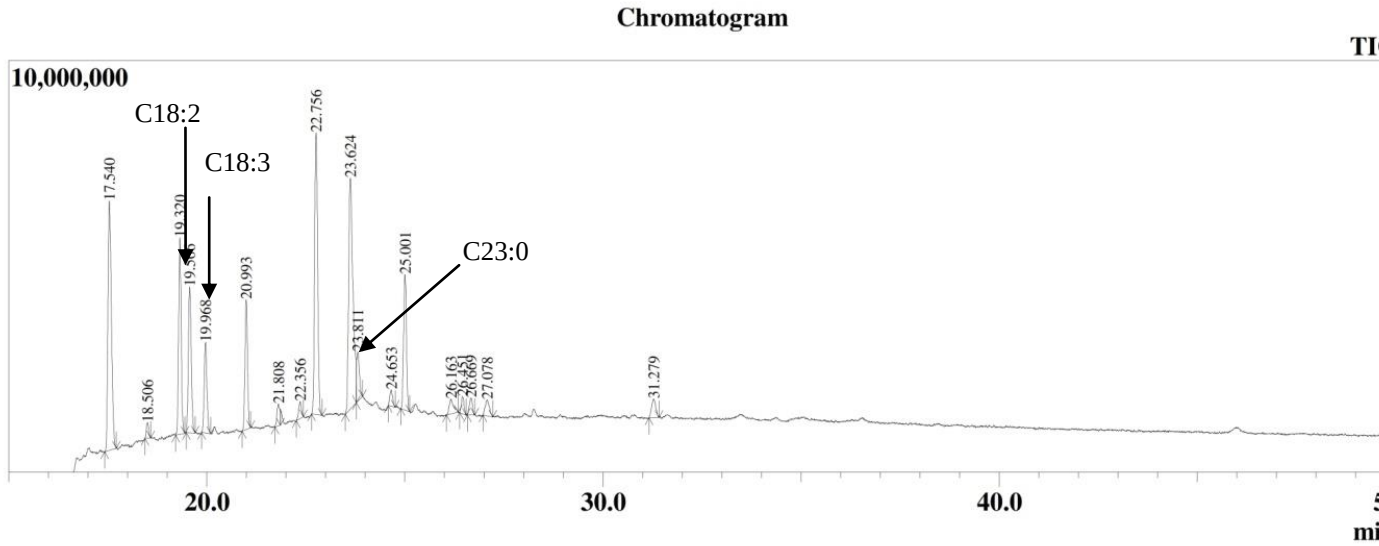


Figure55: Total Ion Chromatogram in EI mode of Callus cultures of *P.oleracea* C18:2= Linoleic acid, C18:3= Linolenic acid, C23:0= Tricosanoic acid

acid and Tricosanoic acid in callus cultures indicated that it can be utilized for the synthesis of odd chain fatty acids.

GC MS reported the presence of long chain fatty acids such as methyl hexacosanoate, methyl octacosanoate and methyl cerebronate in the plant.P.oleracea is a good source of omega 3 fatty as it is able to synthesize long chain fatty acids in both in vivo and in vitro samples.

4.2.2 *P grandiflora*

Betalains are the naturally occurring pigments which have a wide applicability in food industry. *P grandiflora* is rich in betalain and thus its content was evaluated in the in vivo samples. The stability of betalain is a problem as it tends to degrade fast. Generally sodium ascorbate is added to the sample to prevent its degradation. The following experiments were performed first to evaluate the content and second to determine the suitability of sodium ascorbate as a stabilizer. Experiments with sodium ascorbate are generally performed with 50 mM concentration therefore this level of sodium ascorbate was selected for the experiments. The present studies revealed that out of the two components of betalain ie betaxanthin and betacyanin only betacyanin was present while the yellow pigment betaxanthin were absent. Therefore the betalains in the study implies to betacyanin content which was determined by the equation given in Material and method (Section 3.5.3.5).

4.2.2.1 Paper Chromatography of the standard and whole plant of *P grandiflora*

Beet root was taken as the standard source of betalain and the presence of betalain in whole plant of *P grandiflora* was ascertained by paper chromatography. Betalains were extracted from both the samples and the extracts were co chromatographed inproponol: ethanol: water: acetic acid (6:7:6:1) as a mobile phase. The obtained chromatographic pattern revealed the presence of betalain at 0.3 Rf in both the samples confirming the presence of betalains in the plant *P grandiflora*. (Fig. 56a, b, c). Paper chromatography of leaves and root did not separate the bands indicating the absence of the pigment in these plant parts.

4.2.2.2 Effect of sodium ascorbate on the betalains

Sodium ascorbate is an antioxidant which prevents the enzymatic browning and is used as a food additive. The extraction of betalain was carried out in presence or absence of 50 mM sodium

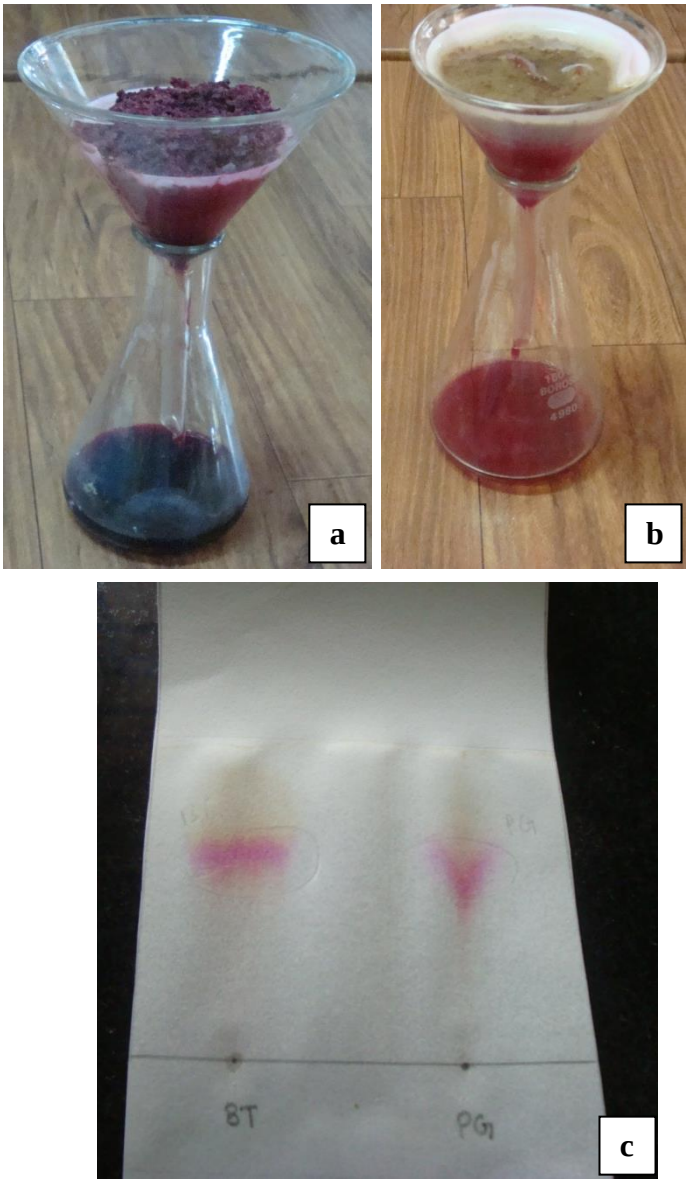


Figure 56: Extraction of betalains (a) Beet root (Standard) (b) Whole plant of *P grandiflora* plant, (c) Paper chromatography of Beet root and whole plant of *P grandiflora*.

ascorbate to determine its effect on the content of betalain. Spectrometric analysis of whole plant revealed that the content of betalain was higher (14.40±0.15) in extract without sodium ascorbate as compared to the extract with sodium ascorbate. Comparing the plant parts, stem had the higher content of betalains in the extract without sodium ascorbate (26.66± 0.19) (Table 19, Fig. 57, 58).

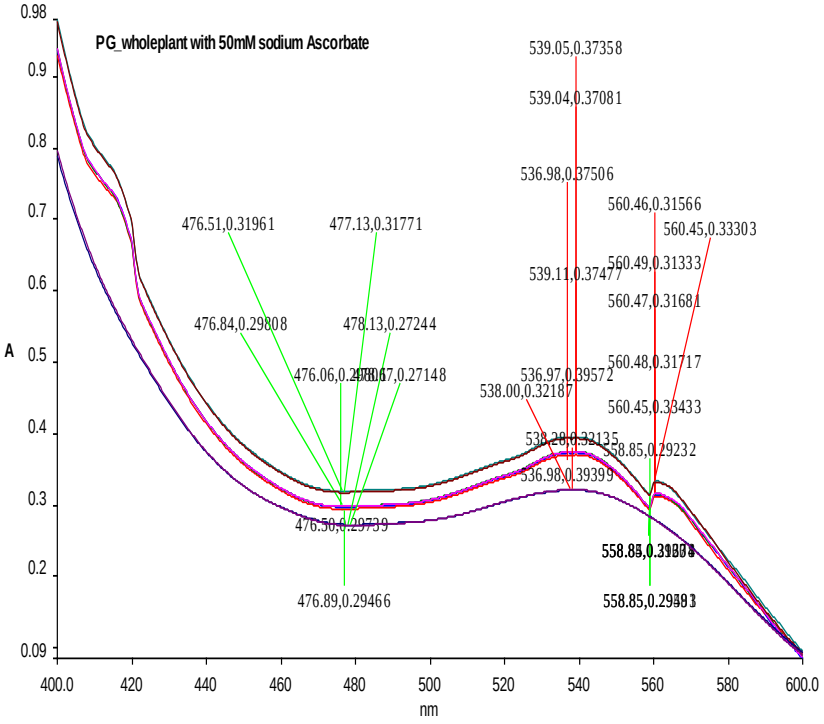


Figure 57: Betalain in whole plant of *P grandiflora* in presence of 50 mM sodium ascorbate.

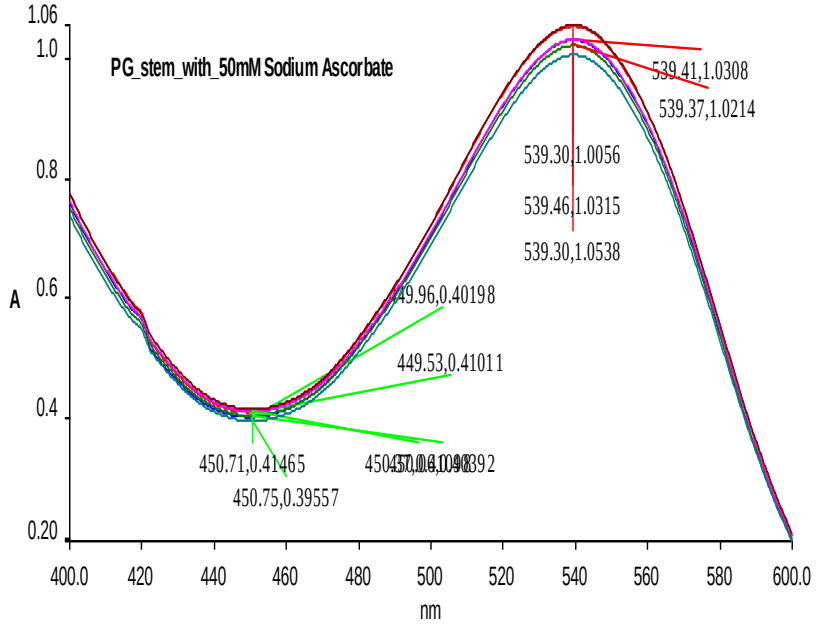


Figure 58: Betalain in stem of *P grandiflora* with 50 mM sodium ascorbate.

The betalain extracts from stem obtained in presence and absence of 50mM sodium ascorbate was left as such for 24 hours after which again their content was determined. It was observed that the sample with sodium ascorbate depicted a stable content as it remained the same (20.13±0.12) while the sample without sodium ascorbate depicted a steep decline to 11.42±0.08. Thus it was inferred that addition of ascorbate to the sample is necessary as it provides stability to the pigment content (Fig. 59).

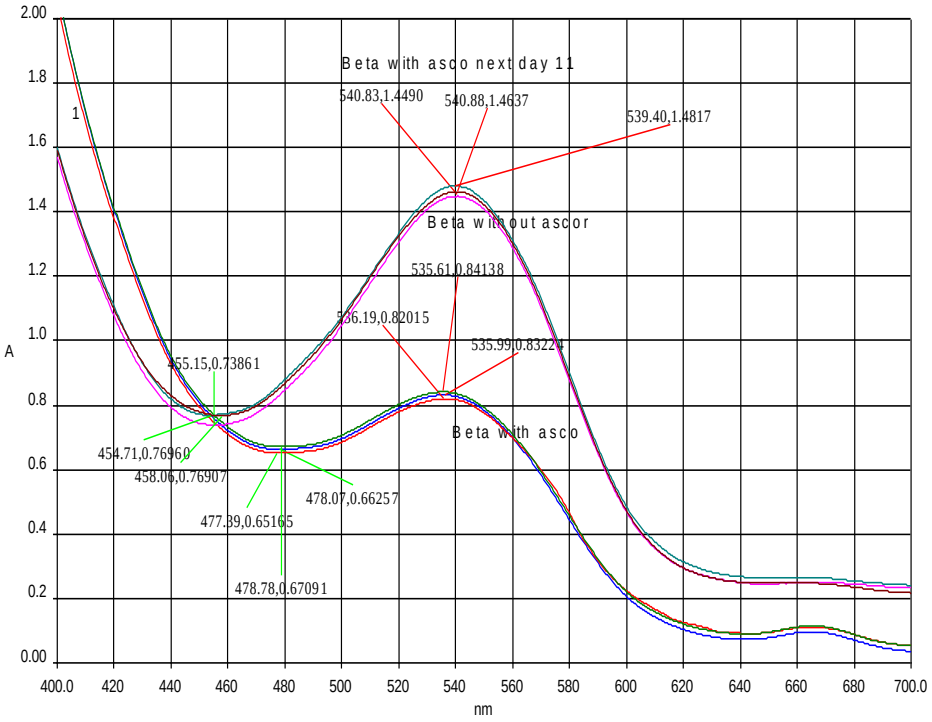


Figure 59: Spectrophotometric analysis of betalain content of stem of *P grandiflora* with and without Sodium ascorbate after 24 hours. The upper curve corresponds to betalain content with 50 mM sodium ascorbate while the lower curve is for betalain content without Sodium ascorbate.

