CHAPTER 4

Results

RESULTS

This chapter describes the results of the experiments carried out in *O.indicum* and *S.suaveolens* which were divided into three sections.

Section I: Seed germination studies

Section II: Regeneration studies

Section III: Synthetic seed studies

4.0 *Oroxylum indicum*

4.1 Section I: Seed germination studies

The experimental design was formulated to understand the germination of the seeds on various planting substrates and to obtain large number of seedlings. The selection of suitable substrate was done by germinating the seeds in different substrates like Soil, Sand, Cocopeat, Cocopeat:soil(1:1),Cocopeat:sand(1:1), Filter paper, MS Basal medium, WPM basal medium and % germination was recorded. As well as the growth parameters were also studied.

4.1.1 Germination of *O.indicum* seeds in different substrates

The seeds of *O.indicum* were soaked in water overnight and next day germinated in substrates like Cocopeat, Cocopeat:soil (1:1), Cocopeat:sand (1:1),filter paper, MS basal medium, WPM basal medium under aseptic conditions, whereas soil and sand substrates were kept under natural conditions. The % germination was recorded for all the different substrates after 4 weeks.

The emergence of radical and plumule from the seeds was observed within a week in all substrates, but at the end of 4 weeks it obtained varied results (Fig.9).

- **Under natural conditions:** Sand and soil were the substrate used for seed germination under natural conditions
  - **Sand:** In sand there was only 44 % germination of seeds observed but the seedlings developed were healthy.
  - **Soil:** The seeds germinated in soil also obtained similar % germination (44%) as that of sand with development of healthy seedlings.

- **Under lab conditions:** Different substrates were placed under lab conditions for evaluating percent seed germination.
  - **Cocopeat:** The cocopeat was filled in root trainers (Fig.10a) and all the seeds placed singly in each well of root trainer got germinated by the end of 4 weeks (100%). Thus the percent germination was recorded maximum in individual cocopeat as compared to the other substrates. This substrate developed healthy seedlings with expanded cotyledonary leaves and well developed roots (Fig 10b).
  - **Cocopeat: Sand (1:1)**: In comparison to above substrates cocopeat:sand(1:1) resulted in less percent germination of seeds(44%) but seedlings developed were healthy.
  - **Cocopeat: Soil (1:1)**: This substrate recorded second highest % germination (90%) and it also developed healthy seedlings with fully expanded cotyledonary leaves.
  - **Filter paper**: The petridish containing filter paper although resulted in 80% germination of seeds but the seedlings were developed with curled cotyledonary leaves. This may be due to less surface area in petridish which do not allow cotyledonary leaves to expand.
  - **MS and WPM basal medium:** This substrate resulted in poor germination of seeds, as MS medium could germinate 20% of seeds only while WPM medium failed to germinate any seeds. In this substrate also the seedlings were developed with curled cotyledonary leaves as in flasks also the surface area was less.

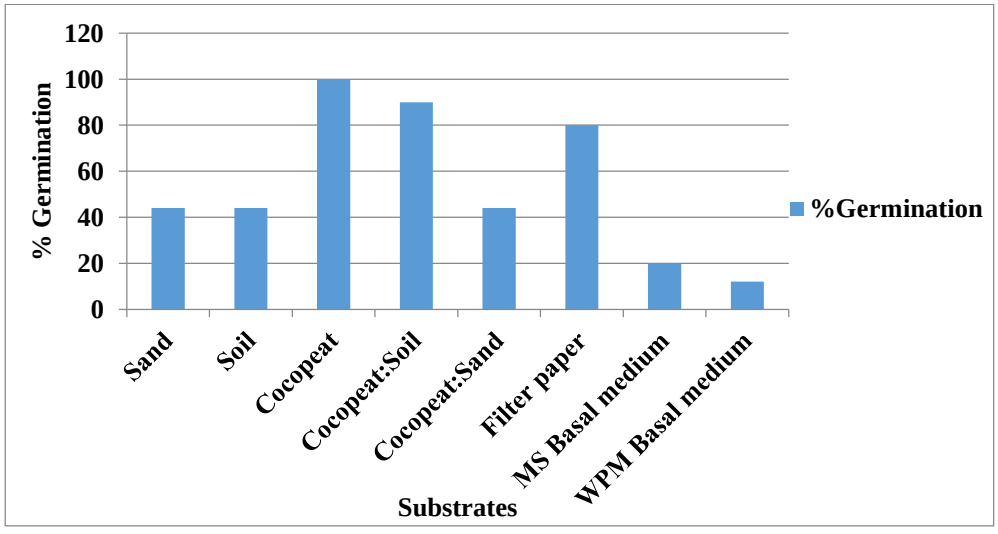

Fig. 9: Germination of *O.indicum* seeds in different substrates

Fig.10: Seed germination in *O.indicum*

a. Root trainer filled with cocopeat
b. Healthy seedlings with expanded cotyledonary leaves in cocopeat after 4 weeks
c. Seedlings in cocopeat : sand(1:1) after 4 weeks
d. Seedlings growth in cocopeat after 4 weeks
e. Seedlings in cocopeat:soil after 4 weeks
f. Single seedling

Therefore in the present experiment the soaking of seeds in water was sufficient for germination of seeds without any treatment and the cocopeat was the optimised substrate for germination of *O.indicum* seeds.

4.1.2 Effect of substrates on growth of *O.indicum* seedlings (Growth parameters)

Since cocopeat proved to be optimum for germination of *O.indicum* seeds the cocopeat and its combination ie.cocopeat, cocopeat: soil (1:1) and cocopeat:sand (1:1) were taken for growth parameters studies. The seeds were germinated in these substrates and the growth of seedlings in terms of length (shoot and root), biomass (Fresh weight and Dry weight), collar diameter were recorded at weekly interval (1^{st},2^{nd}, 3^{rd} and 4^{th} weeks) by selecting 5 seedlings randomly from each substrate.

It was observed that within first week of seed inoculation there was an emergence of radical and plumule only and growth was not enough to take out the seedlings but after 2^{nd} week the growth in seedlings was observed and hence data were recorded. The length of seedlings when measured after second week it was highest in cocopeat:sand (10.1±1.1cms) compared to other two. While after third week seedling length was highest in cocopeat (10.4±1.3cms) and cocopeat:sand (10.3±0.8cms) and was less in cocopeat:soil (8.6±0.6cms). The growth of the seedlings in terms of the length by the end.

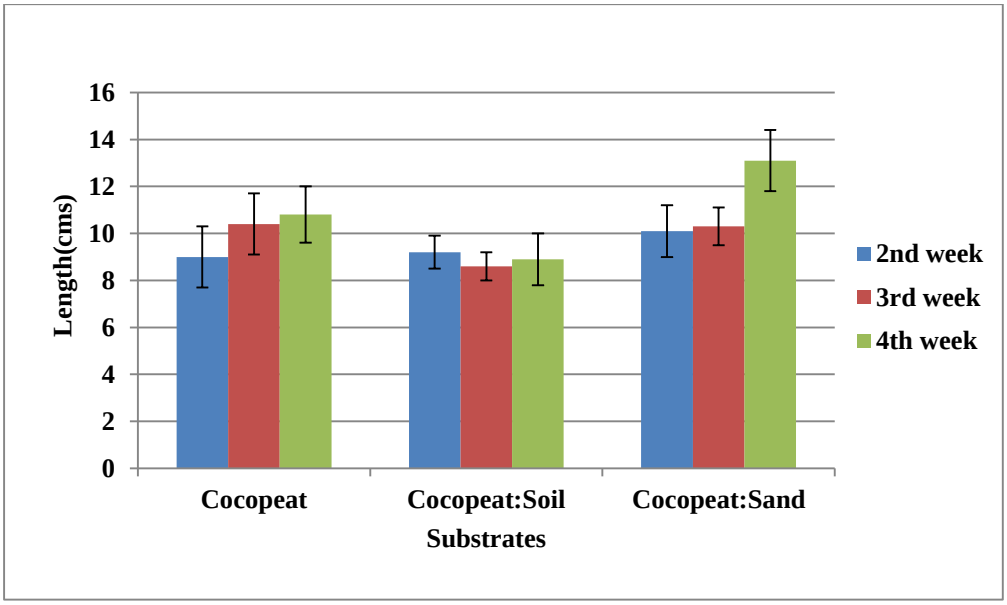

Fig. 11: Effect of different substrates on length of *O.indicum* seedlings

Values represents mean ± S.E of five replicates in each experiment

of 4 weeks increased and was maximum in cocopeat: sand (Fig.10c) (Fig.11) with 13.1 ± 1.2 cms, followed by cocopeat (Fig.10d) with 10.8 ± 1.1cms and in cocopeat: soil it was 8.9 ± 1.1 cms (Fig.10e) (Fig.11).

The seedlings grown in cocopeat:soil recorded maximum collar diameter (1.1±0.1cms) compared to cocopeat and cocopeat:sand after second week. By the end of third week there was slight increase in collar diameter of seedlings grown in cocopeat:soil (1.2 ± 0.03cms) which was maximum when compared to cocopeat and cocopeat:sand. By the end of 4 weeks the collar diameter of seedlings (Fig.12) was nearly same in cocopeat (1.2 ± 0.05cms) and cocopeat:sand (1.2 ± 0.05cms) substrates which was followed by cocopeat:soil (1.08 ± 0.01 cms) (Fig.12).

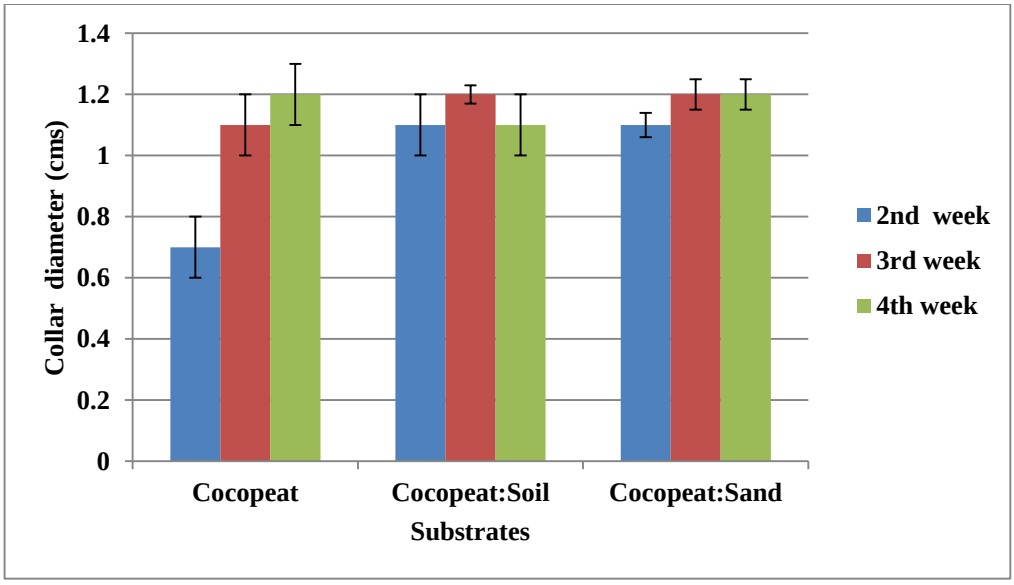

Fig. 12: Effect of different substrates on collar diameter of *O. indicum* seedlings

Values represents mean ± S.E of five replicates in each experiment

The seedlings biomass was maximum in cocopeat:soil after second week with fresh weight (0.6 ± 0.1gms) and dry weight (0.1 ± 0.01 gms) than cocopeat and cocopeat:sand. But after third week seedling biomass was nearly same in all the substrates. The seedling biomass with maximum fresh weight (0.8 ± 0.08gms) was observed in cocopeat:sand mixture, and dry weight to 0.08 ± 0.01 gms after 4th week. In cocopeat the fresh weight was (0.72 ± 0.05 gms) and dry weight was (0.1 ± 0.01 gms) while minimum biomass(FW and DW) was observed in cocopeat:soil (Fig.13).

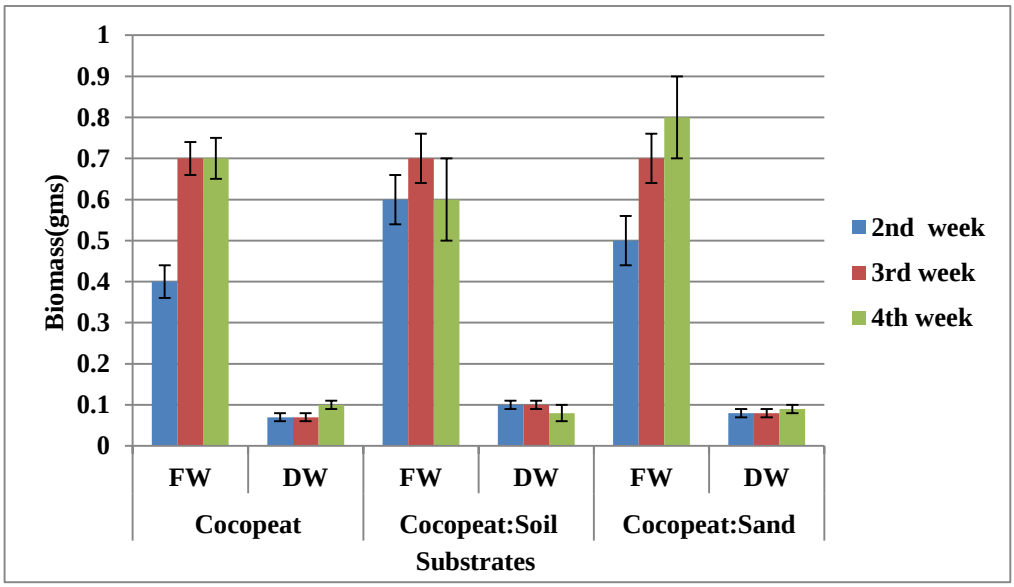

Fig.13: Effect of different substrates on biomass of *O. indicum* seedlings

Values represents mean ± S.E of five replicates in each experiment

FW: Fresh weight, DW: Dry weight

The study reveals that out of the three substrates, cocopeat in combination with sand was suitable in terms of seedling growth ie. length, biomass, collar diameter producing good quality seedlings(Fig.10f). It was optimum planting substrate as this substrate can provide favorable conditions for aeration and water holding capacity.