Table 19: Effect of sodium ascorbate in different samples of *P grandiflora*

Samples	**With sodium ascorbate**	**Without sodium ascorbate**
Whole Plant	12.75± 0.14*	14.40±0.15*
Stem	20.08± 0.15*	26.66± 0.19*
Leaves	-	-
Root	-	-
After 24 hours		
Stem	20.13±0.12*	11.42±0.08*

- = Betalain absent, * = The t test was significant at $p<0.01$.

It was concluded from the experiments that the content of betalains was highest in the stem followed by whole plant while in root and leaves the pigment is absent. Addition of sodium ascorbate was also essential to the samples as it provided stability to the pigment and prevented its degradation even after 24 hours.

4.3 SECTION III MUTATION STUDIES

After the regeneration and metabolite standardization technique for the two species of *Portulaca* were carried out, the next part of the work was to evaluate the effect of chemical mutagen (EMS) on regeneration potential/caulogenesis and their subsequent metabolite profiling. EMS is a potent chemical mutagen which is effective in inducing mutations in a large number of medicinal, horticultural and cereal crops. The variability induced by this mutagen has been instrumental in improving many useful traits in the plants. The genus *Portulaca* as discussed is a rich reservoir of primary and secondary metabolite and the aim of the next series of experiments were to determine the effect of EMS on regeneration and callus induction on *P oleracea* and the subsequent metabolite profiling

4.3.1 *P oleracea*

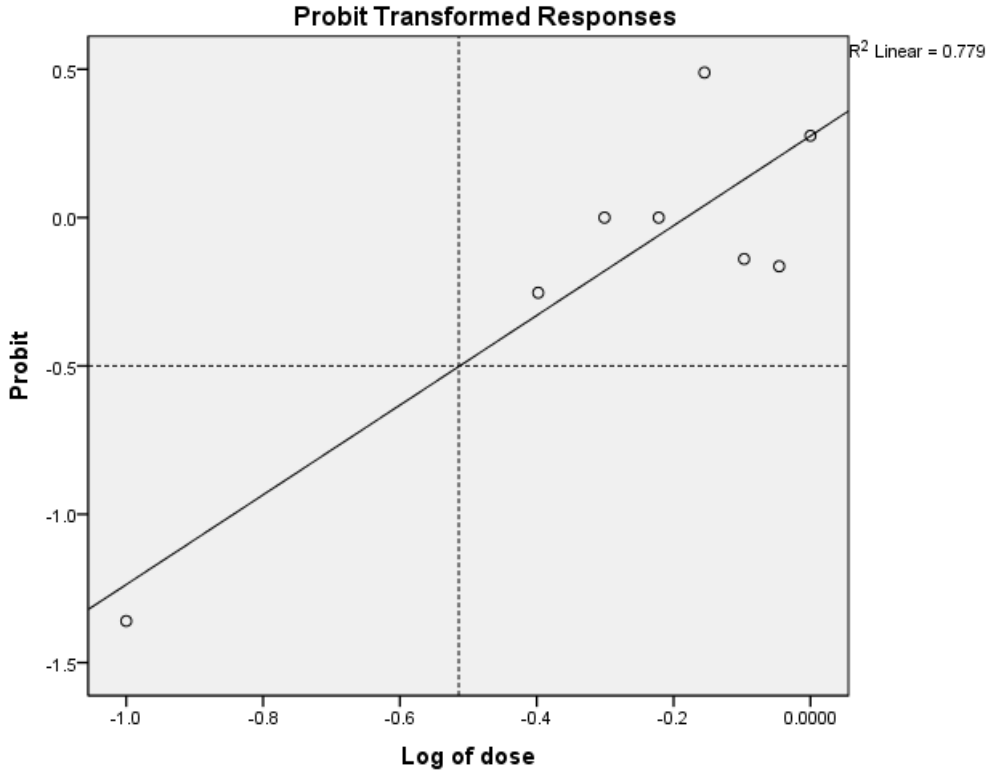
The vitro leaves were treated with EMS and its effect on shoot and callus formation was evaluated. The in vitro shoots and callus regenerated from the treated leaves in their respective protocols were subjected to fatty acid profiling. .

4.3.1.1 Determination of Lethal Concentration 50 (LC50)

For mutation studies it is necessary to determine a LC50 dose where at least 50% of the population can survive the effect of external agent. Probit analysis is one such statistical tool which is utilized to determine LC50 value.

The in vitro leaf explant was treated with a mutagen EMS (0.1-1%) and inoculated on an optimized media for regeneration and the mortality was noted. To determine LC50 dose probit analysis was conducted with respect to dose concentration and mortality number. A graph of log of dose versus probit was obtained and the value of probit 0.5 was -0.514. Since the value is in log so an inverse log of -0.514 was calculated to be 0.3061. Thus the LC50 value calculated from probit analysis was 0.3% EMS. The value of Linear Regression Plot of the probit value against the log dose was R^2 =0.779 (Fig. 60).

Figure 60: Linear Regression Plot of the Probit value against the log (dose), to determine LC50 dose in *P oleracea* leaf explant treated with EMS (0.1-1%).R^2 =0.779



4.3.1.2 Effect of different doses of EMS (0.1-1%) on in vitro leaf explants of *P oleracea*

In vitro leaf explant was treated with different doses of EMS (0.1-1%) and cultured on the optimized media for regeneration (5 µM BA and 10 µM Kin) and callus (2.5 µM BA and 2.5 µM 2,4D) induction.

(a) Treated explant inoculated on shoot regenerating media (5µM BA and 10µM Kin)

EMS treated in vitro leaf explant exhibited a whole spectrum of morphogenesis under the influence of different concentrations of EMS (0.1-1%). The untreated in vitro leaf explant growing on shoot inducing medium served as control. It was observed that at low concentrations of EMS (0.1-0.3%) in vitro shoots were formed through indirect organogenesis. The leaf explant swelled and formed green callus on the margins of explant within 3 weeks. This callus differentiated shoots buds in 4^{th} week and formed shoots by the end of 6^{th} week. After transfer of these shoot clusters to coconut water in 8^{th} week well developed in vitro shoots were obtained at the end of 12 weeks. The number of shoots decreased with increase in concentration of EMS and optimum number of shoots/explant (10.2±0.84) were obtained at 0.2% EMS (Fig. 61a-c, Table 20). It was noted that the number

of shoots formed in treated optimized concentration (0.2% EMS) of leaf explant was less (10.2±0.84) as compared to control (19.18±0.86).

As the concentration was increased to 0.4% EMS, the shoot buds were formed which resulted in clusters of stunted shoots with reddish leaves (Fig. 61d). Further increase in concentration (0.5-0.7%) the explant initiated profuse callus which was green nodular with few shoot buds which grew into stunted shoots as they failed to elongate even after 12 weeks (Fig. 61e, f). At high concentration of 0.8-1% EMS there was vigorous increase in the callus. The number of shoot buds decreased and the callus was green and compact at 0.8% EMS (Fig. 61g). At 0.9% EMS the callus became white green and compact with few shoot buds while at 1% EMS there was profuse initiation of white compact (Fig. 61h, i). Thus the leaf explants initiated in vitro shoots at low concentration of EMS (0.1-0.3%) and gradually lost its organogenic potential such that at high concentrations of EMS (0.9-1%) only profuse callusing was achieved.

During the course of study some morphological variants were also obtained e.g. the size of the leaves formed in various cultures enlarged considerably as in cultures of 0.1% EMS and 0.2% EMS (Fig. 62a(i) and (ii)). The wild and in vitro grown leaves of the plant are ovate however variations in leaf shape was observed in a culture of 0.2% EMS where the leaves developed acicular shape (Fig.62b).Optimum number of in vitro shoots/explant were achieved at 0.2% EMS (10.2±0.84) and a variation in number of shoots/explant was obtained in a culture of 0.2% EMS where the number of shoots increased to 24 shoots however many of which did not elongate (Fig. 62c).It was noted that higher concentrations of EMS suppressed the organogenic potential of the leaf explant and shoot buds with stunted shoots were induced at 0.4% EMS. A variant shoot cultures were obtained at this dose where cluster of in vitro shoot was formed (Fig.62d).At a high concentration of 0.6% EMS the invitro leaf explant induced green nodular callus interspersed with shoot buds and stunted shoots. A variation in the culture was obtained where elongated well developed in vitro shoots were formed at this concentration (Fig.62e).

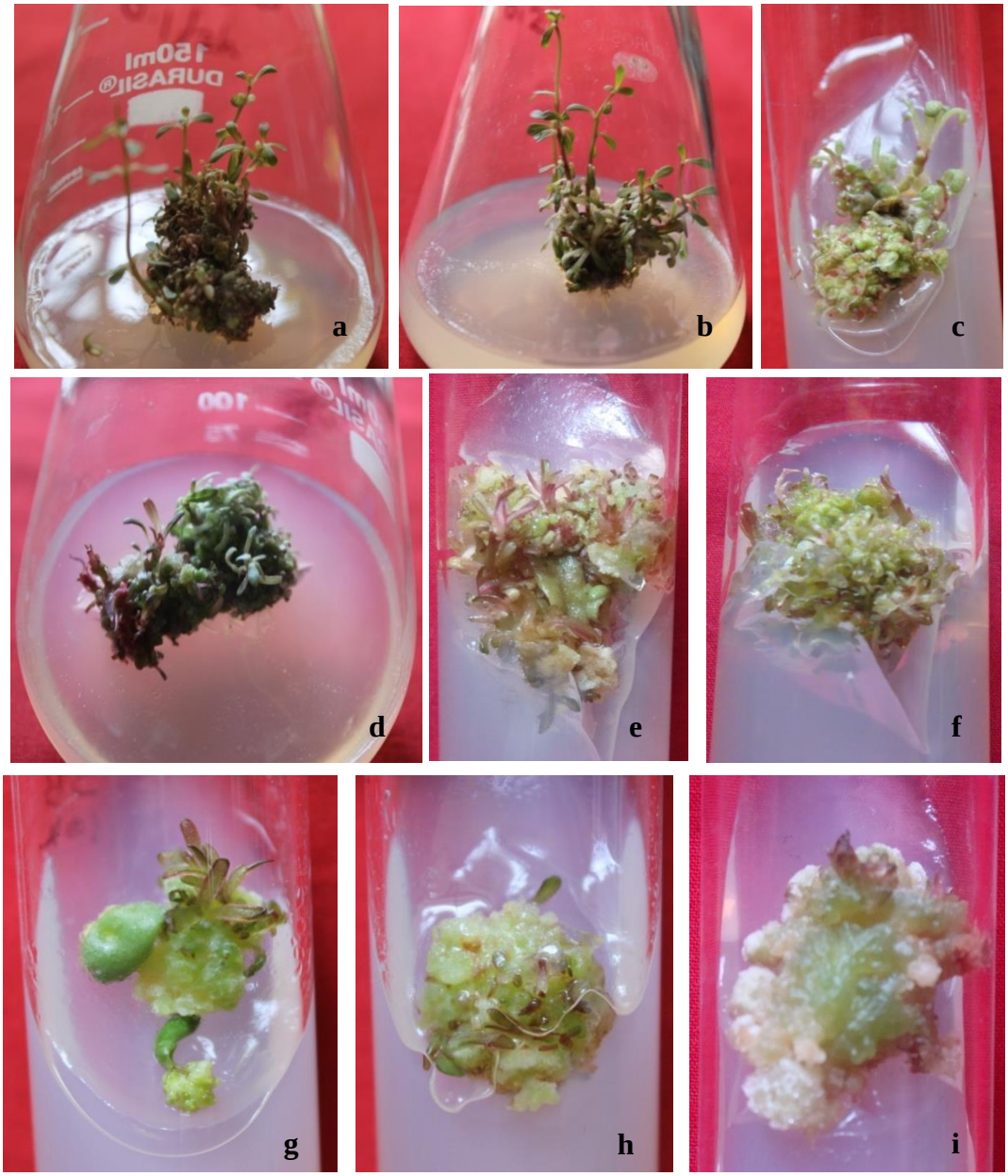


Figure 61: Effect of different concentrations of EMS after 12 weeks (a) Invitro shoots at 0.1%EMS, (b) In vitro shoot formation at 0.2% EMS,(c) The number of in vitro shoot declines at 0.3% EMS, (d)Stunted in vitro shoots at 0.4% EMS,(e and f)Induction of green nodular callus interspersed with shoot buds at0.5% and 0.6% EMS respectively, (g)Compact green callus and shoot buds at 0.8% EMS, (h) greenish white compact callus at 0.9% EMS,(i) White compact callus at 1% EMS.

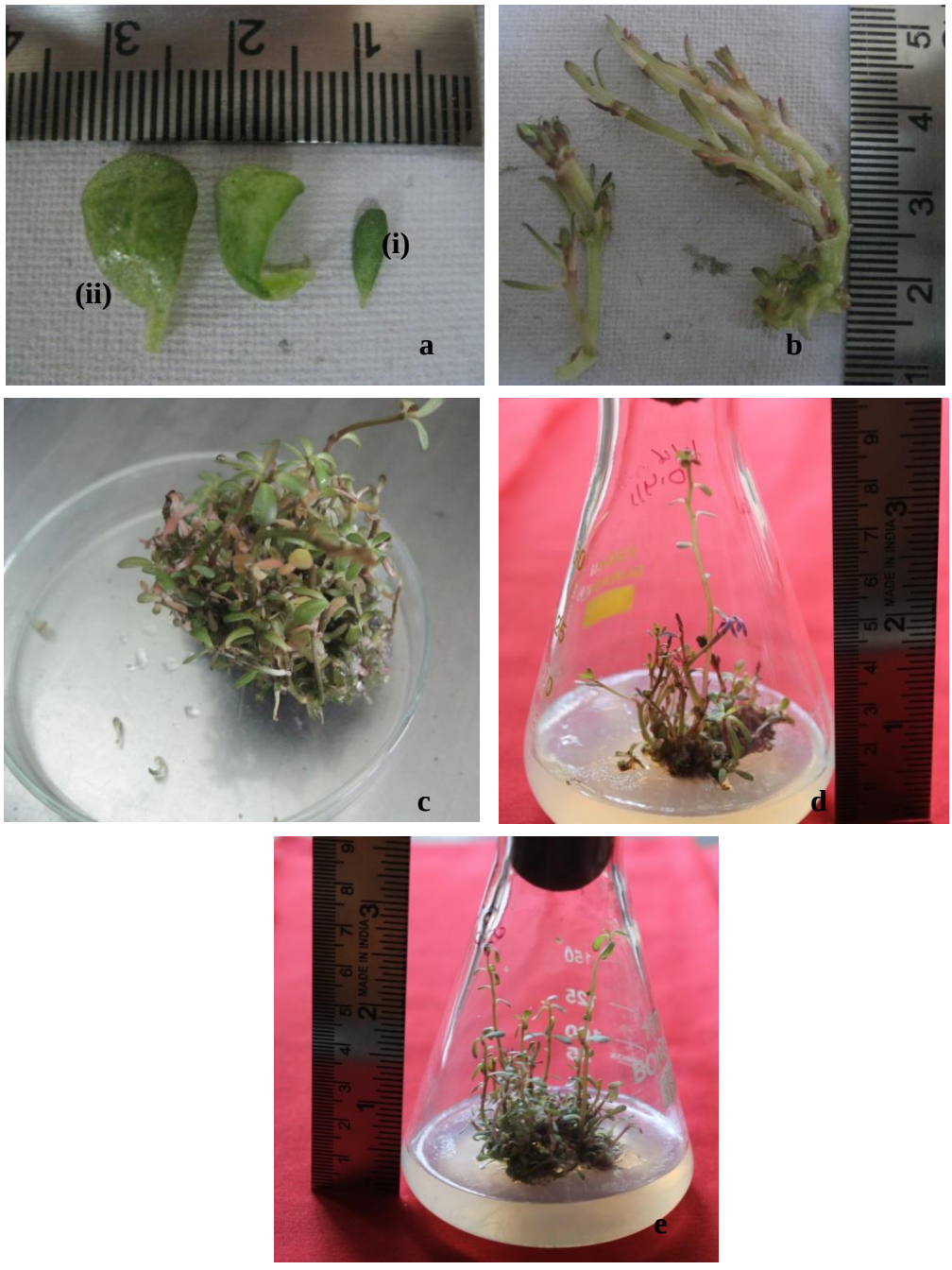


Figure 62: The variations expressed in different doses of in vitro leaf explants cultured on 5µM BA and 10µM Kin. (a) Leaves enlarged in some in vitro raised plants under the influence of 0.1% EMS (i) Control, (ii) treated leaf, (b) a variant plant at 0.2% EMS where the leaves turned acicular, (c) number of in vitro shoots increased at 0.2% EMS. (d) An elongated in vitro plant obtained at 0.4% EMS, (e) In vitro leaf explant formed elongated shoots instead of callus and shoot buds at 0.6% EMS.

Table20: Effect of different doses of EMS on in vitro leaf explants of *P oleracea* after 3 months.

Dose (EMS%)	Response%	Number of Shoots/ explant (*Mean±S.E)
0.1	100	6.2±0.67 b
0.2	90	10.2±0.69 c
0.3	90	3.1±0.73 a

*Means followed by the same letters are statistically not significant with Tukey'sHSD test (n=10) and the value of $p \leq 0.05$.

(b) Treated explants inoculated on callus initiating media (2.5µM BA and 2.5µM 2,4D)

The in vitro leaves treated with different concentrations of EMS (0.1-0.4%) were inoculated on callus inducing medium (2.5 µM BA + 2.5 µM 2,4D) and their effect was analyzed on caulogenesis. The callus induction initiated from the proximal end and soon covered the entire leaf explant and grew at a fast pace. The callus was creamish white, compact and at localized regions it was friable. The fresh weight and dry weight of the callus indicated that there was an increase at 0.1-0.2% EMS after which there was a decline in biomass. Thus optimum callus mass was obtained at 0.2% EMS (18.61±2.68 gm, Fig.63a,b). In the callus inducing medium optimum callus weight of 18.61±2.68 gm/explant was induced at 0.2% EMS. A variant callus culture was obtained where excessive callus induction was obtained and the biomass of the callus was 38 gm. In all the combinations the callus formed was loose and easily fragmented but a variant callus was obtained at 0.3% which was hard and compact (Fig. 63c, d and Fig. 64).

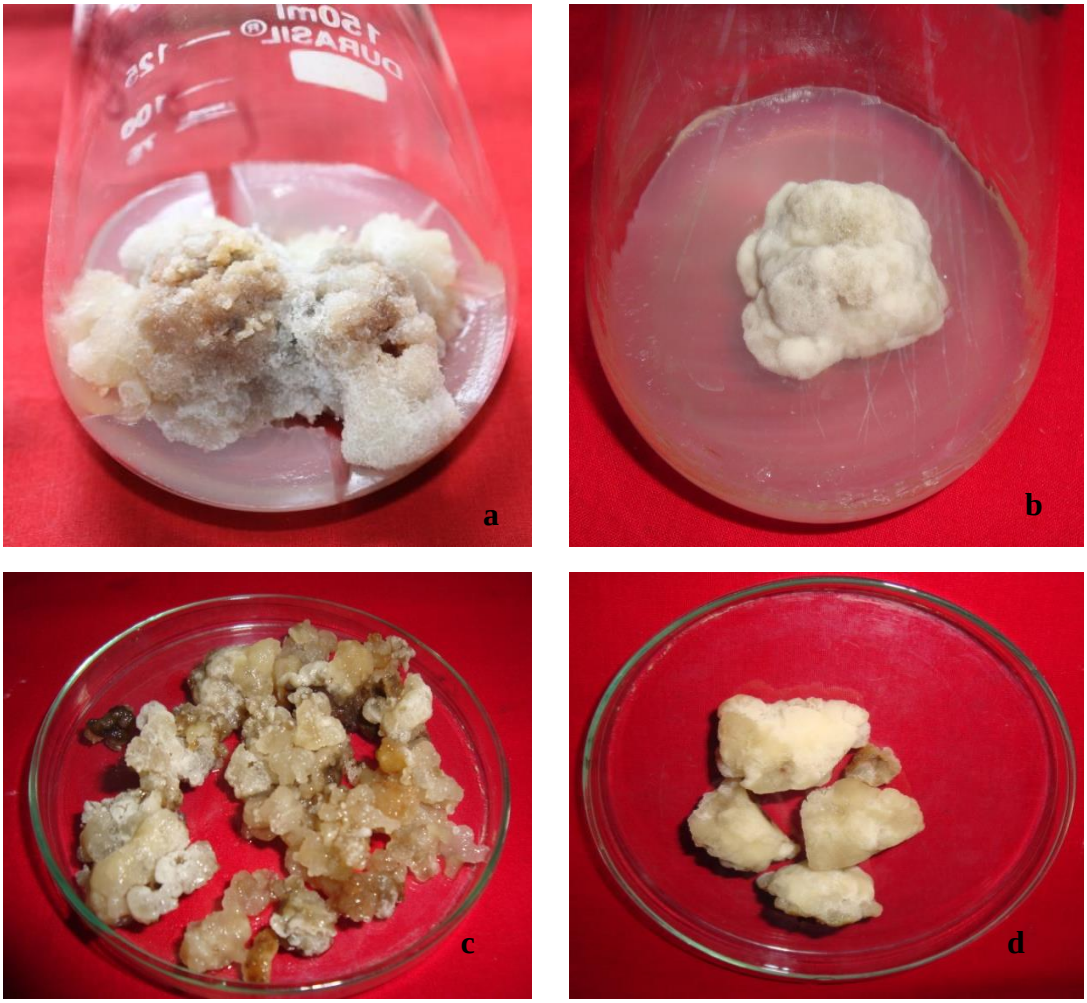
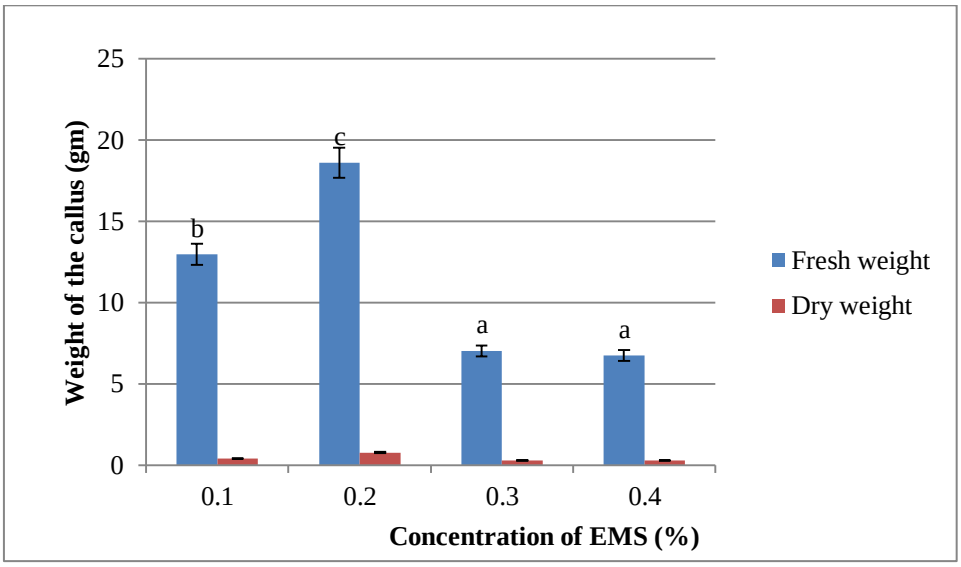


Figure63: Callus induction in EMS treated in vitro leaf explant (a) Optimum callus formation at 2.5 µM BA and 2.5 µM 2,4D from explants treated with 0.2% EMS, (b) The biomass of callus decreased from invitro leaf explant treated with 0.3% EMS. (c) callus cell line variant which formed excessive callus (38 gm) at 2.5 µM BA and 2.5 µM 2,4D from explants treated with 0.2% EMS, (d) A hard compact callus formed at 2.5 µM BA and 2.5 µM 2,4D from explants treated with 0.3% EMS.

Figure 64: Effect of different doses of EMS on callus induction from in vitro leaf explant of *P oleracea* after 3 months.



Means followed by the same letters are statistically not significant with Tukey's HSD test where the value of p= 0.05

4.1.3 Fatty acid fingerprinting of the in vitro cultures obtained after EMS treatment

EMS treated cultures exhibited a whole pattern of morphogenesis where shoots were induced at low concentration and callus was initiated at a high concentration of 0.5-1%. The last part of the study was therefore aimed to assess the influence of EMS on fatty acid synthesis in both in vitro shoots and callus.

(a) Relative content of fatty acid in EMS treated in vitro shoot cultures

The major fatty acid in the untreated in vitro shoots cultures were Palmitic acid, Linolenic acid and Linoleic acid. The in vitro shoot cultures obtained from EMS treated leaf (0.1-0.3%) depicted a variation in the content of fatty acid. Palmitic acid was the major fatty acid in shoot cultures of 0.1 and 0.3% EMS (11%) while its content decreased drastically at 0.2% EMS (1.82±0.16). The content of Linoleic and Linolenic acid was similar at 0.2% dose. Similarly Eicosanoic acid % was 6.01± 0.06 which was high with respect to the doses of 0.1 and 0.3%. It was also noted that untreated in vitro shoots did not synthesize long chain fatty acid but the treated cultures formed long chain fatty acids such as Tricosanoic acid and Tetracosanoic acid (Table 21, Fig. 65,66 and 67).