Overall from the seed germination experiments it can be concluded that cocopeat alone can be a best substrate in terms of seed germination of *O.indicum* which faces difficulties in germination under natural conditions ie. in simple soil. But if cocopeat is used in combination with soil or sand can produce good quality seedlings as it can give better results in terms of collar diameter, seedling length, biomass. Thus cocopeat can be ideal substrate if used individually and in combination.

4.2 Section II: Regeneration studies

Since for regeneration studies large number of seedlings are required the cocopeat substrate was selected for generating maximum seedling explants. Hence the seeds were germinated everytime in cocopeat substrate for getting the same.

To regenerate the *O.indicum* species rapidly the selection of suitable explants and optimisation of medium and PGR was done. Different explants tried were seedling (cotyledonary leaf, cotyledonary node and hypocotyl) and nodal which were placed on

MS medium which is the most commonly used medium and WPM medium which is widely used for woody trees.

The regeneration studies was divided into four stages:

- Establishment of shoot cultures
- Multiplication of shoots
- *In vitro* rooting of microshoots
- Hardening of plantlets

4.2.1 Establishment and multiplication of shoots

In *O.indicum* four different explants were utilised for establishing cultures and their regeneration capacities were evaluated. The explants were placed in MS and WPM medium fortified with different concentration of individual cytokinins and on the basis of this experiment the suitable explants which regenerated into shoots were selected for further studies.

4.2.1.1 Effect of individual cytokinins on shoot induction in MS and WPM medium from cotyledonary leaf explants

The cotyledonary leaf excised (1x1cm) from seedlings were placed horizontally in **MS medium** fortified with the various concentrations of individual cytokinins. Observations after 4 weeks revealed that in presence of BAP at 2µM the leaf curled and there was formation of friable callus (cream in colour) from the cut ends (Fig.14a) and in rest all the concentrations only swelling was observed. Whereas all the Kn and TDZ concentrations failed to induce any morphogenic response in the explants.

WPM medium fortified with individual cytokinins failed to evoke any morphogenic response.

4.2.1.2 Effect of individual cytokinins on shoot induction in MS and WPM medium from hypocotyl explants

The hypocotyl explant (1 cm) was placed horizontally on the surface of MS and WPM medium fortified with individual cytokinins.

In MS medium fortified with different concentrations of individual cytokinin (BAP/Kn/TDZ) only BAP at 20µM was able to induce callus from cut ends at the end of

four weeks (Fig.14b). In presence of Kn in the medium the explant only swelled and failed to differentiate callus or shoot buds. Whereas in WPM medium all the cytokinins at different concentrations failed to induce any response.

Since cotyledonary leaf and hypocotyl explants failed to respond in terms of shoot formation in presence of individual cytokinins, these explants were omitted for any further experimental work.

4.2.1.3 Effect of individual cytokinins on shoot induction in MS and WPM medium from cotyledonary node explants

The cotyledonary node explants (1.5cm) without leaves were excised from seedlings and placed vertically on MS and WPM medium fortified with different individual cytokinins like BAP (2-30 µM) ;Kn(2-30 µM) ; TDZ(0.1-2 µM).

The **MS medium** without PGRs resulted in 50% response only and therefore the medium was fortified with BAP (2-30 µM) concentrations. It was observed that the buds break occurred within two weeks which proliferated into one or two shoots by the end of 4 weeks with varied percent response (Table 8). The lower concentrations of BAP (2µM and 4µM) resulted in 50% response and 67% cultures was obtained in 8µM (Fig.14c) with maximum (1.0 ± 0.3) number of nodes. Further increase in concentrations of BAP at 16 µM there was a decrease in percent response as well as in number of shoots. But at 20 µM there was formation of 1.2 ± 0.4 shoots in 83% cultures (Fig.14d).When BAP, was replaced by Kn there was a poor response at all the concentrations. The percent of shoot formation reached to 17% for 2-8 µM and became nil for higher concentrations and the shoots were very weak and stunted. Similar response was observed for TDZ at 0.1µM concentration and the other concentrations failed to evoke any response.

At the base of nodes in MS medium fortified with BAP (4µM, 16µM, 20µM) a morphogenic callus was formed which differentiated shoot buds. This callus was subcultured separately in the same or other medium.

The explants in **WPM** basal medium depicted poor response as shoots were formed in only 33 % cultures. But when WPM medium was fortified with different concentrations

Fig.14: Morphogenic response in different explants of *O.indicum* in presence of individual cytokinins after 4 weeks
a. Callus formation from cotyledonary leaf explants in MS+BAP (2µM)
b. Callus induction from the cut end of hypocotyl explant in MS+ BAP (20 µM)
c. Single shoot developed from cotyledonary node explant in MS+BAP (8µM)
d. Shoots induction from cotyledonary node explant in MS+BAP (20 µM)
e. Development of long shoot from cotyledonary node explant in WPM+BAP(16 µM)

Table 8. Effect of individual cytokinins on shoot induction from cotyledonary node explants of *O.indicum* after 4 weeks

	MS medium			WPM medium		
Cytokinin (µM)	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
0	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}	33	0.5 ± 0.3^{ab}	0.5 ± 0.3^{ab}
BAP						
2	50	0.7 ± 0.3^{bc}	0.8 ± 0.4^{bc}	33	0.3 ± 0.2^{ab}	0.5 ± 0.3^{ab}
4	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}	50	0.5 ± 0.2^{ab}	0.8 ± 0.4^{ab}
8	67	0.7 ± 0.2^{bc}	**1.0 ± 0.3^{c}**	33	0.3 ± 0.2^{ab}	0.7 ± 0.4^{ab}
16	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}	**50**	**0.6 ± 0.3^{b}**	**1.0 ± 0.6^{b}**
20	**83**	**1.2 ± 0.4^{c}**	0.8 ± 0.2^{bc}	33	0.6 ± 0.2^{b}	0.8 ± 0.7^{ab}
25	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
30	17	0.1 ± 0.2^{a}	0.2 ± 0.2^{ab}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn						
2	17	0.1 ± 0.2^{a}	0.2 ± 0.2^{ab}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
4	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
8	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
16	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
20	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	17	0.3 ± 0.3^{ab}	0.2 ± 0.2^{ab}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
30	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
TDZ						
0.1	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
0.2	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
2	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test

of BAP a varied response was observed (Table 8). The number of shoots were in correlation with percent response, as response increased, the number of shoots also increased and vice versa. At 16µM, 50% cultures responded and formed an average of 0.6 ± 0.3 shoots (Fig.14e) with maximum number of 1.0 ± 0.6 nodes but at higher concentrations (20µM, 25µM and 30µM) there was decrease in percent response. When Kn was added to the medium the cotyledonary node evoked a poor response in terms of

shoot formation. Incorporating TDZ in the medium also resulted in poor response as none of the concentrations induced axillary bud to develop into a shoot and hence the response of this cytokinin was similar to MS medium.

Observations also revealed that at higher concentration of BAP (16µM and 20µM) there was formation of callus at the base of the explants which differentiated shoot buds. This callus was separated and utilised for differentiation of shoots on the same.

Therefore out of all the cytokinins BAP proved to be effective compared to Kn and TDZ in terms of shoot induction in both the medium.

The above results depict a poor morphogenic response in terms of shoot formation for all the concentrations of individual cytokinins in both the media but the shoot cultures were established from cotyledonary node explants and hence further experiments for multiplication of shoots was achieved utilizing the *in vitro* nodes obtained from *in vitro* shoots

4.2.1.3.1 Multiplication of shoots from *in vitro* nodes in presence of individual cytokinins

In this experiment the *in vitro* shoots which developed from cotyledonary node explants were cut into single *in vitro* nodes and utilised for enhancing the number of shoots. The concentrations which had induced shoot formation were selected to transfer these *in vitro* nodes in their respective medium. Observations were recorded for percent response and number of shoots after 8 weeks and 12 weeks or further.

The *in vitro* nodes from cotyledonary node explants were subcultured in **MS medium** supplemented with individual concentrations of BAP (2µM -30µM); Kn(2µM,4µM and 8 µM) and TDZ(0.1µM).

MS medium fortified with different concentrations of BAP resulted in poor response in terms of shoot formation from *in vitro* nodes after 8 weeks. At lower concentrations (2 µM, 4µM and 8µM) the axillary bud failed to respond whereas at BAP (16µM) there was formation of single shoot in 50% cultures with an average of 0.5 ± 0.2 shoots. Increase in BAP to 20µM concentration further decreased the response to 17% (Fig.15).The other two cytokinins (Kn and TDZ) failed to evoke any response.

The *in vitro* nodes were also subcultured in **WPM medium** fortified with different concentrations of BAP (2µM -20µM); Kn (2µM, 8µM, 16µM and 20µM) and TDZ (0.1 µM).

BAP at 4µM induced a single shoot with an average of 0.2 ± 0.2 shoots in 17 % cultures, whereas at 8µM only 0.3 ± 0.2 shoots developed in 33% cultures. But increase in concentration to BAP (16µM,20µM) improved the morphogenic response as 1.7 ± 0.2 number of shoots were formed in 100 % cultures and 1.0 ± 0.4 shoots in 50 % cultures respectively (Fig.15).

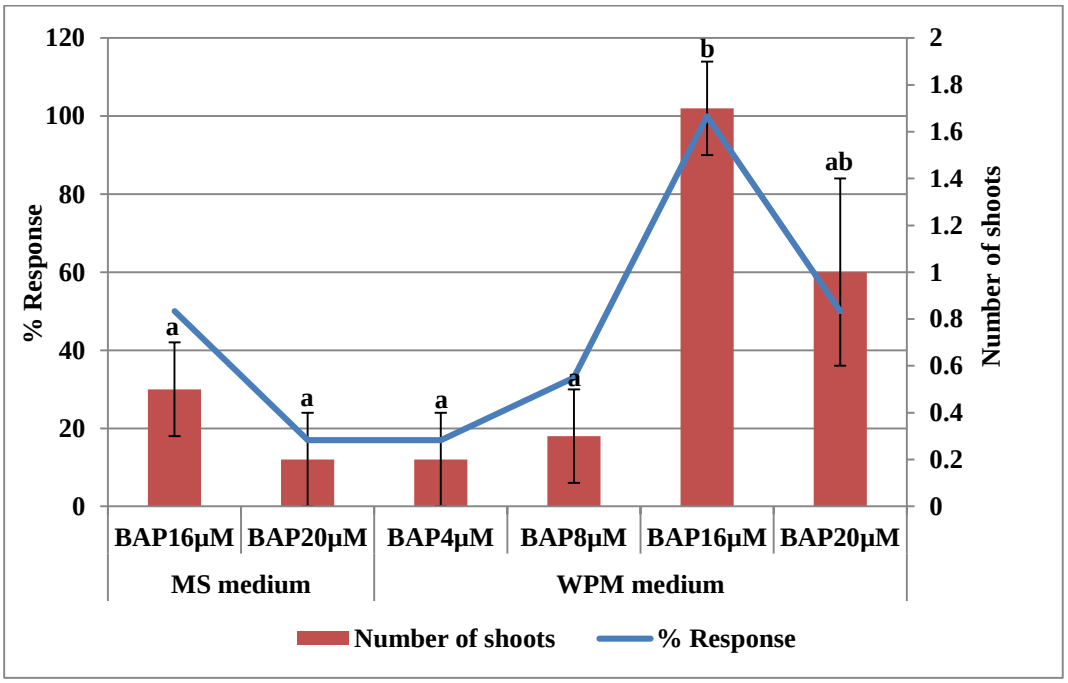

Fig. 15: Effect of individual cytokinins in inducing multiples from *in vitro* nodes of *O.indicum* after 8 weeks

Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

The *in vitro* nodes were subcultured or transferred in respective concentrations of MS and WPM medium but they failed to show any response after 12 weeks except for MS medium fortified with BAP (16µM) there was a slight increase in shoot number with an average of 0.7 ± 0.4 shoots only in 33% cultures and in WPM medium fortified with BAP (8µM) an average of 2.0 ± 0.9 number of shoots were formed with 50% response.

As the cotyledonary node explants were able to form shoot, their regenerative ability was also assessed in presence of two cytokinins and with a combination of cytokinin and auxin.

4.2.1.4 Effect of two cytokinins on shoot induction from cotyledonary node explants of *O.indicum*

The cotyledonary node explants were also placed in MS and WPM medium fortified with combinations of two cytokinins ie. BAP with Kn, BAP with TDZ and Kn with TDZ with different concentrations to evaluate their synergistic effect on number of shoots.

The individual concentrations of BAP (8,16,20µM) which induced a maximum morphogenic response were selected and combined together with different concentrations of Kn (2,4,8,16µM) and TDZ(0.1,0.2,0.25,0.5µM). The % response and number of shoots was recorded after 4 weeks.

❖ **Synergistic effect in MS medium**

- **BAP+Kn**

The cotyledonary node explants when placed in the combinations of BAP (8, 16, 20µM) with Kn (2, 4, 8, 16µM) responded differently. A combination of BAP (8µM) and Kn (8 µM) formed 0.5 ± 0.2 number of shoots in 50% cultures (Fig.16a) having maximum number of 1.3 ± 1.0 nodes. These shoots were long with minute leaves. Further combinations of BAP (16µM) with Kn (2, 4, 8, 16µM) resulted in a poor response in terms of shoot formation. BAP (16µM) and Kn (8µM) was the only combination which formed an average of 0.5 ± 0.3 number of shoots with 1.3 ± 1.0 number of nodes in 33% cultures whereas the other combinations failed to respond. Increase in BAP to 20µM also failed to improve the number of shoots with different concentrations of Kn but there was slight improvement in percent response which reached to 50% when Kn was at 16µM (Table 9).