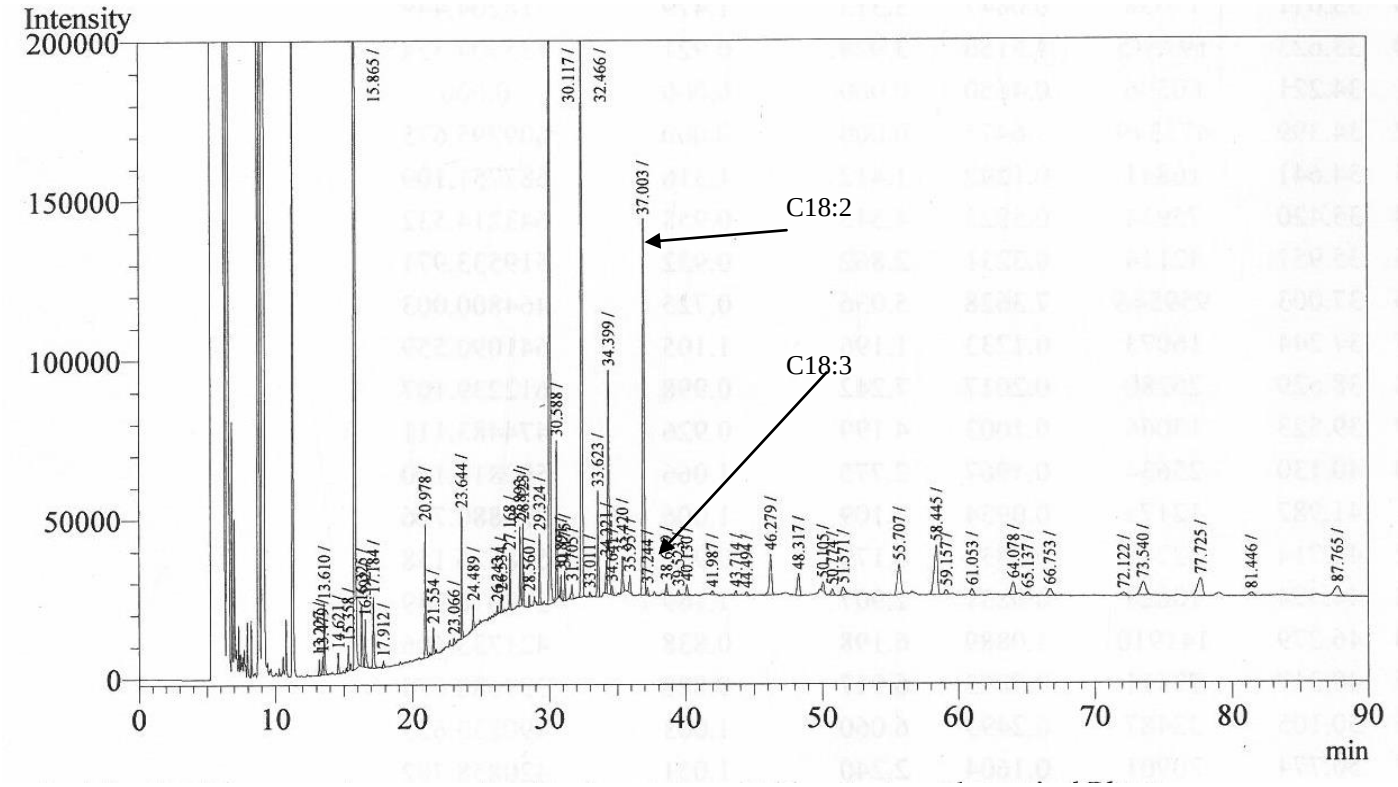


Figure 65: Fatty acid profiling of 0.1% EMS treated shoots of *P oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid

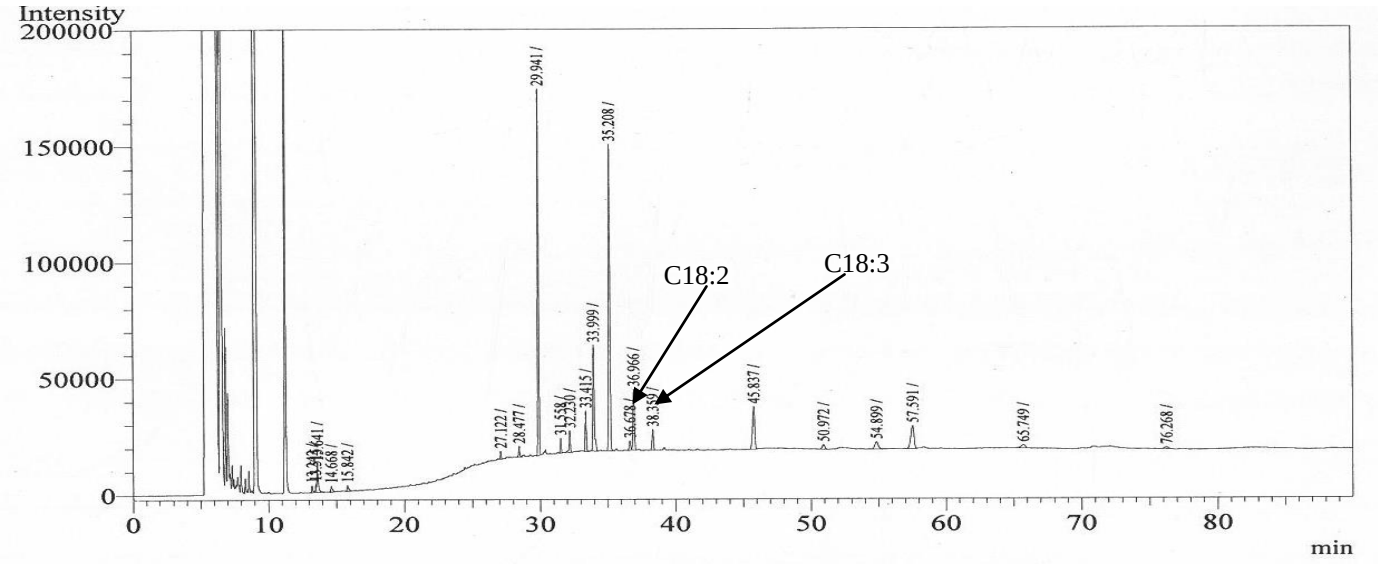


Figure 66: Fatty acid profiling of 0.2% EMS treated shoots of *P oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid

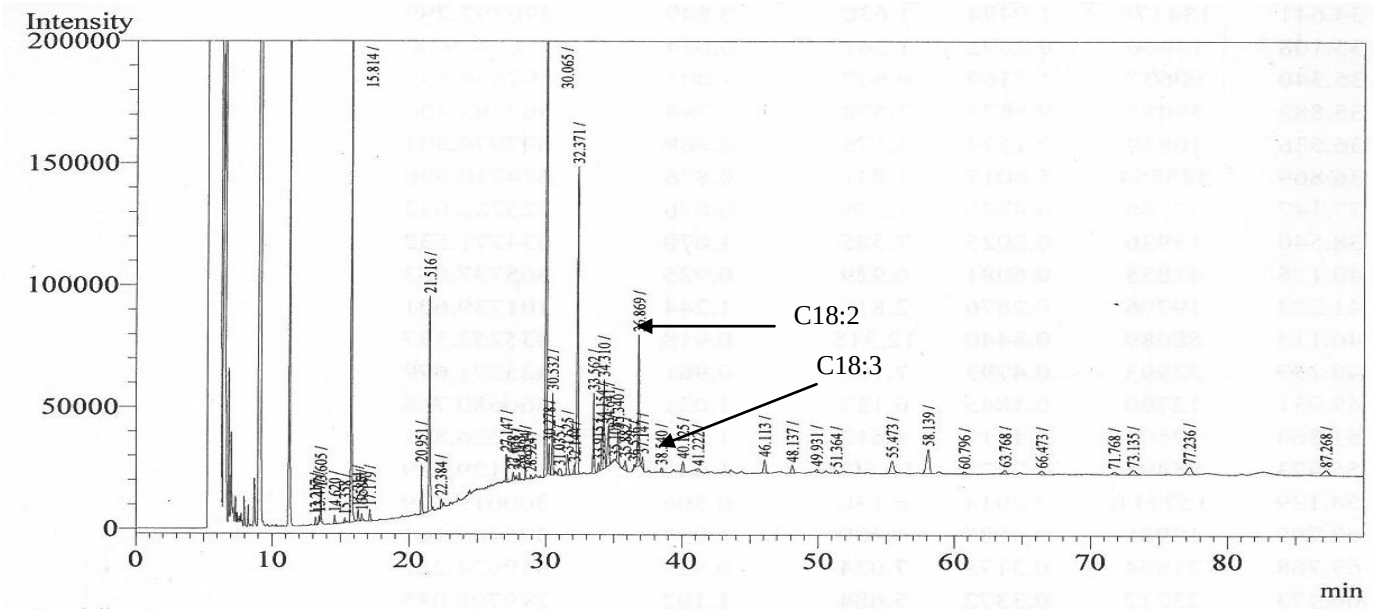


Figure67: Fatty acid profiling of 0.3% EMS treated shoots of *P oleracea*, C18:2= Linoleic Acid, C18:3= Linolenic acid

Table 21: Relative content of fatty acids on EMS treated in vitro cultures of *P oleracea*

Fatty Acids	0.1%EMS Shoot	0.2%EMS Shoot	0.3%EMS Shoot	0.1%EMS callus	0.2%EMS callus	0.3%EMS callus
Palmitic Acid	11.47± 0.61	1.82± 0.16	11.03± 0.32	7.6± 0.34	9.26± 0.69	2.73± 0.47
Heptadecanoic Acid	--	3.15± 0.19	--	--	--	--
Stearic Acid	--	--	3.64± 0.02	--	10.65±1.22	--
Linoleic Acid	7.64± 0.19	1.02±0.62	5.35± 0.28	12.36± 1.16	--	--
Linolenic Acid	0.26±0.04	1.84±0.45	0.26± 0.04	--	--	--
Eicosanoic Acid	1.06± 0.04	6.01± 0.06	0.85± 0.04	4.55± 0.81	3.78± 0.23	--
Docosanoic Acid	2.05± 0.10	0.75± 0.04	0.17± 0.01	0.81± 0.02	1.15± 0.11	2.1± 0.13
Tricosanoic Acid	--	1.97± 0.21	2.27± 0.16	5.12± 0.37	--	--
Tetracosanoic Acid	0.36± 0.03	0.98± 0.15	0.31± 0.06	0.94± 0.14	0.83± 0.04	2.42± 0.35

Values (n=3) are expressed as % of Means ± S.E.

(b) Relative content of fatty acids in EMS treated callus cultures

The in vitro leaf explants was treated with EMS (0.1-0.3%) and inoculated on callus inducing medium. Observations revealed that there was a decline in the variety of fatty acids with increase in concentration of EMS (Table 24). At 0.1% Linoleic acid was the major fatty acid accounting to 12.36±1.16% while it was absent in 0.2 and 0.3% EMS. Linolenic acid is reported to be absent in all the treated doses. Stearic acid is a saturated acid which is extensively used in the preparation of soaps, paints and varnishes. The presence of Stearic acid was not consistent in all the studied doses and the content was also reported to be low. Interestingly at the dose of 0.2% a high content (10.65±1.22) of Stearic acid was obtained which was absent in 0.1 and 0.3% EMS doses. Presence of long chain fatty acids was noted in all the concentrations however callus cultures of 0.1% EMS reported a higher content of Eicosanoic (4.55±0.81) and Tricosanoic acids (5.12±0.37) as compared to treated in vitro shoot cultures (Table 21, Fig. 68,69 and 70).

It was thus concluded from the fatty acid profile of treated calluses that Linoleic acid is the main fatty acid synthesised in this mode of organogenesis while Linolenic acid was completely absent.