- **BAP+TDZ**

The second synergistic combination tried for cotyledonary node explants was of BAP (8/16/20 µM) and TDZ (0.1, 0.2, 0.25, 0.5 µM) at various concentrations. The medium with BAP (8µM) and TDZ (0.1µM, 0.2 µM) increased the percent response to 67%.

But BAP (8µM) with TDZ (0.2µM) resulted in inducing healthy shoots (1.0±0.4) with an average of 1.1 ± 0.5 nodes (Fig.16b). A combination of BAP (8µM) with TDZ (0.5µM) decreased the response to 50% with 0.5 ± 0.2 shoots and 1.2 ± 0.8 number of nodes. Increase in concentration of BAP to 16µM and 20µM combined with all concentrations of TDZ (0.1, 0.2, 0.25, 0.5 µM) resulted in very poor response in terms of number of shoots and nodes (Table 9).

- **Kn+TDZ**

The interaction of Kn (2, 4, 8µM) with TDZ (0.1, 0.2, 0.25, 0.5µM) was also evaluated and it was observed that all the combinations of these two cytokinins was unable to improve the response for shoot formation(Table 9).

Thus in MS medium fortified with BAP (8µM) with TDZ (0.2µM) resulted in maximum shoot induction with 67% response and 1.0 ± 0.4 number of shoots among all the different combinations.

❖ **Synergistic effect in WPM medium**

Similar combinations of cytokinins were also tried for WPM medium.

- **BAP+Kn**

In WPM medium when BAP was added with Kn, the cotyledonary node formed only one or two shoots, which were long and healthy with many nodes. A combination of BAP 8 µM with 2µM and 4µM of Kn resulted in poor response but it increased when Kn reached to 8µM as 83% cultures responded and a maximum of 1.2 ± 0.3 number of shoots with 2.1 ± 0.7 number of nodes were formed(Fig.16c). Increased BAP (16µM) concentration with Kn at 2, 4, 8, 16µM resulted in 50% response but there was formation of callus at the base of explant placed in BAP(16µM) with Kn(8µM) combination (Fig.16d). Further combinations of BAP (20µM) with Kn (2, 4, 8, 16µM) failed to form shoots (Table 9).

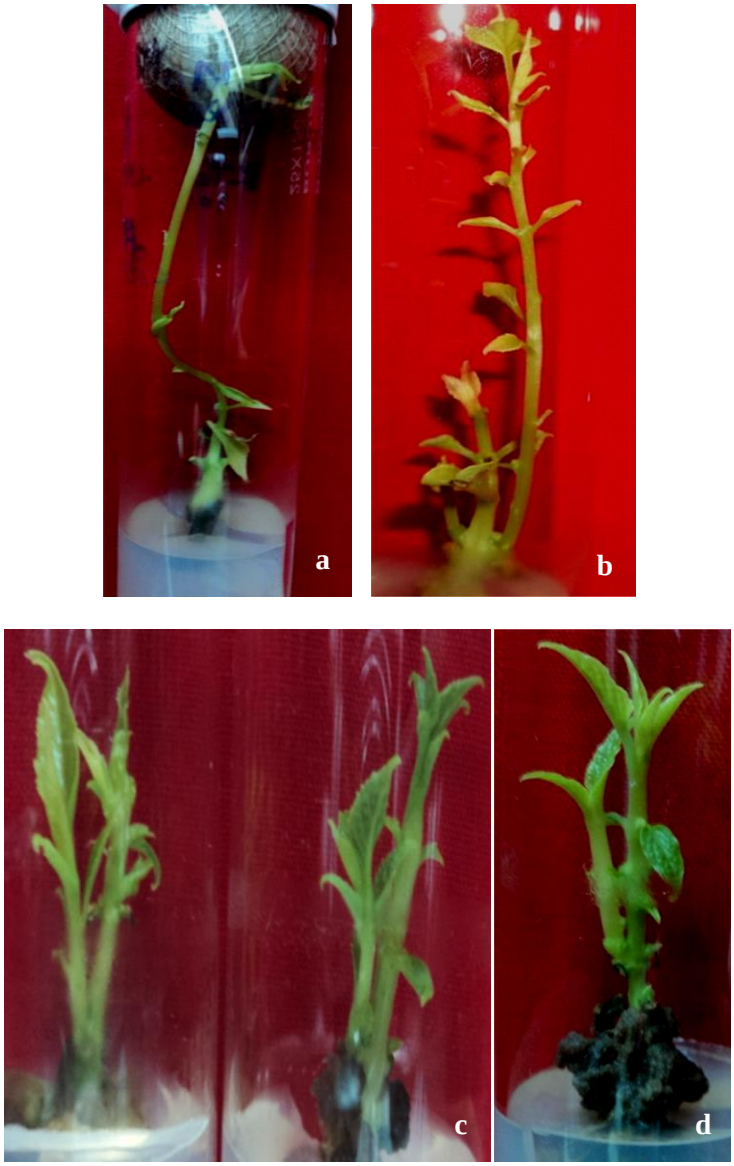

Fig.16:Shoot induction from cotyledonary node explants of *O.indicum* in prescence of two cytokinins after 4 weeks

a. Development of single long shoot with minute leaves in MS +BAP(8µM) + Kn(8µM)
b. Healthy shoots developed in MS +BAP(8µM) +TDZ(0.2µM)
c. Shoots developed in WPM +BAP(8µM) +Kn(8µM)
d. Two small shoots formation in WPM+BAP(16µM) +Kn(8µM)with slight callus at its base

Table.9 Effect of two cytokinins on shoot induction from cotyledonary node explants of *O.indicum* after 4 weeks

Cytokinins (µM)			MS medium			WPM medium		
			% Response	Number of shoots*	Number of *in vitro* nodes*	% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**						
8	2	-	17	0.3±0.3^{b}	0.3±0.3^{a}	33	0.3±0.2^{ab}	0.8±0.5^{ab}
8	4	-	17	0.2±0.2^{ab}	0.2±0.2^{a}	17	0.2±0.2^{a}	1.0±1.0^{b}
8	**8**	-	50	0.5±0.2^{bc}	1.3±1.0^{a}	**83**	**1.2±0.3^{c}**	**2.1±0.7^{c}**
8	16	-	17	0.2±0.2^{ab}	0.8±0.8^{a}	17	0.2±0.2^{a}	0.2 ±0.2^{ab}
16	2	-	0	0.0±0.0^{a}	0.0±0.0^{a}	17	0.2±0.2^{a}	0.2±0.2^{ab}
16	4	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
16	8	-	33	0.5±0.3^{bc}	1.3±1.1^{a}	50	0.8±0.5^{bc}	0.7±0.3^{ab}
16	16	-	0	0.0±0.0^{a}	0.0±0.0^{a}	50	0.8±0.5^{bc}	0.5±0.2^{ab}
20	2	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
20	4	-	33	0.3±0.2^{b}	0.3±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
20	8	-	17	0.3±0.3^{b}	0.5±0.5^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
20	16	-	50	0.5±0.2^{bc}	0.5±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
8	-	0.1	67	0.6±0.2^{bc}	0.7±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
8	-	**0.2**	**67**	**1.0±0.4^{c}**	1.1±0.5^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
8	-	0.25	50	0.7±0.3^{bc}	0.8±0.4^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
8	-	0.5	50	0.5±0.2^{bc}	1.2±0.8^{a}	17	0.3±0.3^{ab}	0.5±0.5^{ab}
16	-	0.1	17	0.2±0.2^{ab}	0.5±0.5^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
16	-	0.2	17	0.2±0.2^{ab}	0.2±0.2^{a}	50	0.5±0.2^{ab}	0.5±0.2^{ab}
16	-	0.25	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
16	-	0.5	50	0.5±0.2^{bc}	0.8±0.5^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
20	-	0.1	33	0.3±0.2^{b}	0.8±0.7^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
20	-	0.2	17	0.2±0.2^{ab}	0.7±0.7^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
20	-	0.25	17	0.2±0.2^{ab}	0.3±0.3^{a}	17	0.2±0.2^{a}	0.2±0.2^{ab}
20	-	0.5	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	2	0.1	17	0.2±0.2^{ab}	0.2±0.2^{a}	0	0.0±0.0 ^{a}	0.0±0.0^{a}
-	2	0.2	0	0.0±0.0^{a}	0.0±0.0^{a}	17	0.2±0.2^{a}	0.2±0.2^{ab}
-	2	0.25	17	0.2±0.2^{ab}	0.2±0.2^{a}	17	0.2±0.2^{a}	0.2±0.2^{ab}
-	2	0.5	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	4	0.1	50	0.5±0.2^{bc}	0.5±0.2^{a}	33	0.3±0.2^{ab}	0.5±0.3^{ab}
-	4	0.2	33	0.3±0.2^{b}	0.3±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	4	0.25	33	0.3±0.2^{b}	0.3±0.2^{a}	17	0.2±0.2^{a}	0.2±0.2^{ab}
-	4	0.5	17	0.2±0.2^{ab}	0.3±0.3^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	8	0.1	50	0.5±0.2^{bc}	0.7±0.3^{a}	17	0.2±0.2^{a}	0.2±0.2^{ab}
-	8	0.2	17	0.2±0.2^{ab}	0.7±0.7^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	8	0.25	17	0.2±0.2^{ab}	0.2±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	8	0.5	50	0.5±0.2^{bc}	0.5±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}

*****Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

- **BAP+TDZ ; Kn + TDZ**

The synergistic combinations of BAP (8, 16, 20µM) with TDZ (0.1, 0.2, 0.25, 0.5µM) and Kn (2,4,8µM) with TDZ (0.1, 0.2, 0.25,0.5µM) induced a poor response in terms of number of shoots and number of nodes (Table 9).

Therefore in WPM medium fortified with BAP (8µM) and Kn (8µM) resulted in maximum response (83%) and number of shoots (1.2 ± 0.3) among all the different combinations used.

4.2.1.4.1 Multiplication of shoots from *in vitro* nodes in presence of two cytokinins

All the combinations of cytokinins which had a synergistic effect in inducing a morphogenic response (after 4 weeks) in terms of shoot formation were considered for subculturing. The single nodes were excised from these shoots and respective combinations of MS and WPM media and their effect was evaluated after 8, 12, 16, 24 and 32 weeks.

In all the combinations which had induced a response for shoot formation failed to induce a similar response after 8 weeks from *in vitro* nodes. Instead callus with shoot buds differentiated at the base of *in vitro* shoots in few synergistic combinations which were utilised separately and assessed for shoot bud differentiation.

Since combination of two cytokinins failed to induce multiples the synergistic effect of cytokinins and auxins was tried for enhancement of shoots.

4.2.1.5 Effect of cytokinins and auxins on shoot induction from cotyledonary node explants

As in earlier experiment few combinations of two cytokinins were able to form a shoots the studies for evaluating the effect of cytokinin and auxin together on shoot number was also tried as their interaction is known to enhance shoot number. In this study auxins like IAA(0.1,0.5 and 1µM); IBA(0.1,0.5 and 1µM);NAA(0.1,0.5 and 1µM) were added with those concentrations of BAP/Kn/TDZ which had resulted in giving the highest percent response in terms of shoot formation(after 4 weeks).

Shoot induction in MS medium

In MS medium the cotyledonary node were placed in media fortified with BAP (20µM) with different auxins and it regenerated into an average of 0.8±0.2 number of healthy

shoots in 83% cultures at IAA(0.1µM). Combination of BAP with IBA resulted in poor response, whereas with NAA only 0.1µM evoked a maximum number of shoots (1.7 ± 1.0) in 50% cultures which was highest with respect to the other combination (Table 10). Presence of NAA at 0.1µM and 1µM in the medium also induced a morphogenic

Table 10. Effect of cytokinins and auxins on shoot induction from cotyledonary node explants of *O.indicum* in MS medium after 4 weeks

Cytokinins (µM)			Auxins (µM)			% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**	**IAA**	**IBA**	**NAA**			
20	-	-	**0.1**	-	-	**83**	0.8 ± 0.2^{a}	1.8 ± 0.7^{c}
20	-	-	0.5	-	-	33	0.3 ± 0.2^{a}	0.3 ± 0.2^{ab}
20	-	-	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20	-	-	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20	-	-	-	0.5	-	33	0.3 ± 0.2^{a}	0.8 ± 0.7^{abc}
20	-	-	-	1	-	33	0.5 ± 0.3^{a}	0.8 ± 0.7^{abc}
20	-	-	-	-	**0.1**	50	**1.7 ± 1.0^{b}**	1.3 ± 0.6^{bc}
20	-	-	-	-	0.5	17	0.2 ± 0.2^{a}	0.7 ± 0.7^{abc}
20	-	-	-	-	1	33	0.5 ± 0.3^{a}	0.3 ± 0.2^{ab}
-	8	-	0.1	-	-	50	0.5 ± 0.2^{a}	1.0 ± 0.5^{abc}
-	8	-	0.5	-	-	17	0.2 ± 0.2^{a}	0.2 ± 0.2^{ab}
-	8	-	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.1	-	33	0.3 ± 0.2^{a}	0.3 ± 0.2^{ab}
-	8	-	-	0.5	-	17	0.2 ± 0.2^{a}	0.2 ± 0.2^{ab}
-	8	-	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	-	0.1	67	0.7 ± 0.2^{a}	0.8 ± 0.3^{abc}
-	8	-	-	-	0.5	33	0.3 ± 0.2^{a}	0.5 ± 0.3^{ab}
-	8	-	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	0.1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	0.5	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	1	-	-	17	0.2 ± 0.2^{a}	0.2 ± 0.2^{a}
-	-	0.1	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	-	0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test

callus at the base of explants which had the capacity to differentiate shoot buds.

When Kn (8µM) was added along with auxins, it showed a poor response in all the combinations except with NAA (0.1µM) which resulted in 66% response with 0.7 ± 0.2 number of shoots (Table 10). Combination of TDZ (0.1µM) with different concentrations of IAA/NAA/IBA also resulted in poor response (Table 10).

Out of all the combinations BAP (20µM) with IAA (0.1µM) resulted in maximum number (0.8±0.2) of healthy shoots in 83% cultures.

Shoot induction in WPM medium

Similar to MS medium the single concentration of BAP, Kn and TDZ which had induced a maximum response were selected and combined with auxins (IAA, NAA and IBA).

It was observed that WPM medium supplemented with BAP (16µM) and auxins (IAA/IBA/NAA) at different concentrations failed to enhance the response in terms of shoot formation. Single weak stunted shoots developed with small leaves which proves that these PGRs. Whereas all the combinations of Kn with auxin proved that these PGRs failed to induce any response and TDZ with auxin also resulted in poor response after 4 weeks (Table 11).

Thus combining cytokinin and auxin failed to induce a synergistic response from cotyledonary node explants in MS and WPM medium.

4.2.1.5.1 Multiplication of shoots from *in vitro* nodes in presence of cytokinins and auxins

The *in vitro* shoots which developed from cotyledonary node explants in combinations of cytokinins and auxins (at the end of 4 weeks) were excised into single nodes and subcultured in MS and WPM medium with respective concentrations. Observations revealed that *in vitro* nodes were unable to proliferate and develop into shoots in presence of an auxins and thus further enhancement of shoots failed in both the media.

Table 11. Effect of cytokinins and auxins on shoot induction from cotyledonary node explants of *O.indicum* in WPM medium after 4 weeks

Cytokinins (µM)			Auxins (µM)			% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**	**IAA**	**IBA**	**NAA**			
16	-	-	0.1	-	-	17	0.3 ± 0.3^{a}	0.5 ± 0.5^{ab}
16	-	-	0.5	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	-	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	-	-	0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	-	-	-	1	17	0.2 ± 0.2^{a}	0.8 ± 0.8^{b}
-	8	-	0.1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	0.5	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	-	0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	0.1	-	-	33	0.3 ± 0.2^{a}	0.3 ± 0.2^{ab}
-	-	0.1	0.5	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	1	-	-	33	0.3 ± 0.2^{a}	0.3 ± 0.2^{a}
-	-	0.1	-	0.1	-	17	0.2 ± 0.2^{a}	0.2 ± 0.2^{ab}
-	-	0.1	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	-	0.1	33	0.3 ± 0.2^{a}	$0.3\ \pm0.2^{ab}$
-	-	0.1	-	-	0.5	17	0.2 ± 0.2^{a}	0.3 ± 0.3^{a}
-	-	0.1	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test

Thus from all of the above observations it can be concluded that the cotyledonary node explants were able to form shoots in both MS and WPM medium fortified with individual cytokinins, combinations of cytokinins and cytokinin with auxins. But the cotyledonary node

or the *in vitro* node were able to develop mostly into single shoot in only few cultures. Therefore this explant failed to develop multiple shoots. Similar work in regeneration studies was conducted using nodal explants.

4.2.1.6 Effect of individual cytokinins on shoot induction in MS and WPM medium from nodal explants

Initially the response of nodal explants (1.5cm) was also evaluated in presence of individual cytokinins like BAP/Kn/TDZ in MS and WPM medium

The explants were placed in **MS** basal **medium** which was considered as control resulted in 50% response but when BAP was incorporated in the medium at different concentrations the buds break was observed within two weeks for mostly all the concentrations tried. BAP (16 µM) induced the axillary bud to proliferate into single shoot (Fig.17a) in 67% cultures and at 20µM a maximum number (1.7 ± 0.4) of shoots were formed with 1.5±0.7 number of nodes in 83% of cultures(Fig.17b). Replacing BAP with Kn in the medium able to induce a morphogenic response only at 8µM in 33 % cultures. Similarly in presence of TDZ response in terms of shoot formation was observed at 0.1µM and 2µM in 17 % cultures(Table 12).

Similar to cotyledonary node the nodal explants obtained poor response in **WPM medium** without PGRs, but when medium was fortified with BAP concentrations it induced bud break and formed shoots. BAP at 16µM induced response only in 33% cultures but the shoot which developed were healthy with large leaves (Fig.17c).Increase in BAP to 20µM induced 83% cultures to form shoots (1.3 ± 0.4) with 1.2 ± 0.3 number of nodes within four weeks (Fig.17d). The medium fortified with Kn concentration had a similar effect on explants as observed in MS medium with 8 µM inducing response in only 33% in cultures. Presence of TDZ in the medium improved the response in most of the concentrations tried and a maximum of 67% was achieved at 0.5µM and 2µM. TDZ at 0.5µM formed healthy shoots with large leaves (Fig.17e) and the number of shoots had an average of 1.3 ± 0.5 *in vitro* nodes(Table 12).

The morphogenic response which was obtained through nodal explants was best among all the explants tried as healthy shoots grew in both MS and WPM medium.

Table 12.Effect of individual cytokinins on shoot induction from nodal explants of *O.indicum* after 4 weeks

Cytokinin (µM)	MS medium			WPM medium		
	% Response	Number of shoots*	Number of *in vitro* nodes*	% Response	Number of shoots*	Number of *in vitro* nodes*
0	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{ab}	33	0.5 ± 0.3^{ab}	0.5 ± 0.3^{ab}
BAP						
2	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
4	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{ab}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
8	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{ab}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
16	67	0.7 ± 0.2^{b}	0.7 ± 0.2^{b}	**83**	$\mathbf{1.3\pm0.4^{c}}$	$\mathbf{1.2\pm0.3^{bc}}$
20	**83**	$\mathbf{1.7\pm0.4^{c}}$	$\mathbf{1.5\pm0.7^{c}}$	33	0.5 ± 0.3^{ab}	0.3 ± 0.2^{a}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
30	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{ab}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn						
2	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
4	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
8	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{a}
16	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
20	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
30	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
TDZ						
0.1	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.2	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{ab}
0.25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	17	0.2 ± 0.2^{ab}	0.3 ± 0.3^{a}
0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	67	0.7 ± 0.2^{bc}	1.3 ± 0.5^{c}
1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
2	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	67	0.8 ± 0.3^{bc}	0.7 ± 0.2^{ab}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq0.05$ according to Duncan's Multiple range test

Similar to cotyledonary node, the nodal explants were able to form shoots in both the media in presence of BAP and suitable to establish shoot cultures after 4 weeks and hence were taken up for further studies.

Fig.17 Shoot induction from nodal explants of *O.indicum* in presence of individual cytokinins after 4 weeks

a. Formation of single shoot in MS+BAP(16µM)
b. Devlopment of healthy shoot in MS+BAP(20µM)
c. Single shoot formation with large leaves in WPM+BAP(16µM)
d. Induction of a single shoot with small leaves in WPM+BAP(20µM)
e. Single shoot with large leaves in WPM+TDZ(0.5µM)

4.2.1.6.1 Multiplication of shoots from *in vitro* nodes in presence of individual cytokinins

The *in vitro* shoots obtained from nodal explants (at the end of 4 weeks) were also excised into single nodes and were subcultured in MS and WPM medium with respective concentrations. Observations were recorded for percent response and number of shoots after 8 weeks and further.

The *in vitro* nodes were subcultured in MS medium fortified with BAP (2, 4, 16, 20 and 30µM); Kn (8µM); TDZ(0.1 and 2µM) as these concentrations had induced a response.

The *in vitro* nodes could form single shoots only with an average of 0.2 ± 0.2 shoots in MS+BAP (20µM) with 17% response and 0.5 ± 0.2 shoots were formed in MS+TDZ(2µM) with 50% response after 8 weeks. The rest all the concentrations failed to develop shoots.

The *in vitro* nodes of these shoots when transferred to the same medium ie. BAP (20µM) and TDZ (2µM) failed to develop shoots by the end of 12 weeks. Whereas in WPM medium none of the concentrations were able to induce proliferation in buds to form shoots even after 8 weeks.

Since nodal explants were unable to form multiples in presence of individual cytokinins they were placed in MS and WPM medium fortified with two cytokinins.

4.2.1.7 Effect of two cytokinins on nodal explants

The nodal explants were also placed in MS and WPM medium fortified with combinations of two cytokinins ie. BAP with Kn, BAP with TDZ and Kn with TDZ with different concentrations to study their effect on shoot number.

- **Synergistic effect in MS medium**

- **BAP+Kn**

The first combination, BAP(8µM) with Kn (2,4,8,16µM) resulted in forming an average of 1.5 ± 0.6 number of shoots and 1.2 ± 0.3 number of nodes in 83% cultures when both cytokinins were at 8µM (Fig.18a). Increase in BAP to 16µM with different concentrations of Kn induced a maximum response at 16µM as 67% percent cultures were able to form 1.0 ± 0.4 number of shoot. But this number of shoots increased to 2.0 ± 0.6 when BAP was at 20µM with Kn(8µM) in 83 % cultures (Fig.18b)(Table 13).

A combination of BAP (20µM) with different concentrations of Kn (2, 4, 8 and 16µM) callus with shoot buds was induced from the base of explants which was transferred separately in respective fresh media.

- **BAP+TDZ**

When BAP(8/16/20µM) was combined with TDZ(0.1,0.2,0.25,0.5µM) concentrations ,the medium fortified with BAP(8µM) and TDZ(0.1µM) obtained maximum of 83 % response with 1.0 ± 0.3 mean number of shoots and 1.8 ± 0.6 number of nodes whereas in BAP(8µM) with TDZ(0.2µM) combination maximum 1.3 ± 0.5 number of shoots were obtained in 67% cultures (Fig.18c).When the level of concentration BAP was increased to 16µM ,20µM and combined with all the concentrations of TDZ resulted in poor response. There was only slight improvement in BAP (20µM) with TDZ (0.1µM and 0.2µM) having 50% response (Table 13).

A combination of BAP (20µM) with TDZ (0.1, 0.25, 0.5µM) resulted in formation of callus at the base of the explants within two weeks. This callus became nodular and regenerated shoot buds which covered the entire explants at its base.

- **Kn+TDZ**

In the last combination of cytokinin, Kn was coupled with TDZ(0.1,0.2,0.25,0.5µM) concentrations, the Kn(2µM) and Kn (4µM) combinations resulted in poor response while the percent response increased to 83% when Kn(8µM) was added with TDZ(0.5µM)(Table 13).

Out of all the combinations of cytokinins tried in MS medium the BAP (8µM) with Kn (8µM) resulted in maximum 83% response and 1.5 ± 0.6 shoots.

❖ **Synergistic effect in WPM medium**

The synergistic effect of cytokinins in WPM medium was as follows:

Table 13. Effect of two cytokinins on shoot induction from nodal explants of *O.indicum* after 4 weeks

Cytokinins (µM)			MS medium			WPM medium		
			% Response	Number of shoots*	Number of *in vitro* nodes*	% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**						
8	2	-	17	0.2±0.2^{ab}	0.2±0.2^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
8	4	-	17	0.2±0.2^{ab}	0.3±0.3^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
8	8	-	83	1.5±0.6^{de}	1.2± 0.3^{bc}	17	0.3±0.3^{ab}	0.5±0.5^{abc}
8	16	-	33	0.3±0.2^{ab}	0.5±0.3^{ab}	33	0.3±0.2^{ab}	0.3±0.2^{ab}
16	2	-	17	0.2±0.2^{ab}	1.0±1.0^{abc}	67	1.5±0.7^{cd}	0.8±0.2^{abc}
16	**4**	-	0	0.0±0.0^{a}	0.0±0.0^{a}	**100**	**2.0±0.4^{d}**	**3.3±0.5^{e}**
16	8	-	0	0.0±0.0^{a}	0.0±0.0^{a}	50	0.7±0.3^{abc}	0.7±0.3^{abc}
16	16	-	67	1.0±0.4^{bcd}	0.9±0.3^{abc}	50	0.7±0.3^{abc}	1.3±0.6^{abc}
20	2	-	33	1.2±0.7^{cde}	1.0±0.6^{abc}	17	0.2±0.2^{ab}	0.5±0.5^{abc}
20	4	-	33	0.3±0.2^{ab}	0.7±0.5^{abc}	50	1.0±0.5^{abcd}	1.3±0.6^{abc}
20	**8**	-	**83**	**2.0±0.6^{e}**	**1.7±0.4^{c}**	67	1.2±0.6^{bcd}	1.5±0.6^{bc}
20	16	-	50	0.7±0.3^{abcd}	0.5±0.2^{ab}	50	0.8±0.4^{abc}	1.3±0.7^{abc}
8	-	0.1	83	1.0±0.3^{bcd}	1.8±0.6^{c}	33	0.5±0.3^{abc}	0.8 ±0.5^{abc}
8	-	0.2	67	1.3±0.5^{cde}	1.7±0.8^{c}	33	0.7±0.5^{abc}	0.6±0.4^{abc}
8	-	0.25	17	0.2±0.2^{ab}	0.7±0.7^{abc}	50	0.5±0.2^{abc}	1.7±0.8^{cd}
8	-	0.5	33	0.3±0.2^{ab}	0.3±0.2^{ab}	100	1.2±0.2^{bcd}	3.0±0.4^{de}
16	-	0.1	17	0.3±0.3^{ab}	0.3±0.3^{ab}	33	0.3±0.2^{ab}	0.3±0.2^{ab}
16	-	0.2	33	0.3±0.2^{ab}	0.3±0.2^{ab}	33	0.3±0.2^{ab}	1.0±0.7^{abc}
16	-	0.25	17	0.2±0.2^{ab}	0.2±0.2^{ab}	17	0.5±0.5^{abc}	0.4±0.4^{abc}
16	-	0.5	0	0.0±.0.0^{a}	0.0±0.0^{a}	83	1.2±0.3^{bcd}	1.3±0.4^{abc}
20	-	0.1	50	0.8±0.5^{abcd}	0.5±0.2^{ab}	50	1.2±0.7^{bcd}	0.8±0.4^{abc}
20	-	0.2	50	0.3±0.3^{ab}	0.3±0.3^{ab}	50	0.8±0.4^{abc}	1.5±0.7^{bc}
20	-	0.25	33	0.5±0.3^{abc}	0.3±0.2^{ab}	33	0.5±0.3^{abc}	0.7±0.5^{abc}
20	-	0.5	33	0.5±0.3^{abc}	0.8±0.7^{abc}	83	1.0±0.4^{abcd}	1.4±0.6^{bc}
-	2	0.1	0	0.0±0.0^{a}	0.0±0.0^{a}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	2	0.2	0	0.0±0.0^{a}	0.0±0.0^{a}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	2	0.25	17	0.2±0.2^{ab}	0.2±0.2^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	2	0.5	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	4	0.1	17	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	4	0.2	17	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
-	4	0.25	17	0.2±0.2^{ab}	0.2±0.2^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	4	0.5	17	0.2±0.2^{ab}	0.2±0.2^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	8	0.1	33	0.3±0.2^{ab}	0.3±0.2^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	8	0.2	33	0.3±0.2^{ab}	0.5±0.3^{ab}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	8	0.25	67	0.7±0.2^{abcd}	0.7±0.2^{abc}	17	0.2±0.2^{ab}	0.2±0.2^{ab}
-	8	0.5	83	0.8±0.2^{abcd}	1.0±0.3^{abc}	0	0.0±0.0^{a}	0.0±0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test