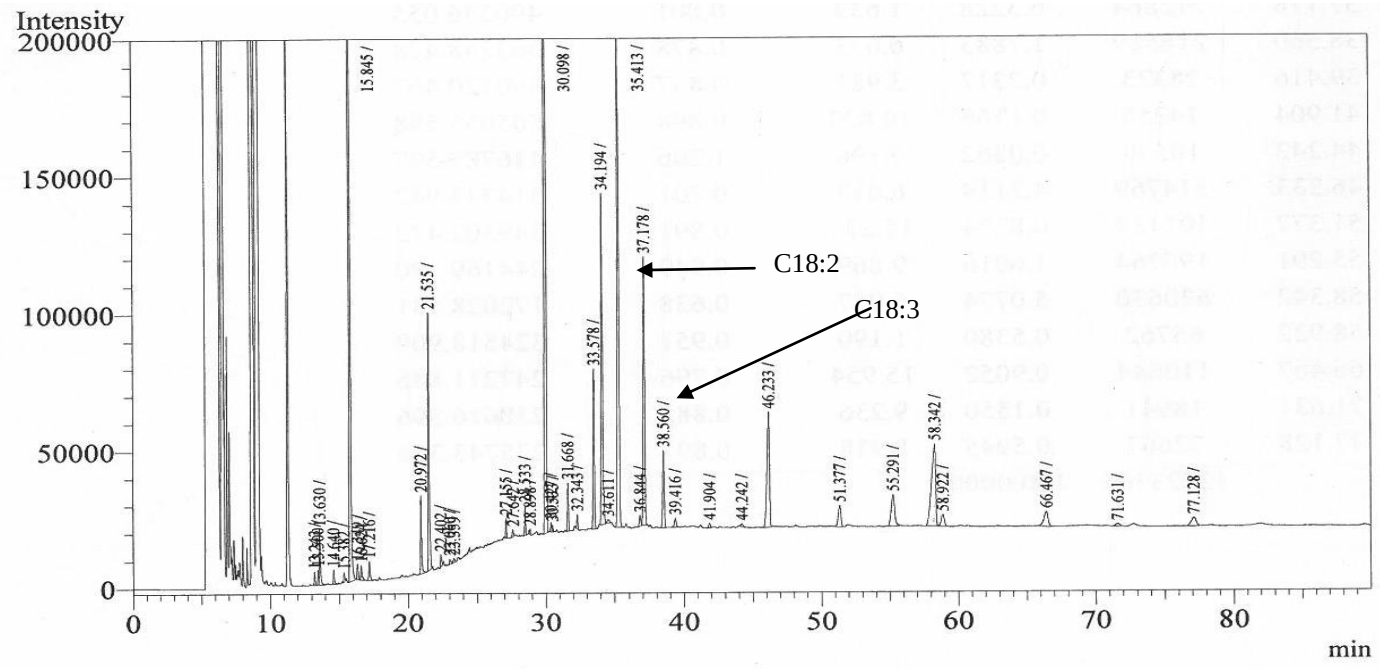


Figure 68: Fatty acid profiling of 0.1% EMS treated callus of *P oleracea*, 18:2= Linoleic acid, 18:3= Linolenic acid

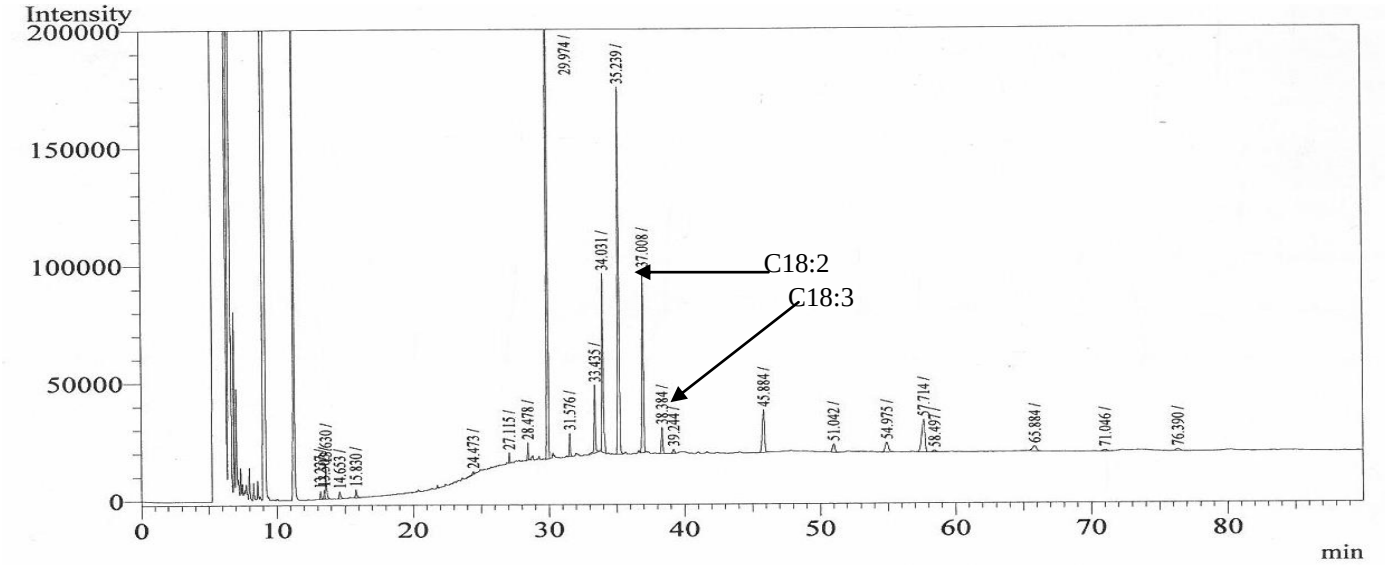


Fig 69: Fatty acid profiling of 0.2% EMS treated callus of *P oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid

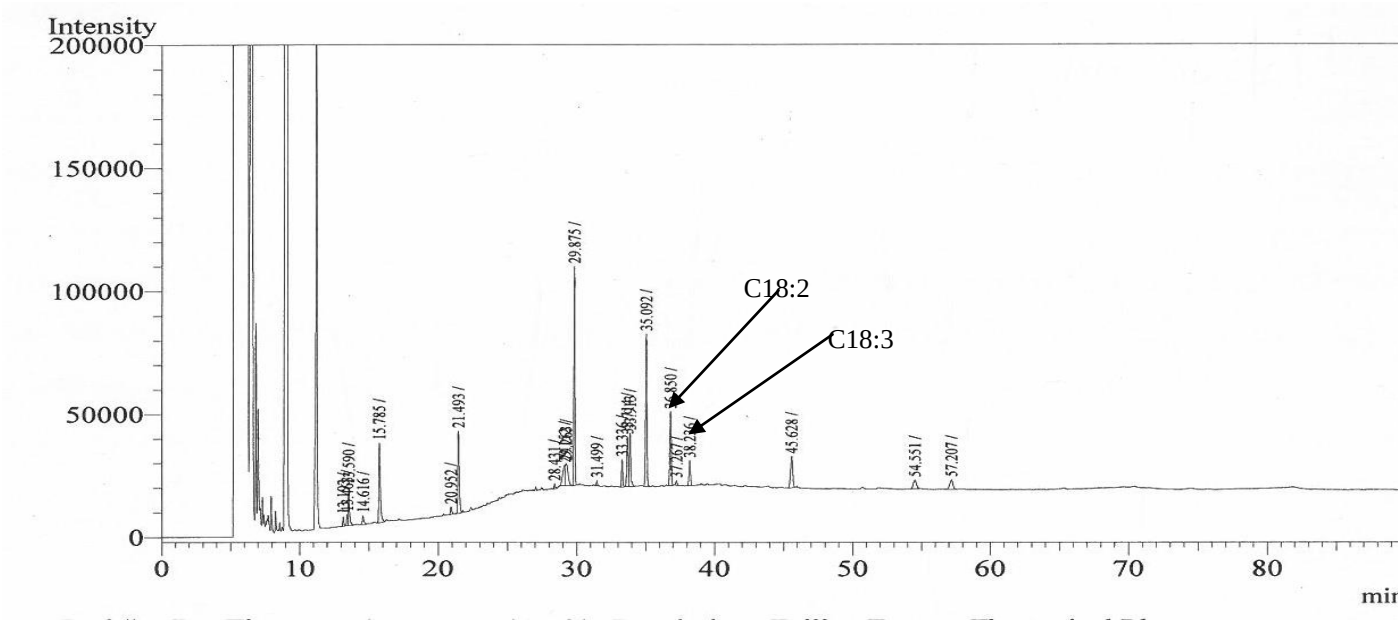


Figure 70: Fatty acid profiling of 0.3% EMS treated callus of *P oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid

Due to the EMS treatment the synthesis of some fatty acid increases as was noted in 0.2% EMS concentration where there was an increased production of Stearic acid. These results suggested that variability can be induced in the fatty acid profiling which can be stabilised and enhanced by different manipulations.

During the course of study morphological variant (at 0.4 and 0.6%) were obtained as discussed. The fatty acid profile of these variants was also determined. Omega fatty acids were not detected in the shoot cultures of 0.4% EMS and presence of Trans Elaidic acid is reported which was not synthesised in other cultures studied (Fig. 71).

A wide range of fatty acids were detected in the variant shoot culture of 0.6% EMS. Both Linoleic and Linolenic acids were synthesized in the shoot culture however Stearic acid was the prominent fatty acid (10.77±0.28) followed by Palmitic and Linolenic acid (5%). Long chain fatty acids from Docosanic to Hexacosanoic acid were present in this shoot cultures (Table 22, Fig. 72).

Table 22: Fatty acid profiling of variant in vitro shoot cultures at 0.4 and 0.6% EMS of *P.oleracea*

Fatty Acids	0.4% EMS Shoot	0.6% EMS Shoot
Palmitic Acid	4.19±0.35	5.66±0.29
Stearic Acid	2.19±0.31	10.77±0.28
Linolenic Acid	-	5.1±0.24
Linoleic acid	-	1.24±0.45
Eicosanoic Acid	-	0.54±0.09
Trans Elaidic Acid	3.62±0.28	-
Docosanoic Acid	0.58±0.09	0.73±0.03
Tricosanoic Acid	-	0.5±0.03
Tetracosanoic Acid	0.19±0.02	1.04±0.04

Values (n=3) are expressed as % of Means ± S.E

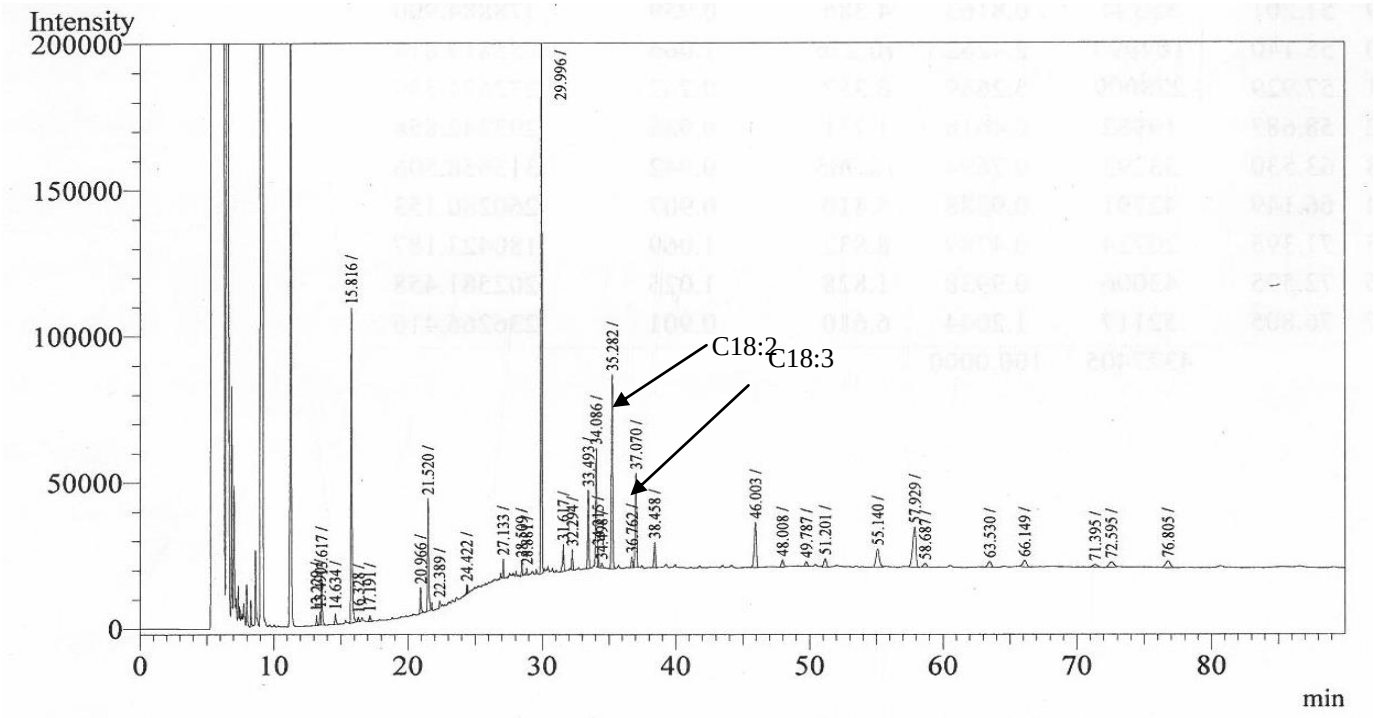


Figure 72: Fatty acid profile of 0.6% EMS treated shoot cultures of *P oleracea*, C18:2= Linoleic acid, C18:3= Linlenic acid