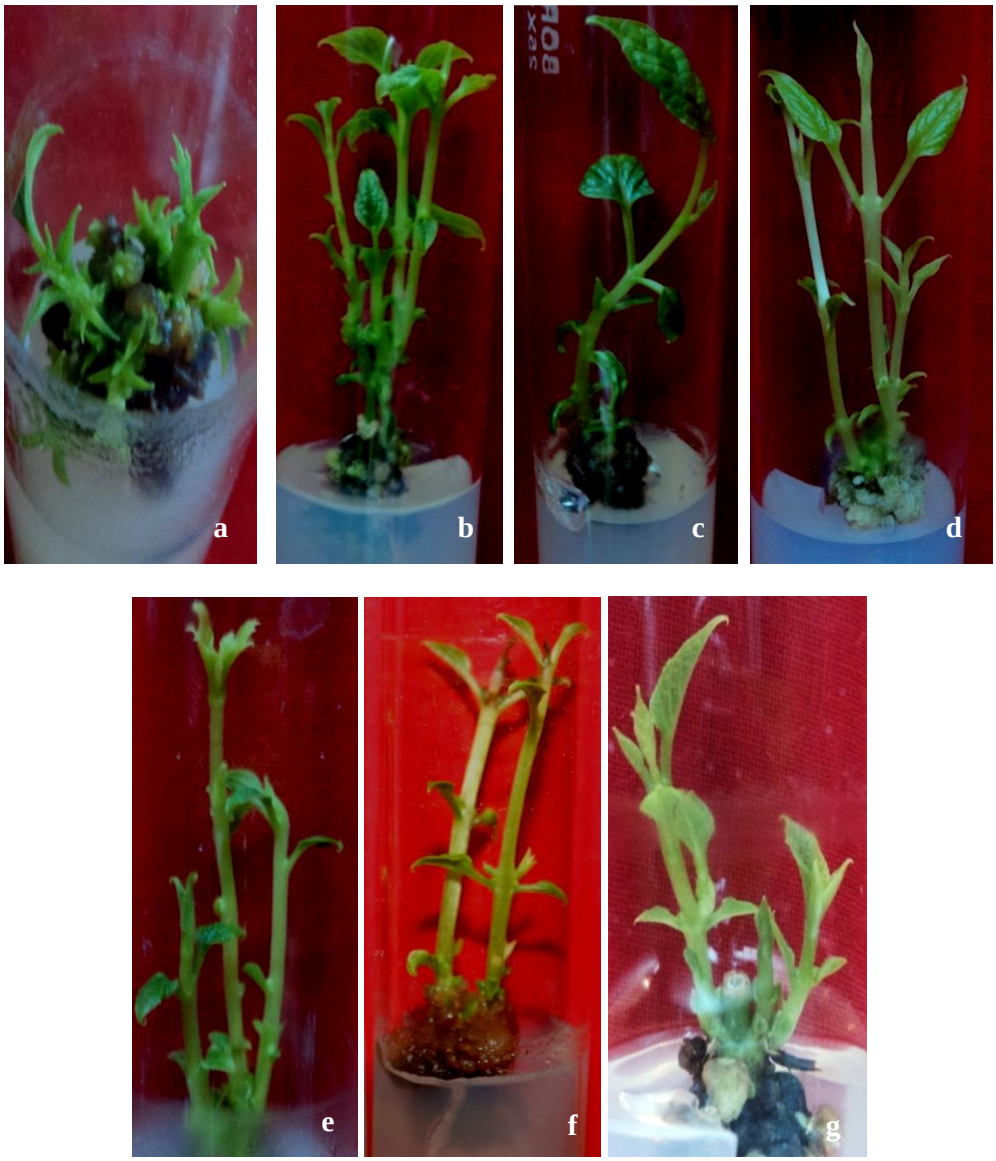

Fig. 18: Shoot induction from nodal explants of *O.indicum* in presence of two cytokinins after 4 weeks

a. Small shoots developed in MS+BAP(8 µM)+Kn(8 µM)

b. Shoots developed in MS+ BAP(20µM) +Kn(8µM)

c. Single shoot with large leaves in MS+ BAP(8µM) +TDZ(0.2µM)

d. Healthy shoots developed in WPM+BAP(16µM)+Kn(2µM)

e. Healthy shoots developed in WPM+BAP(16µM) +Kn(4µM)

f. Two shoots developed from the axil of nodes in WPM+BAP(20µM)+Kn(8 µM)

g. Small multiples developed in WPM+BAP(8µM) +TDZ(0.5µM)

- **BAP+Kn**

When BAP and Kn were added in combination with different concentrations in WPM medium BAP at 8µM combination with Kn (2, 4, 8, 16µM) evoked very less percent response and number of shoots. Whereas BAP at 16µM when combined with Kn (2µM), resulted in developing healthy number of shoots (1.5 ± 0.7) with 67% response (Fig.18d). Increase in level of Kn at 4µM a 100% response was observed with an average of 2.0 ± 0.4 number of long healthy shoots (Fig.18e) with many nodes (3.3 ± 0.5) but the response decreased when BAP (16µM) was added with Kn at 8µM and 16µM. Further increase in level to BAP 20µM with Kn (2, 4, 8, 16µM) also resulted in formation of healthy shoots. A maximum number of shoots (1.2 ± 0.6) and nodes (1.7 ± 0.4) were formed in presence of BAP (20µM) and Kn(8 µM) combination(Fig.18f)(Table13).

- **BAP+TDZ**

BAP and TDZ combination also resulted in similar response with formation of healthy shoots at varied concentrations. Out of the different combinations of BAP (8µM) with TDZ(0.1,0.2,0.25,0.5µM) maximum 100 % response was observed in BAP(8µM) +TDZ(0.5 µM) with highest number of nodes (3.0 ± 0.4) (Fig.18g).Whereas in BAP (16µM) and BAP (20µM)combinations with all TDZ concentrations, 83% response was observed at TDZ(0.5 µM) but the number varied(Table 13) .

- **Kn+TDZ**

All the combinations of Kn (2, 4 and 8µM) with TDZ (0.1,0.2,0.25µM) obtained very poor response in terms of shoot formation in WPM medium(Table 13) as the shoots formed were very weak.

Hence a maximum of 100 % response with 2.0±0.4 number of shoots was obtained in WPM medium fortified with BAP (16µM) with Kn(4µM) out of all the combinations.

4.2.1.8.1 Multiplication from *in vitro* nodes in presence of two cytokinins

The *in vitro* shoots which developed from nodal explants in combinations of cytokinins (at the end of 4 weeks) were excised in single nodes and subcultured in MS and WPM medium fortified with respective concentrations for further enhancement of shoots. Observations were recorded for percent response and number of shoots after 8 weeks and in further passages.

By the end of 8 weeks it was observed that out of various combinations, the MS medium fortified with BAP(8/20µM) with TDZ(0.25µM), the *in vitro* axillary buds were able to respond (Fig.19) while rest all combinations failed to respond. In BAP(8µM) with TDZ(0.25 µM) combination 33% response was obtained with an average of only 1.7±1.1 shoots (Fig.20a) after 8 weeks which was slightly more from the number obtained at the end of 4 weeks. Comparatively in BAP (20µM) with TDZ (0.25µM) percent response was more (67%) but number of shoots were less (1.0 ± 0.4) (Fig.19).

Whereas in WPM medium fortified with BAP(8µM) and Kn(8µM), BAP(16µM) and Kn(16µM) ,BAP(16µM) with TDZ(0.5µM),BAP(20µM) with TDZ(0.1µM,0.2µM) combinations only induced response after 8 weeks (Fig.19).

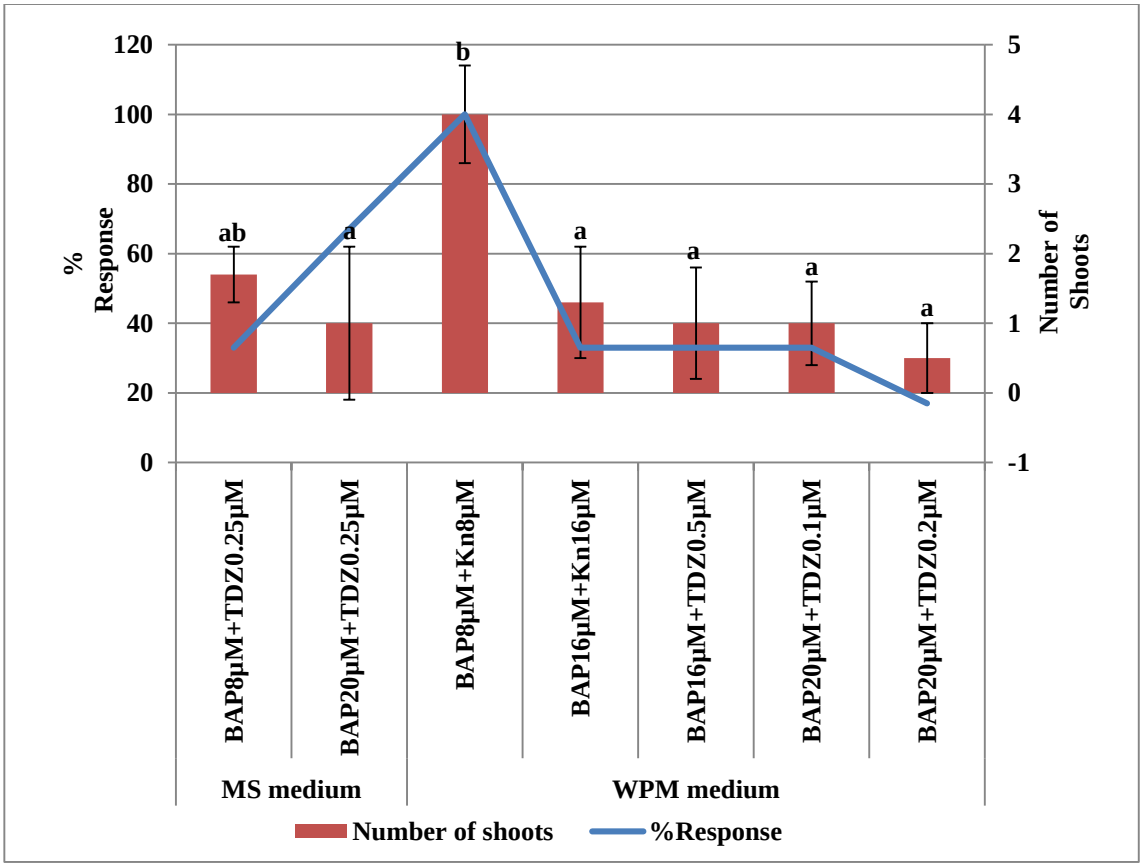

Fig. 19: Effect of two cytokinins in inducing multiples from *in vitro* nodes of *O.indicum* after 8 weeks

Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

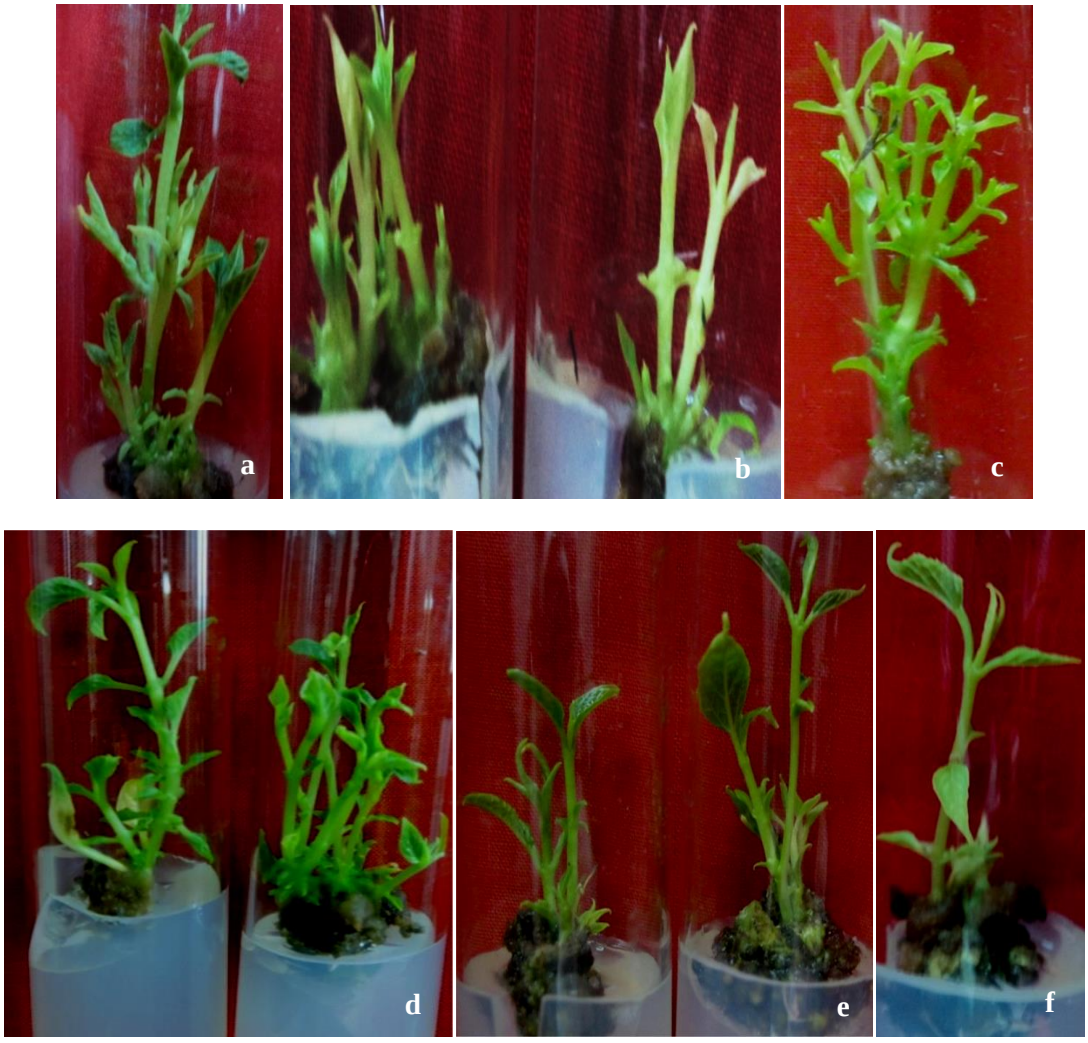

Fig.20: Multiple shoots developed from *in vitro* nodes of *O.indicum* in presence of two cytokinins

a. Induction of shoots in MS+BAP(8µM) +TDZ(0.25µM) after 8 weeks
b. Shoot developed in WPM + BAP(8µM) +Kn(8µM) after 8 weeks
c. Shoots developed in MS+BAP(8µM) +TDZ(0.25µM) after 12 weeks
d. Healthy multiples developed in MS+BAP(8µM) +TDZ(0.25µM) after 16 weeks
e. One or two shoots developed in WPM+BAP(8µM) +Kn(8µM) after 12 weeks
f. Development of shoots in WPM+BAP(8µM)+Kn(8µM) after 16 weeks

In each combination there was formation of one or two shoots except in WPM fortified with BAP (8µM) and Kn (8µM) where 100% response was observed with an average number (4.0 ± 0.7) of shoots with 3-4 nodes (Fig.19) (Fig.20b).

In MS medium fortified with BAP(20µM) with TDZ(0.25µM), the shoots failed to grow after 10 weeks and whereas in BAP(8µM) with TDZ(0.25µM) there was 100% response observed and the number enhanced to an average of 5.0 ± 1.3 healthy shoots by end of 12 weeks (Fig.20 c and d). The shoots were again excised into single nodes and were subcultured in the same medium but after 16 weeks there was only slight increase in number observed with an average of 5.3 ± 1.5 healthy shoots (Fig.20e) (Fig.21).

The *in vitro* nodes which were transferred to WPM medium fortified with BAP (16µM) and Kn (16µM) or TDZ(0.5µM),BAP(20µM) with TDZ(0.1,0.2µM) combinations were subcultured but failed to form the shoots after 12 weeks. Only in medium fortified with BAP (8µM) and Kn(8µM) 100% culture responded by the end of 12 weeks but a decreased in shoot number to 3.0 ± 0.4 was observed(Fig.20e) (Fig.21).

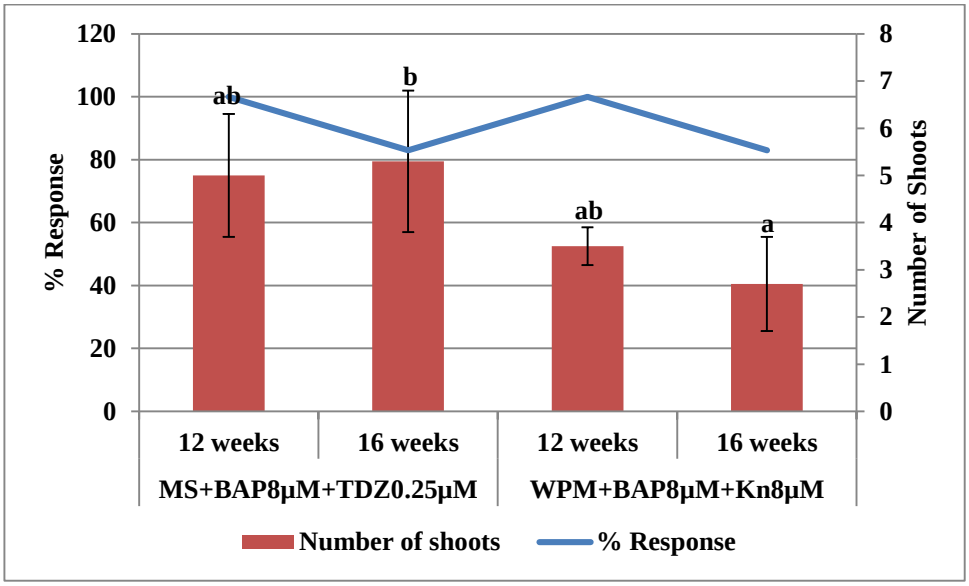

Fig.21: Number of shoots from *in vitro* nodes of *O.indicum* after 12 and 16 weeks in MS and WPM medium

Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

These shoots were divided into single *in vitro* nodes and subcultured in the same combination but there was decreased in the number 2.7 ± 1.0 as well as percent response to 83% by the end of 16 weeks(Fig.20f).

The combinations in which shoots were formed upto 16 weeks were MS medium supplemented with BAP (8µM) and TDZ(0.25µM) and WPM medium fortified with BAP(8 µM) and Kn(8µM). Thus in the same *in vitro* nodes were placed and observations were recorded for further passages ie. after 24 and 32 weeks.

In MS medium fortified with BAP(8µM) and TDZ(0.25µM) combination for every passage 100% response was obtained but there was reduction in shoot number as it reached to(2.2 ± 0.3) after 24 weeks and again increased to 3.2 ± 0.6 after 32 weeks. These shoots subcultured in the same medium get elongated with an average shoot length (2.2 ± 0.3 cms) after 32 weeks (Fig.21).In WPM medium supplemented with BAP(8µM) and Kn(8µM) combination the shoots were subcultured but failed to survive in subsequent passages.

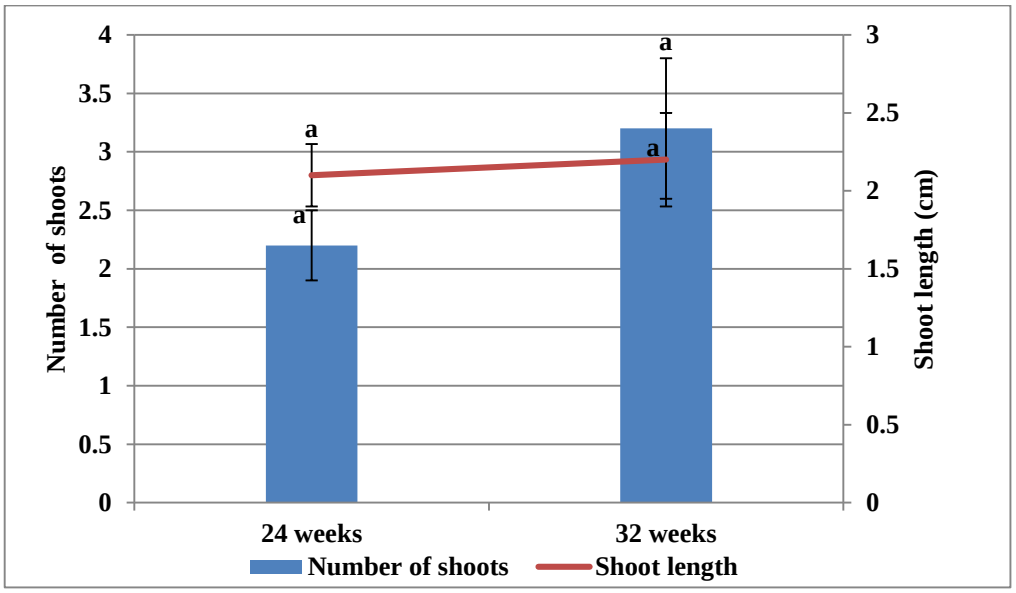

Fig.22: Average number of shoot and shoot length of *O.indicum* in MS+BAP(8µM) +TDZ(0.25µM) combination after 24 and 32 weeks

Values represents mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

4.2.1.8 Effect of cytokinins with auxins on nodal explants

Similar to cotyledonary node explants the cytokinin and auxin interaction was also tried for nodal explants. The auxins (IAA/IBA/NAA) were added with individual concentrations of cytokinins (BAP/Kn/TDZ) and were evaluated for their effect on formation of number of shoot and *in vitro* nodes.

Shoot induction in MS medium

The nodal explants were placed in medium fortified with cytokinin and auxins, and after 4 weeks the response was evaluated. The BAP (20µM) with different concentration of IAA resulted in single shoot formation, replacing IAA with IBA proved to be effective comparatively in terms of number of shoots and nodes. BAP at 20µM with IBA (0.5 µM)induced 83 % response resulting in long shoots which had maximum number of nodes (2.8±1.0) (Fig.23a).Increase in IBA to 1µM similar response was obtained but the shoots developed were maximum with 1.2±0.3 number (Fig.23b) among all the combinations used. When BAP was added with different concentrations of NAA all the combinations failed to improve the response in terms of shoots (Table 14). In BAP (20µM) with NAA (0.1, 0.5µM), BAP (20µM) with IBA (0.1, 0.5, 1µM) combinations callus with shoot buds were produced at the base of the nodes which were transferred to fresh medium.

When Kn (8µM) and TDZ(0.1µM) was added with different concentrations of auxins (IAA/NAA/IBA) it resulted in poor response in terms of percent shoot formation (Table 14).

Thus in MS medium fortified with BAP (20µM) and IBA (0.1µM) resulted in maximum number of shoots (1.2 ± 0.3) and nodes (1.8 ± 0.7) out of all the combinations used.

Shoot induction in WPM medium

In WPM medium the BAP (16µM) combination with auxin proved to be very effective in terms of number of shoot and nodes after 4 weeks. When BAP (16µM) was added with different concentrations of IAA it resulted in forming healthy shoots but leaves of lower nodes were minute compared to upper node leaves. These shoots were with more than

Fig.23: Shoot induction from nodal explants of *O.indicum* in presence of cytokinins and auxins after 4 weeks

a. Induction of long shoot in MS+BAP(20µM) +IBA(0.5µM)
b. Shoot with small compound leaves in MS+BAP(20µM)+IBA(1µM)
c. Development of more than one shoots in WPM+BAP(16µM) +IAA(1µM)
d. Multiple shoots developed inWPM+BAP(16µM)+IBA(0.1µM)
e. Two shoots developed in WPM+BAP(16µM) +IBA(1µM)
f. Shoots developed in WPM+BAP(16µM)+NAA(0.5µM)

Table 14. Effect of cytokinins and auxins on shoot induction from nodal explants of *O.indicum* in MS medium after 4 weeks

Cytokinins (µM)			Auxins (µM)			% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**	**IAA**	**IBA**	**NAA**			
20	-	-	0.1	-	-	33	0.6 ± 0.5^{ab}	0.5 ± 0.3^{ab}
20	-	-	0.5	-	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
20	-	-	1	-	-	50	0.7 ± 0.5^{ab}	1.2 ± 0.7^{bc}
20	-	-	-	0.1	-	50	0.5 ± 0.2^{ab}	0.7 ± 0.3^{abc}
20	-	-	-	0.5	-	83	1.0 ± 0.3^{ab}	2.8 ± 1.0^{d}
20	-	-	-	**1**	-	**83**	$\mathbf{1.2\pm0.3^{b}}$	$\mathbf{1.8\pm0.7^{cd}}$
20	-	-	-	-	0.1	17	0.8 ± 0.8^{ab}	0.3 ± 0.3^{ab}
20	-	-	-	-	0.5	33	1.2 ± 1.0^{b}	0.5 ± 0.3^{ab}
20	-	-	-	-	1	33	0.3 ± 0.2^{ab}	0.7 ± 0.4^{abc}
-	8	-	0.1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	0.5	-	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
-	8	-	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.5	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
-	8	-	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	-	0.1	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
-	8	-	-	-	0.5	33	0.3 ± 0.2^{ab}	0.5 ± 0.3^{ab}
-	8	-	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	0.1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	0.5	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	1	-	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
-	-	0.1	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	1	-	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
-	-	0.1	-	-	0.1	33	0.3 ± 0.2^{ab}	0.7 ± 0.4^{abc}
-	-	0.1	-	-	0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test

two nodes which were with short internodes. BAP at 16µM with IAA at 0.1µM resulted in 83% response with 2.2 ± 1.0 number of shoots and 2.4 ± 0.6 nodes. Increase in IAA to 1µM resulted in 100% cultures with slightly higher (2.3 ± 0.7) number of shoots (Fig.23c). When BAP (16 µM) was added with IBA (0.1, 0.5,1µM) there was formation of healthy shoots with

many nodes but internodal length was less. IBA at 0.1 µM resulted in 2.2 ± 0.3 number of healthy shoots in 100 % cultures (Fig.23d) whereas in increased to IBA (0.5µM) 83% response was obtained with an average number of 1.7 ± 0.6 shoots.