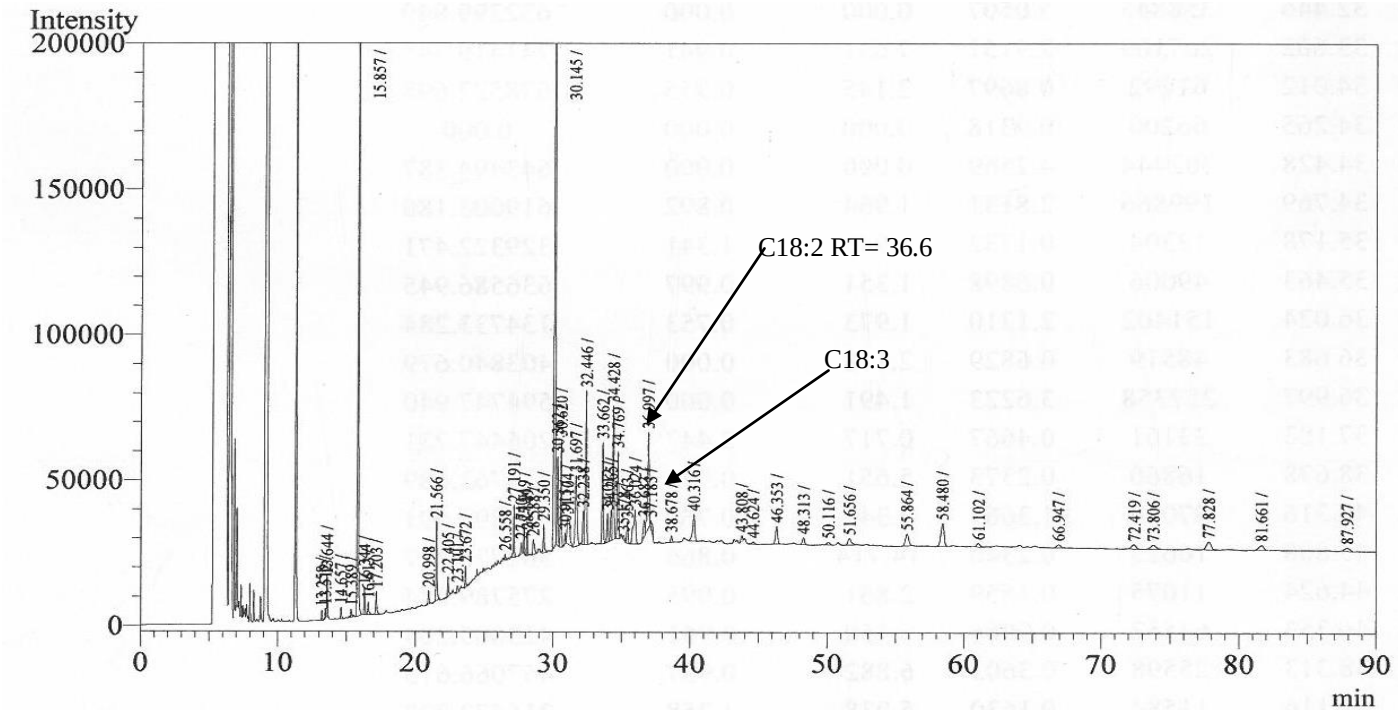


Figure 72: Fatty acid profile of 0.4% EMS treated shoot cultures of *P oleracea*, C18:2= Linoleic acid, C18:3= Linolenic acid

4.3.1.5 Mass Spectrometry of the fatty acids present in EMS treated samples.

GCMS studies on the treated in vitro shoot cultures confirmed the presence of fatty acids reported in GC analysis. Besides this 0.1%EMS dose also synthesised 16 Octadecenoic Acid. Callus cultures of 0.2% EMS also synthesized cyclo propanoic acid such as Methyl 9,10 methylene hexadecanoate, Methyl 9,10 methylene octadecanoate and Octdecanoic acid 9,10,12 trimethoxy methyl ester. The fatty acid profile of 0.3% EMS dose was simple and GSMS profile also reported the presence of 10,13 Octadecadienoic acid methyl ester and an isomer of Docosanoic Acid (Fig. 73,74and 75).

GCMS studies of treated callus cultures revealed that the culture synthesized cyclo propanoic acid such as cyclo propane octanoic acid, 2 hexyl methyl ester and cyclo propane octanoic acid. Besides this long chain fatty acids such as Docosanoic, Tetracosanoic and Hexacosanoic acid were also synthesized (Fig. 76,77 and 78).

In vitro shoot variant (0.4, 0.6% EMS) synthesized cyclo propanoic acid such as cyclo propane octanoic acid, 2 hexyl methyl ester and cyclo propane octanoic acid. Besides this long chain fatty acids such as Docosanoic, Tetracosanoic and Hexacosanoic acid were also synthesized (Fig. 79 and 80). The mass spectra of Linoleic acid and Linolenic acid was obtained from shoot cultures treated with 0.1% EMS (Fig. 81 and 82)

Thus it was concluded in the present experiments that the treated callus cultures had the potential to synthesize long chain fatty acids and industrially important Stearic acid while for omega fatty acids shoot cultures were a better option. GCMS studies also study revealed that under the influence of EMS the in vitro shoot as well as callus are capable for synthesizing long chain fatty acids and some novel fatty acids such as cyclo propanoic acid which has industrial application.

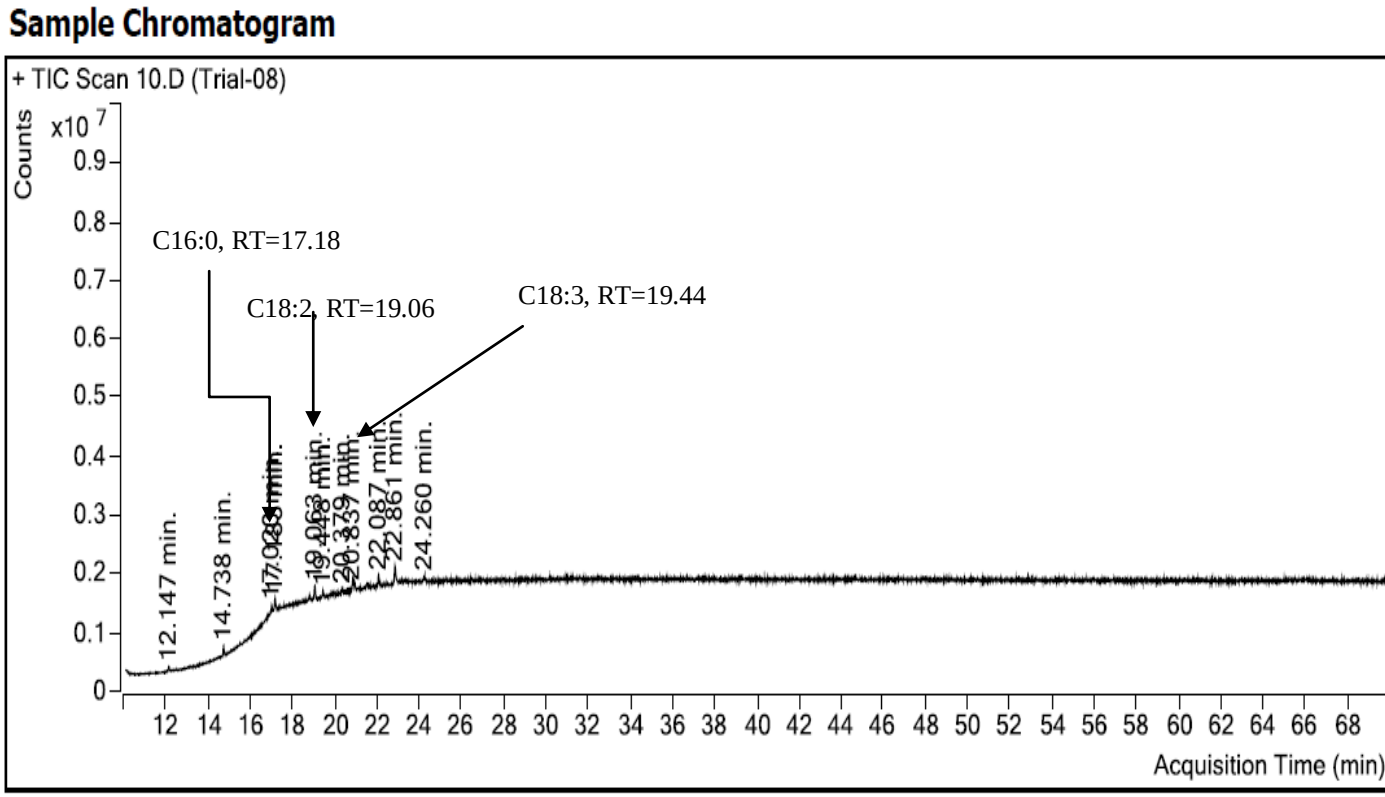


Figure 73: Total Ion Chromatogram of fatty acids present in 0.1% EMS treated shoots of *P oleracea*, C16:0= Palmitic acid, C18:2= Linoleic acid, C18:3= Linolenic acid

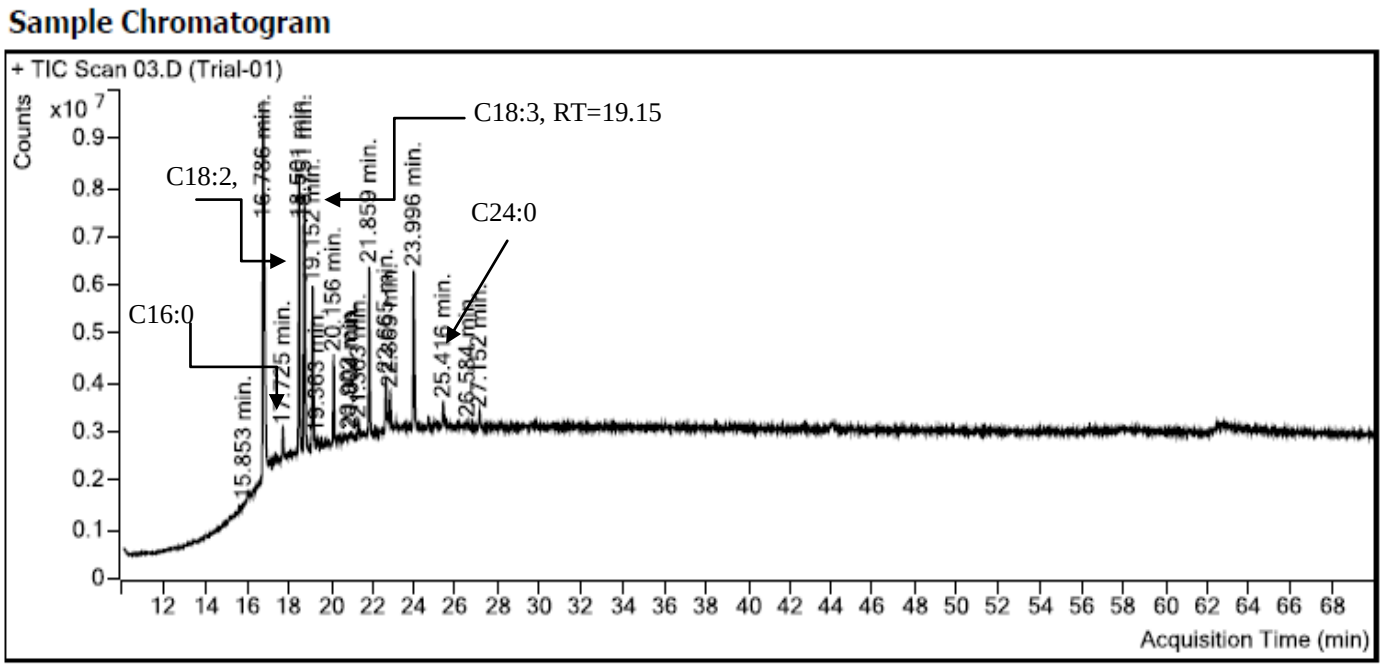


Figure 74: Total Ion Chromatogram of fatty acids present in 0.2% EMS treated shoots of *P oleracea*, C16:0= Palmitic acid, C18:2= Linoleic acid , C18:3= Linolenic acid

Sample Chromatogram

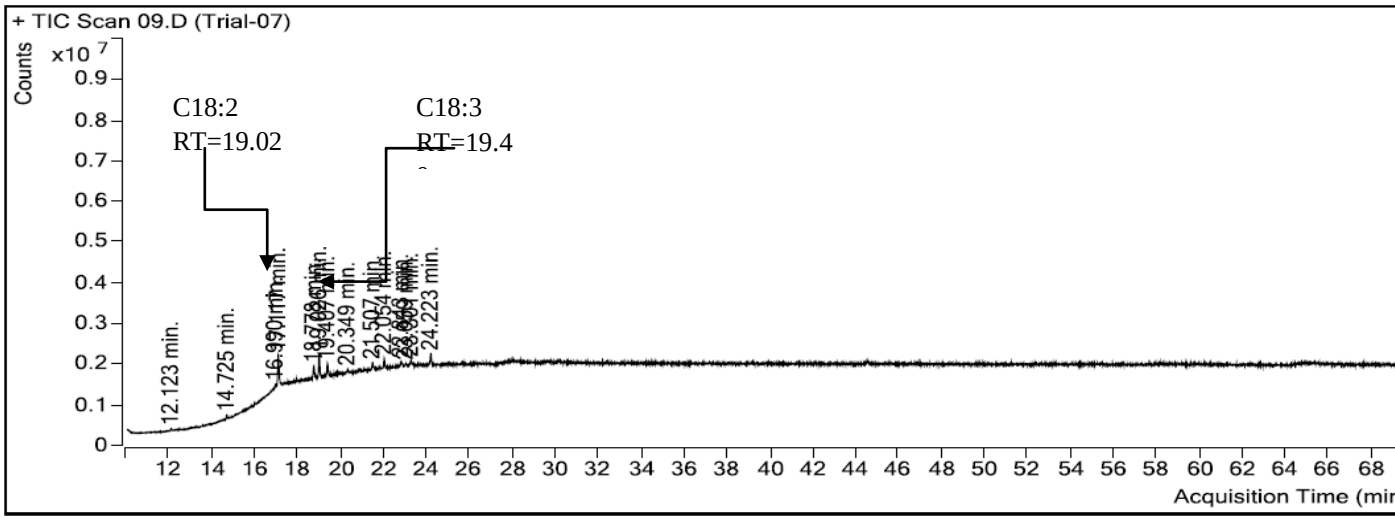


Figure 75: Total Ion Chromatogram of fatty acids present in 0.3% EMS treated shoots of *P.oleracea,* C18:2= Linoleic acid, C18:3= Linoleic acid

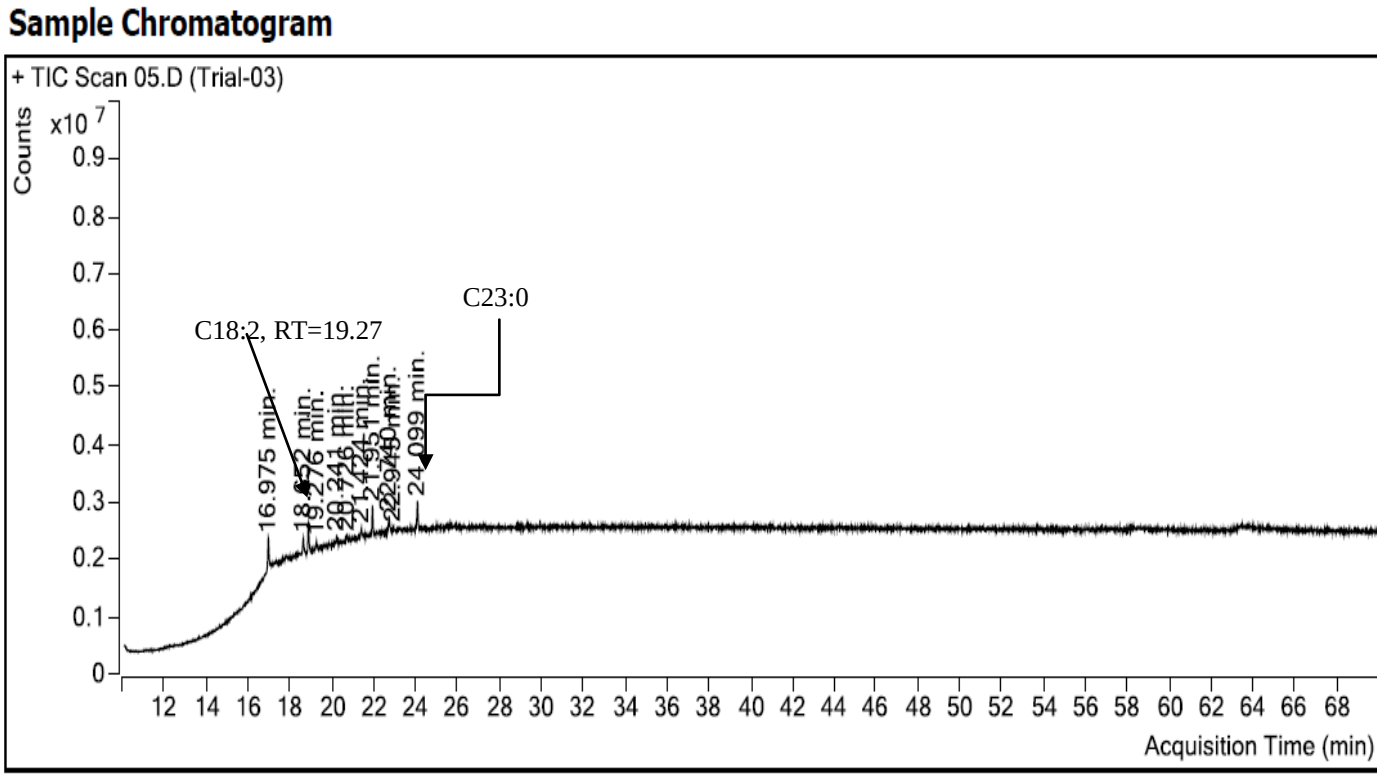


Figure 76: Total Ion Chromatogram of fatty acids present in 0.1% EMS treated callus of *P oleracea*,C18:2= Linoleic acid, C23:0=Tricosanoic acid

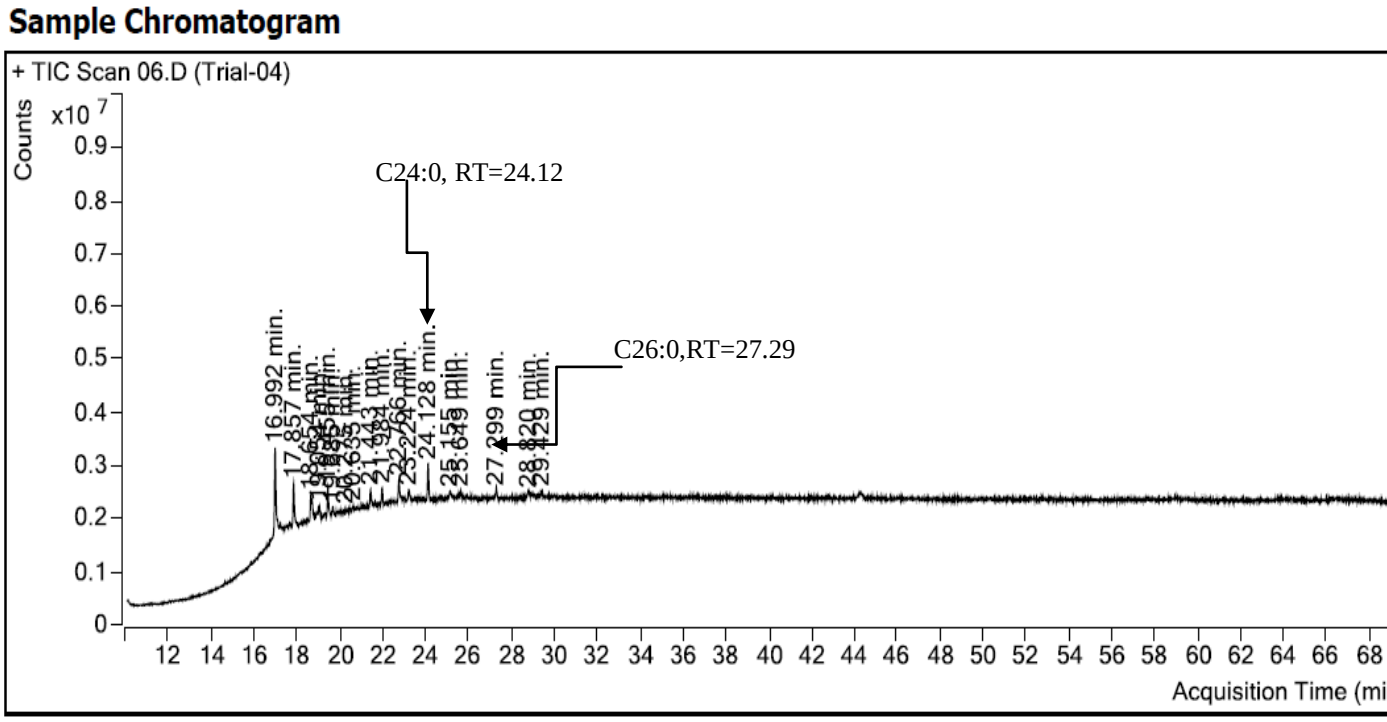


Figure 77: Total Ion Chromatogram of fatty acids present in 0.2% EMS treated callus of *P oleracea,* C24:0= Tetracosanoic acid, C26:0= Hexacosanoic acid.

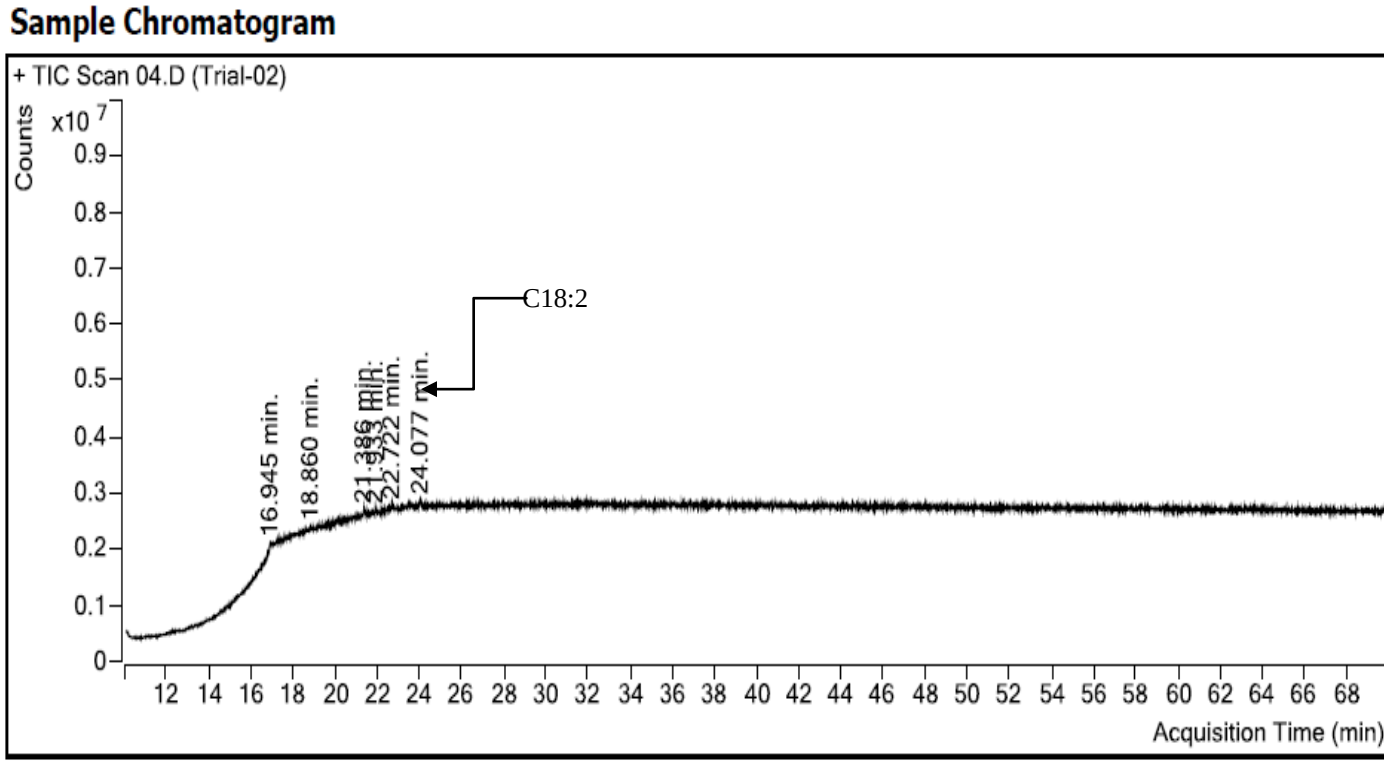


Figure 78: Total Ion Chromatogram of fatty acids present in 0.3% EMS treated callus of *P oleracea*, C18:2= Linoleic acid.

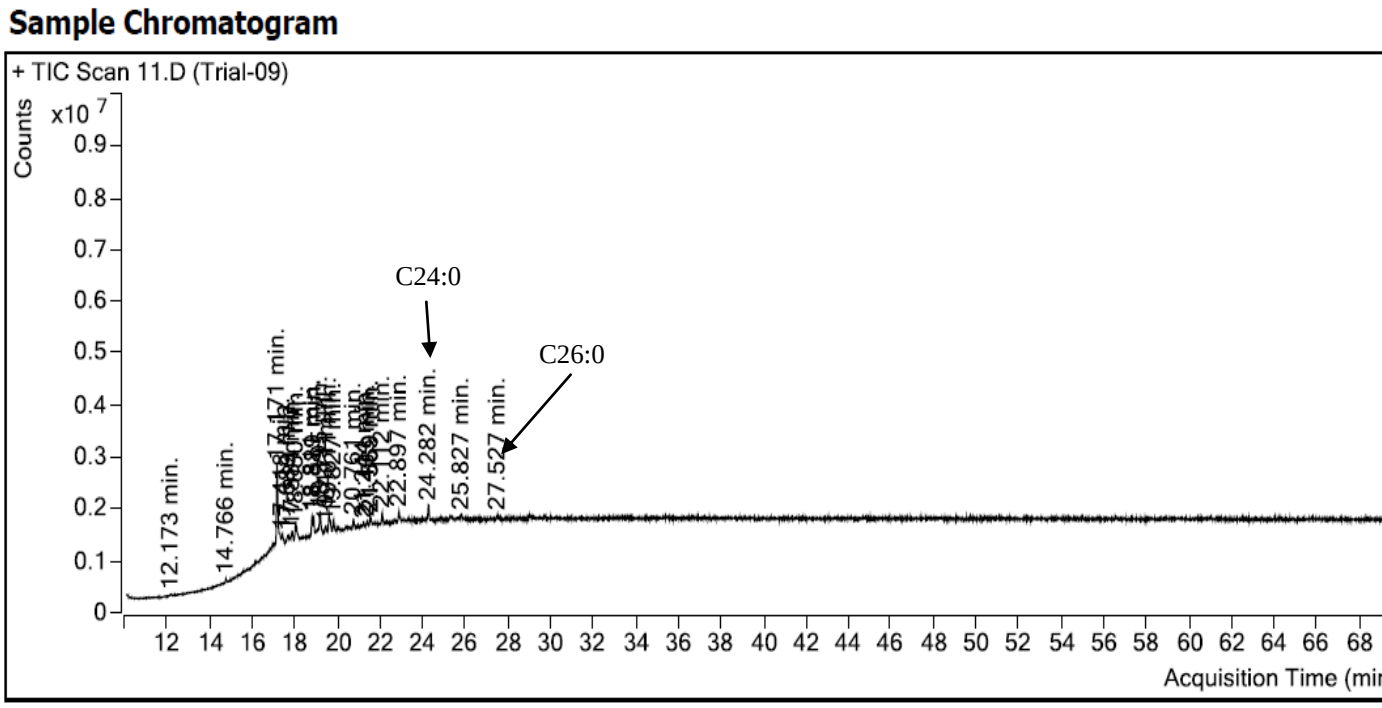


Figure 79: Total Ion Chromatogram of fatty acids present in 0.4% EMS treated shoots of *P oleracea*, C24:0= Tetracosanoic acid and C26:0= Hexacosanoic acid