Table 15. Effect of cytokinins and auxins on shoot induction from nodal explants of *O.indicum* in WPM medium after 4 weeks

Cytokinins (µM)			Auxins (µM)			% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**	**IAA**	**IBA**	**NAA**			
16	-	-	0.1	-	-	83	2.2 ± 1.0^{ef}	2.4 ± 0.6^{fg}
16	-	-	0.5	-	-	100	$1.7\pm0.3d^{ef}$	2.3 ± 0.4^{fg}
16	-	-	**1**	-	-	**100**	$\mathbf{2.3\pm0.7^{f}}$	$\mathbf{2.6\pm0.2^{fg}}$
16	-	-	-	0.1	-	100	2.2 ± 0.3^{ef}	1.8 ± 0.2^{def}
16	-	-	-	0.5	-	83	1.7 ± 0.6^{def}	1.9 ± 0.6^{ef}
16	-	-	-	1	-	83	1.2 ± 0.3^{bcde}	3.1 ± 0.4^{g}
16	-	-	-	-	0.1	83	1.0 ± 0.3^{abcd}	1.2 ± 0.3^{bcde}
16	-	-	-	-	0.5	100	1.3 ± 0.2^{cdef}	2.0 ± 0.4^{ef}
16	-	-	-	-	1	50	0.7 ± 0.3^{abcd}	1.7 ± 0.9^{cde}
-	8	-	0.1	-	-	17	0.2 ± 0.2^{ab}	0.3 ± 0.3^{ab}
-	8	-	0.5	-	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
-	8	-	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.1	-	33	0.3 ± 0.2^{abc}	0.5 ± 0.3^{ab}
-	8	-	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	1	-	33	0.3 ± 0.2^{abc}	0.7 ± 0.4^{abc}
-	8	-	-	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	-	0.5	33	0.3 ± 0.2^{abc}	0.5 ± 0.3^{ab}
-	8	-	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.5	0.1	-	-	17	0.2 ± 0.2^{ab}	0.3 ± 0.3^{ab}
-	-	0.5	0.5	-	-	33	0.2 ± 0.2^{ab}	0.5 ± 0.3^{ab}
-	-	0.5	1	-	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.5	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.5	-	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.5	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.5	-	-	0.1	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
-	-	0.5	-	-	0.5	50	0.7 ± 0.3^{abcd}	0.8 ± 0.4^{abcd}
-	-	0.5	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test

Further increase to IBA (1µM) decreased the number of shoots but increase in number of (3.1 ± 0.4) nodes (Fig.23e) .When BAP (16µM) was coupled with different concentrations of NAA healthy shoots were formed. NAA at 0.1µM induced shoots with long internodal length having many nodes and upper node leaf were large in size compared to lower nodes but at NAA (0.5µM) an average of 1.3 ± 0.2 shoots developed in 100% cultures (Fig.23f).Similarly in MS medium incorporating Kn(8µM) with different auxins obtained poor response as single shoot were formed with only one or two nodes. TDZ at 0.5 µM with different auxin concentrations also evoked poor response in terms of percent shoot formation (Table 15).

Therefore in WPM medium out of all the combinations use BAP (16µM) and IAA(1µM) resulted in maximum number of shoots(2.3 ± 0.7) and 2.6 ± 0.2 nodes in 100% cultures.

4.2.1.8.1 Multiplication of shoots from *in vitro* nodes in presence of cytokinins and auxins

In vitro nodes developed from combinations of cytokinin and auxins (after 4 weeks) were also subcultured and the observations were recorded after 8 weeks and in subsequent passages.

In MS medium fortified with the various concentrations of cytokinin and auxin combinations no further growth was observed after 8 weeks. Presence of BAP (20µM) with IBA (0.5µM) (Fig.24a), and with IBA (1µM) where shoots were formed with an average of 1.3±0.9 and 1.0±0.0 respectively (Fig.25).Similarly in WPM medium many combinations failed to respond only BAP at 16 with IAA (0.5µM), IAA (1µM) and BAP (16µM) with IBA(0.1µM) (Fig.24 b, c and d) resulted in shoots formation after 8 weeks. A maximum of 3.5 ± 0.7 number of healthy shoots were formed in presence of IBA at 0.1µM (Fig.25).

Fig.24: Multiples developed from *in vitro* nodes of *O.indicum* in presence of cytokinin and auxin

a. Small multiple shoots developed in MS+BAP(20µM)+IBA(0.5µM) after 8 weeks

b. Shoots developed in WPM+BAP(16 µM) +IAA(0.5µM) after 8 weeks

c. Small shoots developed in WPM+BAP(16µM) +IAA(1µM) after 8 weeks

d. Healthy shoot developed in WPM+ BAP(16µM) +IBA(0.1µM) after 8 weeks

e. and f. Healthy multiples developed in MS+BAP(20µM) +IBA(0.5µM) after 12 weeks

g. Shoots developed in MS+BAP(20µM) +IBA(0.5µM) after 16 weeks

h. Shoots developed in WPM+BAP(16µM) +IAA(1µM) after 12 weeks

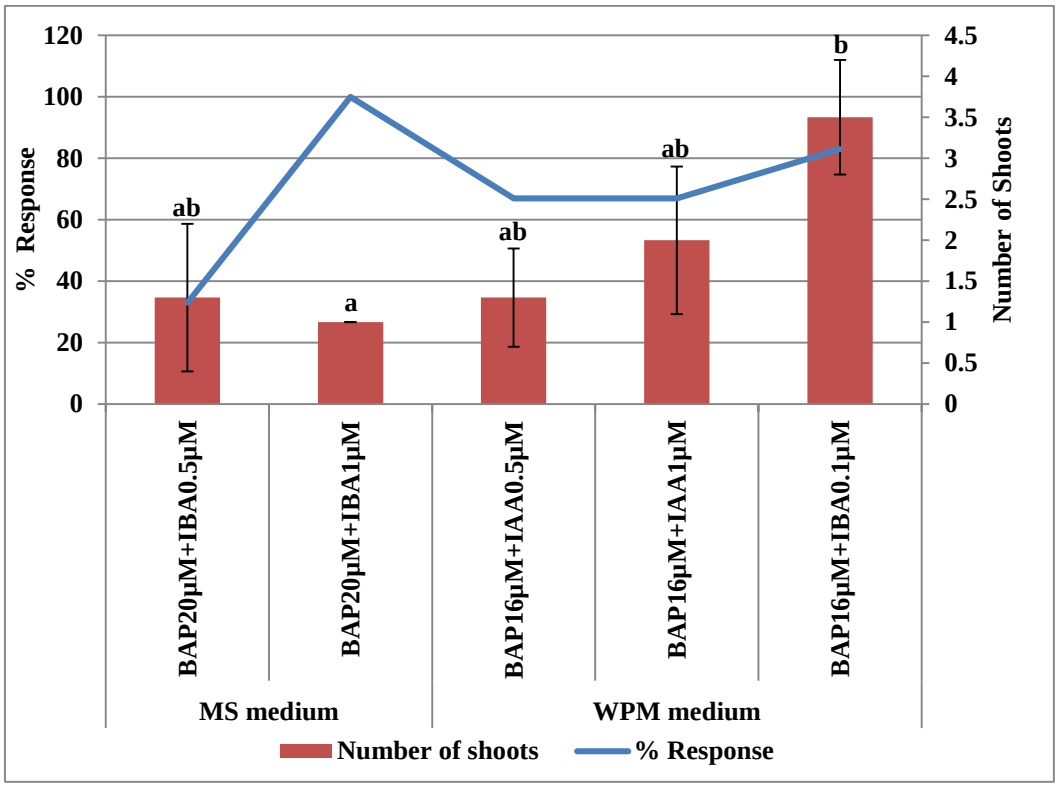

Fig. 25: Effect of cytokinins and auxins in inducing multiples from *in vitro* nodes of *O.indicum* after 8 weeks

Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

In the next subculture after 12 weeks there was an increase in number of shoots formed with an average of 3.7 ± 1.1 in MS medium fortified with BAP(20µM) and IBA(0.5µM) combination while the rest of the combinations failed to respond (Fig.24 e and f) within 12 weeks(Fig.26). In medium fortified with BAP (20µM) and IBA (0.5µM) the shoots number got drastically reduced to one or two shoots per node by the end of 16 weeks (Fig.24g).

In WPM medium fortified with BAP (16µM) and IAA(0.5µM) combination the number remained the same while in BAP(16µM) and IAA(1µM) there was an increase in number of shoots reaching upto 4.0 ± 1.7 after 12 weeks (Fig.24h) which reduced by the end of 16 weeks(Fig.26).

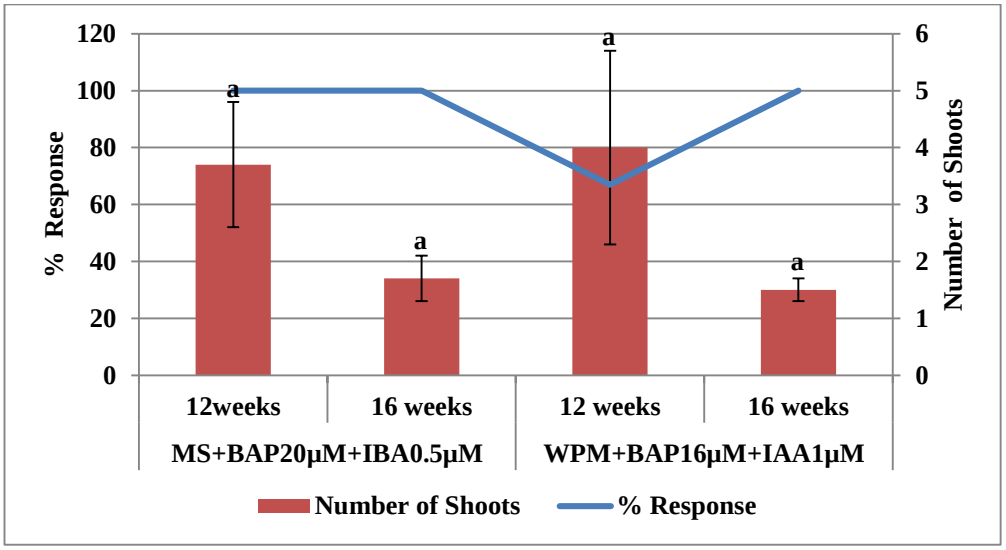

Fig.26: Effect of MS+BAP(20µM) +IBA(0.5 µM) and WPM + BAP(16µM) +IAA(1µM) on inducing multiples in *O.indicum* after 12 and 16 weeks

Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

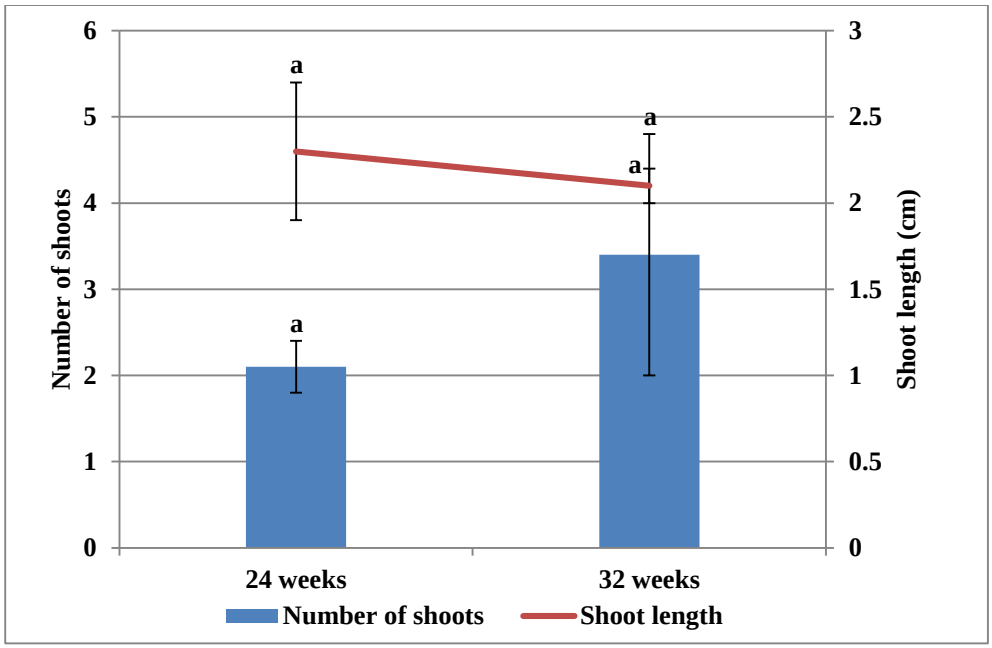

Fig. 27: Average shoot number and shoot length of *O.indicum* in MS+ BAP(20 µM)+IBA(0.5 µM) combination

Values represents mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

The shoots in MS medium with BAP (20µM) and IBA(0.5µM) and WPM medium with BAP(16µM) and IAA(1µM) combinations were again subcultured. After 24 weeks in WPM medium fortified with BAP (16µM) and IAA (1µM) combination shoots failed to survive and whereas in MS medium with BAP (20µM) and IBA (0.5µM) there was again an increase in number of shoots was observed (Fig.27) with an average shoot length reaching 2.3 ± 0.4 cms after 24 weeks but decreased to 2.1 ± 0.1 cm after 32 weeks.

From all the above experiments concludes that the nodal explants was the most suitable explants in regenerating shoots as well as forming healthy multiples in MS and WPM medium.