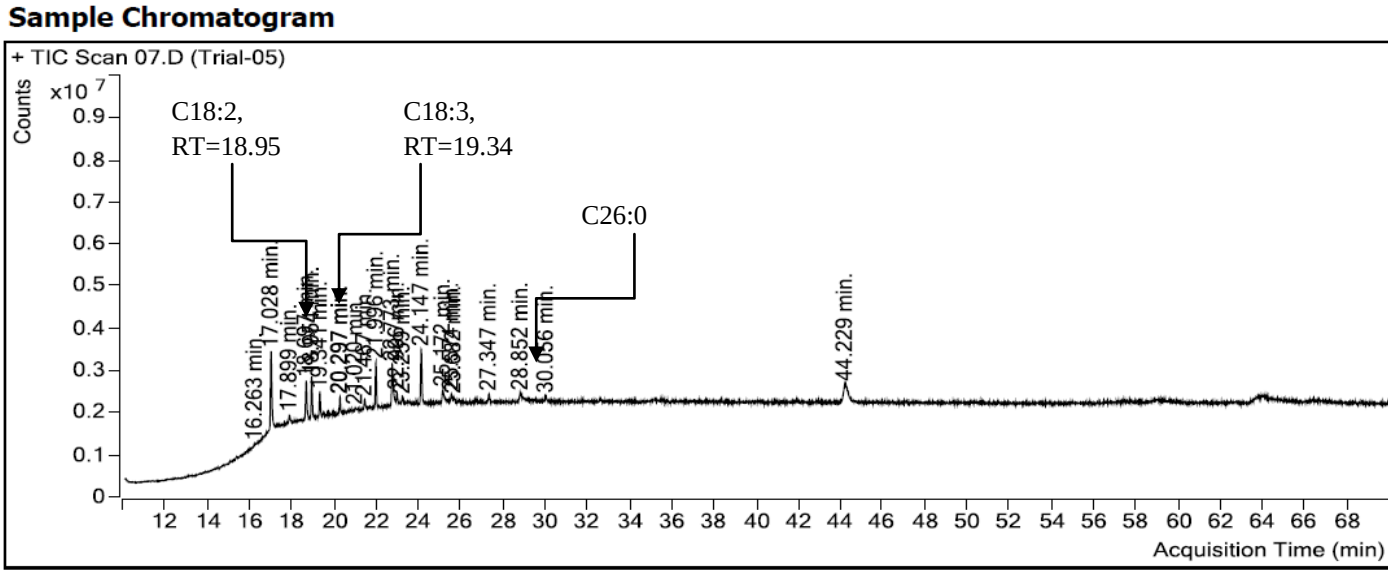


Figure80: Total Ion Chromatogram of fatty acids present in 0.6% EMS treated shoots of *P oleracea,* C18:2= Linoleic acid, C18:3= Linolenic acid, C26:0= Hexacosanoic acid

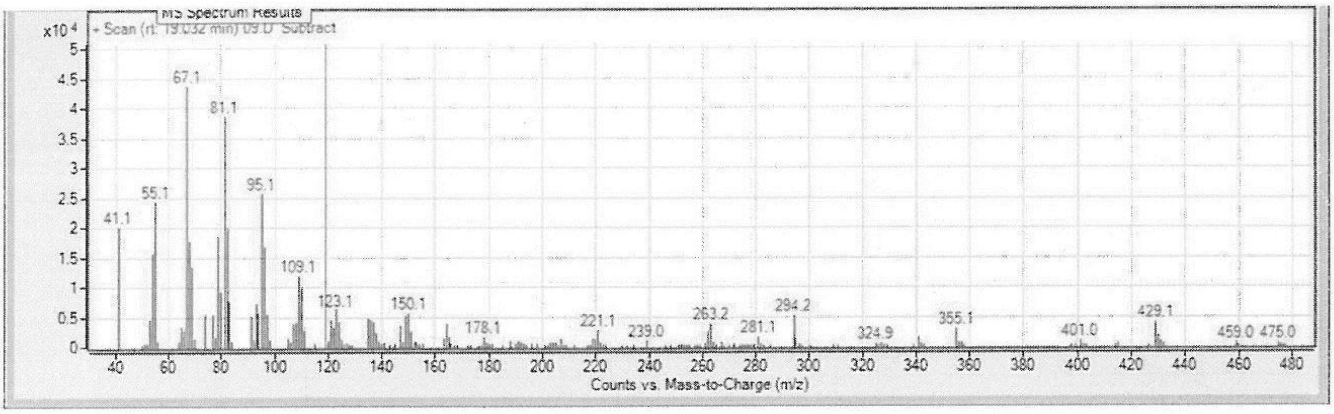


Figure 81: Mass Spectra of Linoleic Acid (9,12 Octadecadienoic Acid, methyl ester) of 0.1% EMS shoot cultures of *P.oleracea*

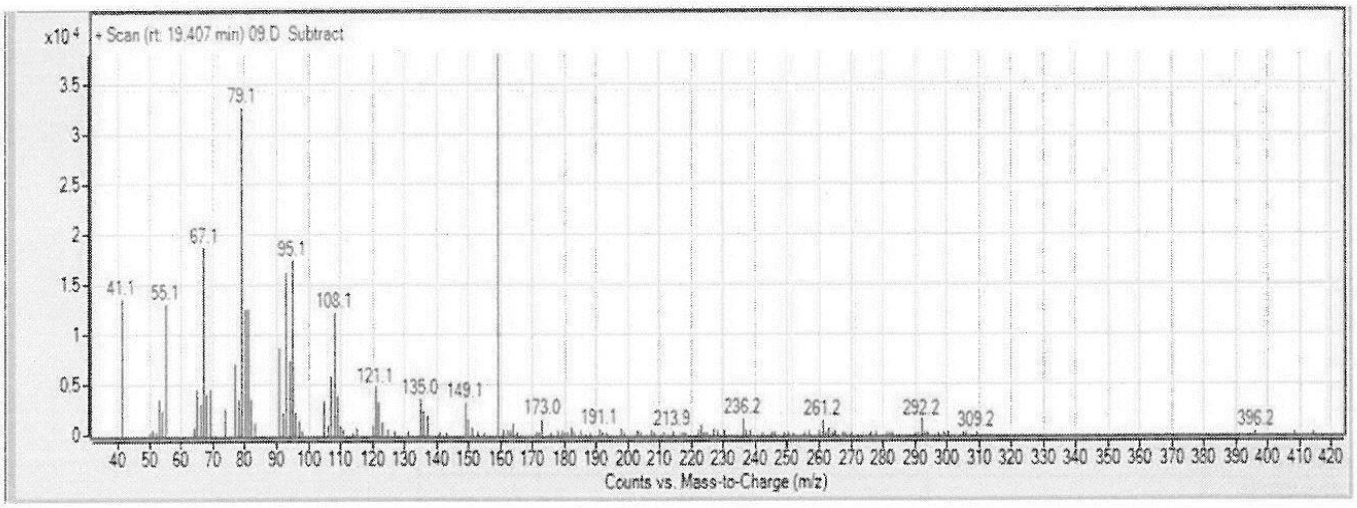


Figure 82: Mass Spectra of Linolenic Acid (9,12,15 Octadecatrienoic Acid , methyl ester) in 0.1% EMS shoot cultures of *P.oleracea*

4.3.1.6 Histological study of in vitro shoot cultures initiated from EMS treated explants

The explants treated with different concentrations of EMS (0.1-1%) formed in vitro shoots at low concentrations (0.1-0.3%) beyond which it induced callus of varying texture and morphology. At 0.1% EMS callus differentiated along with shoot buds after 8 weeks which indicated indirect mode of regeneration (Fig. 83a). These meristemoids eventually developed into shoots at 0.3% EMS (Fig. 83b). As the concentration of EMS increased to 0.5% the regenerative capacity was lost. At a very high concentration of 1% only dividing cells were observed which differentiated xylem and phloem cells (Fig. 83c, d). Anatomically it was confirmed that explants under the influence of EMS differentiated shoots at low concentrations while at high concentrations callus was induced.

Thus in the present investigation regeneration protocol for the P oleracea and P grandiflora was determined, followed by the analysis of their metabolite content. Under the influence of the chemical mutagen EMS it was observed that the in vitro cultures have the potential to synthesise long chain fatty acids.

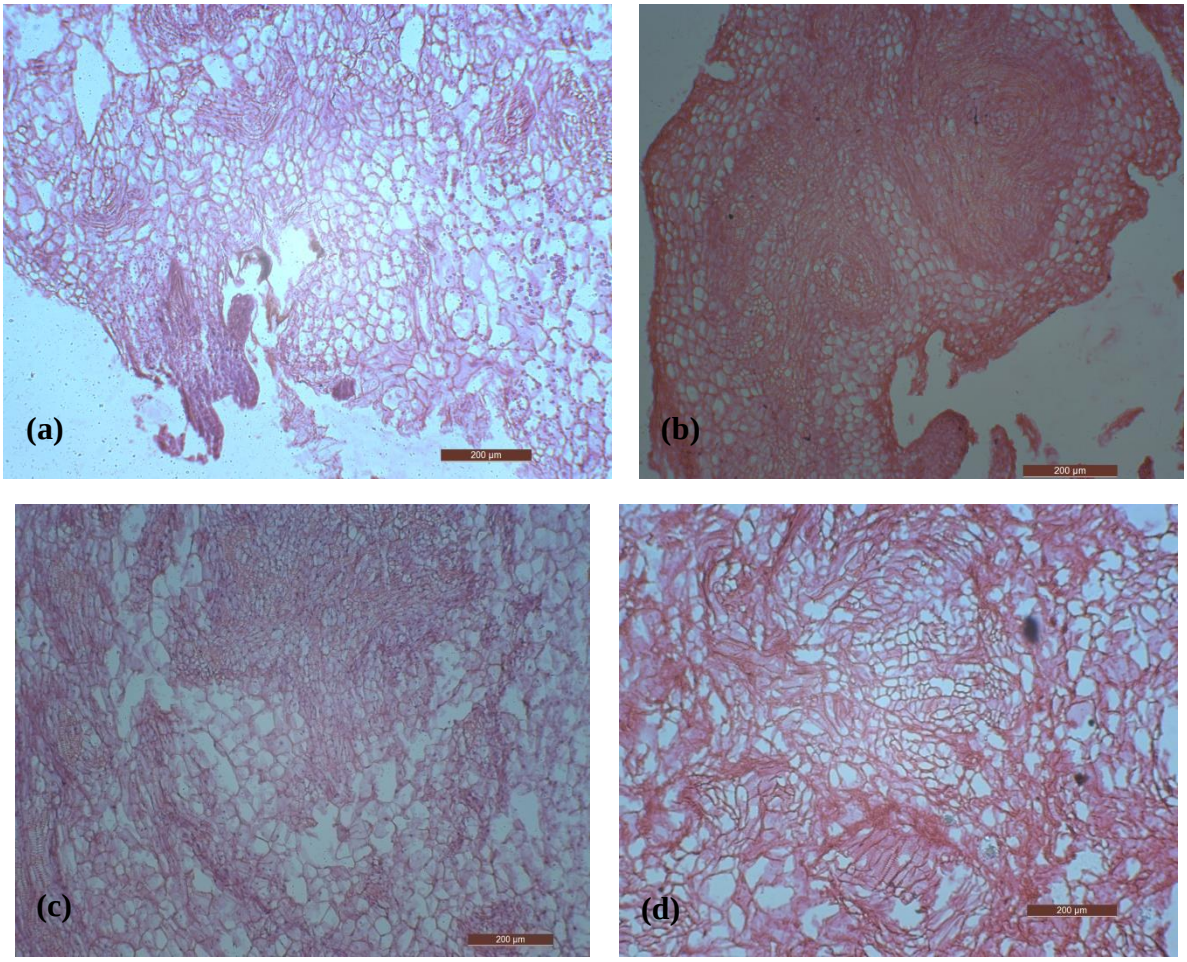


Figure 83: Effect of different concentrations of EMS on leaf explants of *P oleracea* after 8 weeks (a) Differentiation of shoot buds at 0.1% EMS, (b) Meristemoid formation at 0.3% EMS, (c) Actively dividing meristematic cells at 5% EMS, (d) Parenchymatous cells formation at 1% EMS.