The overall results from nodal explants concludes that BAP was the most effective cytokinin individually and in combinations in terms of shoot induction and multiplication in both the media. In MS medium fortified with BAP (20µM), BAP (8µM) with TDZ (0.25µM) and BAP (20µM) with IBA (0.5µM) was the best combinations in inducing maximum number of shoots and in WPM medium fortified with BAP(16µM) individually and in combination with cytokinin (BAP16µM with Kn 4µM) and auxin(BAP16µM with IAA1µM) was the effective concentration in inducing maximum number of shoots.

4.2.2 Indirect organogenesis

In MS and WPM medium fortified with various PGRs there was formation of morphogenic callus at the base of cotyledonary node and nodal explants. This callus differentiated shoot buds and hence was excised and placed on their respective media for enhancing the number of shoots.

- **Cotyledonary node explants**

In this explant there was formation of morphogenic callus in presence of individual cytokinin, combinations of cytokinin and cytokinin with auxins after 4 weeks.

4.2.2.1 Individual cytokinin

In presence of individual cytokinin a morphogenic callus was induced below *in vitro* shoots which developed from cotyledonary node explants in MS medium fortified with BAP at 4µM and 20µM whereas in WPM medium it was induced in presence of BAP at 16µM and 20µM.

In MS medium fortified with BAP (4µM) the basal callus with shoot buds (Fig.28a) was subcultured initially in the same medium with respective concentrations. After 8 weeks it was observed that there was further differentiation of shoot buds but they failed to elongate into shoots. Therefore the clumps of shoot buds was transferred to another medium fortified with BAP (4µM) and GA_3 (1µM) as GA_3 is known to elongate shoots. Supplementing the medium with GA_3 allowed the shoot buds to grow into shoots. Transferring this differentiating callus to same medium every four weeks enhanced the number of shoots to 19.3 ± 2.4 after 16 weeks (Fig.28b; Fig 29).The morphogenic callus reduced its potency to regenerate large number of shoots after 24 weeks and 32 weeks.

Similarly in presence of BAP at 20µM there was formation of slight callus with shoot buds below the *in vitro* shoots(Fig.28c) which was subcultured in lower concentration ie. 8µM and after 8 weeks of transfer there was an increase in shoot buds. Therefore in the further subculture clumps of shoot buds were transferred to medium with BAP at 8 µM and in medium fortified with BAP (8µM) and GA_3 (1µM). It was observed that in the subsequent passages ie. by the end of 12,16 and 24 weeks in both the combinations there was an increase in shoot number(Fig.28d;Fig.29).

Similarly in WPM medium fortified with BAP (16µM) callus with shoot buds differentiated below the *in vitro* shoots and was subcultured by adding silver nitrate (20mg/l) to evaluate its effect on enhancing the shoot number. Observation revealed that there was formation of shoot buds only which failed to elongate into shoots by the end of 8 weeks.

The morphogenic callus which developed in presence of BAP at 20µM initially differentiated many shoot buds by 8 weeks, but when subcultured formed an average of 5.6 ± 2.0 number of shoots by the end of 12 weeks (Fig.28e). These shoot clumps when transferred to same medium a two fold increase in number of shoots (10.0 ± 0.0) was observed by the end of 16 weeks but after 24 weeks the number decreased to 3.0 ± 1.3 (Fig.29).The shoots which developed were small and hence, in order to elongate the shoots they were transferred to medium fortified with BAP (20µM) and GA_3 (1µM). It was observed that the shoots formed were long but devoid of leaves (Fig.28f).

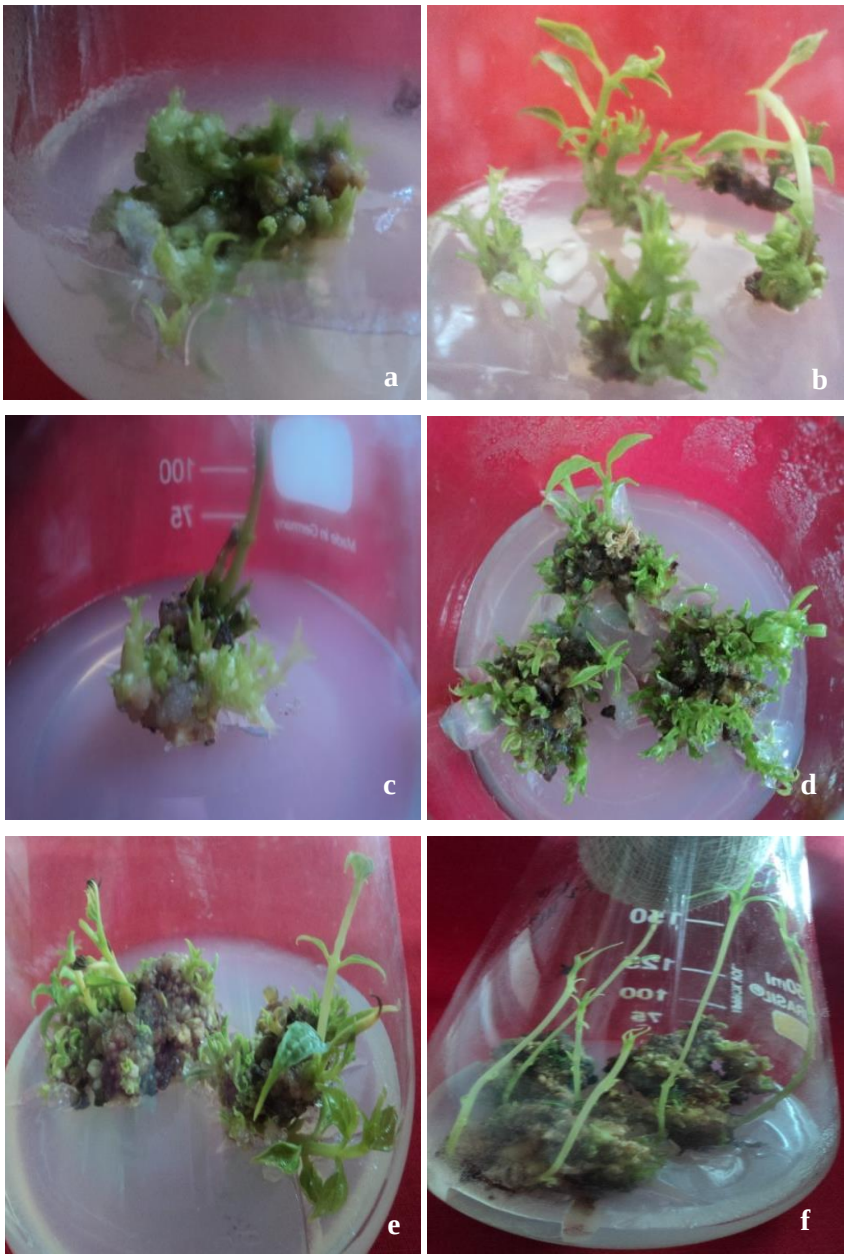

Fig.28: Shoot differentiation from morphogenic basal callus in *O.indicum*

a. Callus with shoot buds at the base of nodes in MS+BAP(4µM) after 4 weeks.

b. Differentiation of shoots in MS+BAP (4µM) +GA_3 (1µM) after 16 weeks

c. Callus with shoot buds induced at base of nodes in MS+BAP(20µM) after 4 weeks

d. Shoots developed in MS+BAP(8µM)+GA_3(1µM) after 12 weeks

e. Shoots developed in WPM+BAP(20µM) after 12 weeks

f. Shoots of WPM+BAP(20µM) transfer to WPM+BAP(20µM) +GA_3(1µM) get elongated after 32 weeks

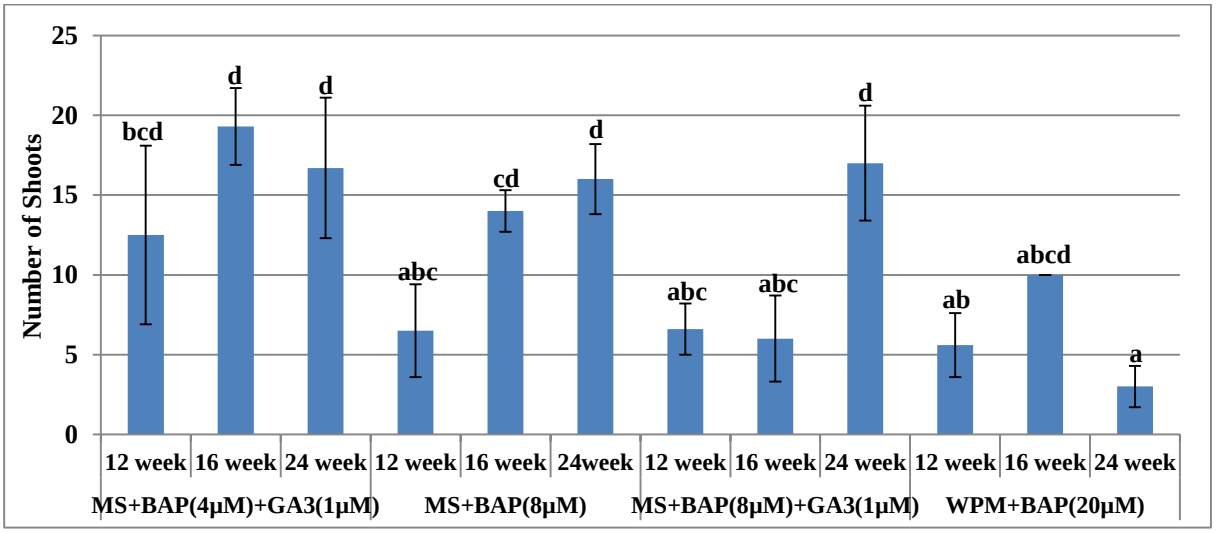

Fig.29: Multiple shoots formation from morphogenic callus in MS and WPM medium fortified with different PGRs after 12, 16, 24 weeks in *O.indicum*

Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

4.2.2.2 Synergistic effect of cytokinins

The basal callus with shoot buds developed from cotyledonary nodes were observed (after 4 weeks) in the combinations: MS medium with BAP (8µM) and TDZ (0.25µM), BAP (20µM) with TDZ (0.1µM, 0.2µM). This morphogenic callus lost its potency to regenerate to differentiate shoots after 8 weeks.

In WPM medium also morphogenic callus developed in WPM medium fortified with BAP (16µM) with Kn (8µM, 16µM), BAP (8µM) and TDZ (0.5µM) were transferred in respective media. But only in medium fortified with BAP (16µM) and Kn (8µM) combination an average number of 2.5 ± 2.5 shoots were formed after 8 weeks. The shoots get elongated in the same medium and new shoots also develop. But after second subculture the shoots failed to survive by the end of 12 weeks.

4.2.2.3 Effect of cytokinins and auxins

The combinations of cytokinins and auxin which had resulted in formation of basal morphogenic callus in *in vitro* shoots developed from cotyledonary node explants in previous studies are MS medium fortified with BAP (20µM) with NAA (0.1µM) and BAP(20µM)

with NAA1µM). This morphogenic callus was excised and transferred to the same medium and an average of 3.0 ± 0.0 shoots were formed after 8 weeks. These shoot clumps were again subcultured in the same medium which resulted in differentiating many shoot buds and few shoots but the number decreased after 12 weeks and further they failed to survive.

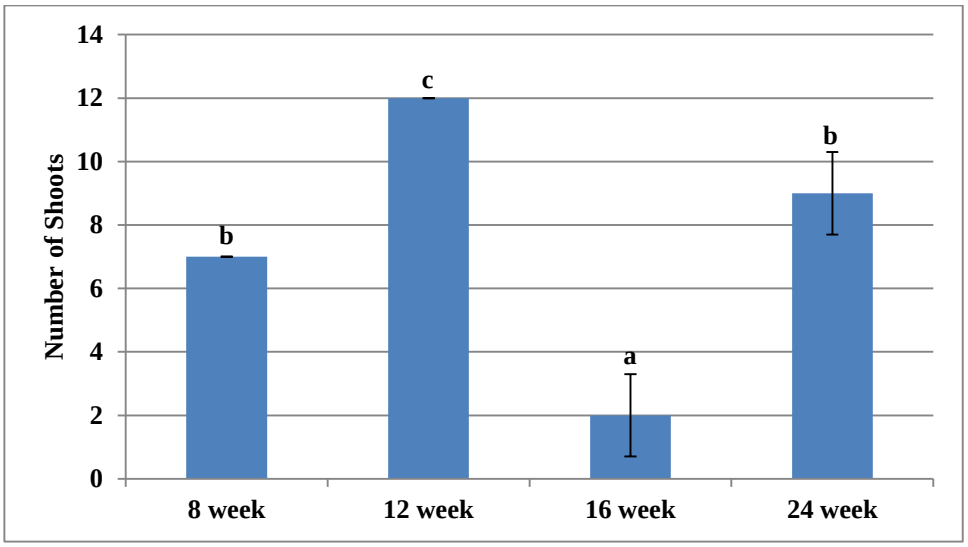

Fig. 30: Average number of shoots from morphogenic callus in MS+ +BAP (20µM) +NAA(1µM) after 8,12,16,24 weeks in *O.indicum*

Values represents mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

Whereas the morphogenic callus in MS medium fortified with BAP (20µM) and NAA (1µM) after 8 weeks was able to differentiate shoot buds of which few of them elongated into shoots (7.0 ± 0.0) in number. In the next subculture ie. by the end of 12 weeks the number increased to 12.0 ± 0.0. This shoot clumps along with callus when further subcultured every four week there was a decrease in number of shoots after each passage (Fig.30) (Fig.31 a-d). Therefore results of indirect organogenesis revealed that the morphogenic callus developed below in vitro shoots formed from cotyledonary node explants had the potency to differentiate into shoots. The highest number of shoots (19.3 ± 2.4) were developed in MS medium fortified with BAP (4 µM) and GA_3 (1 µM).

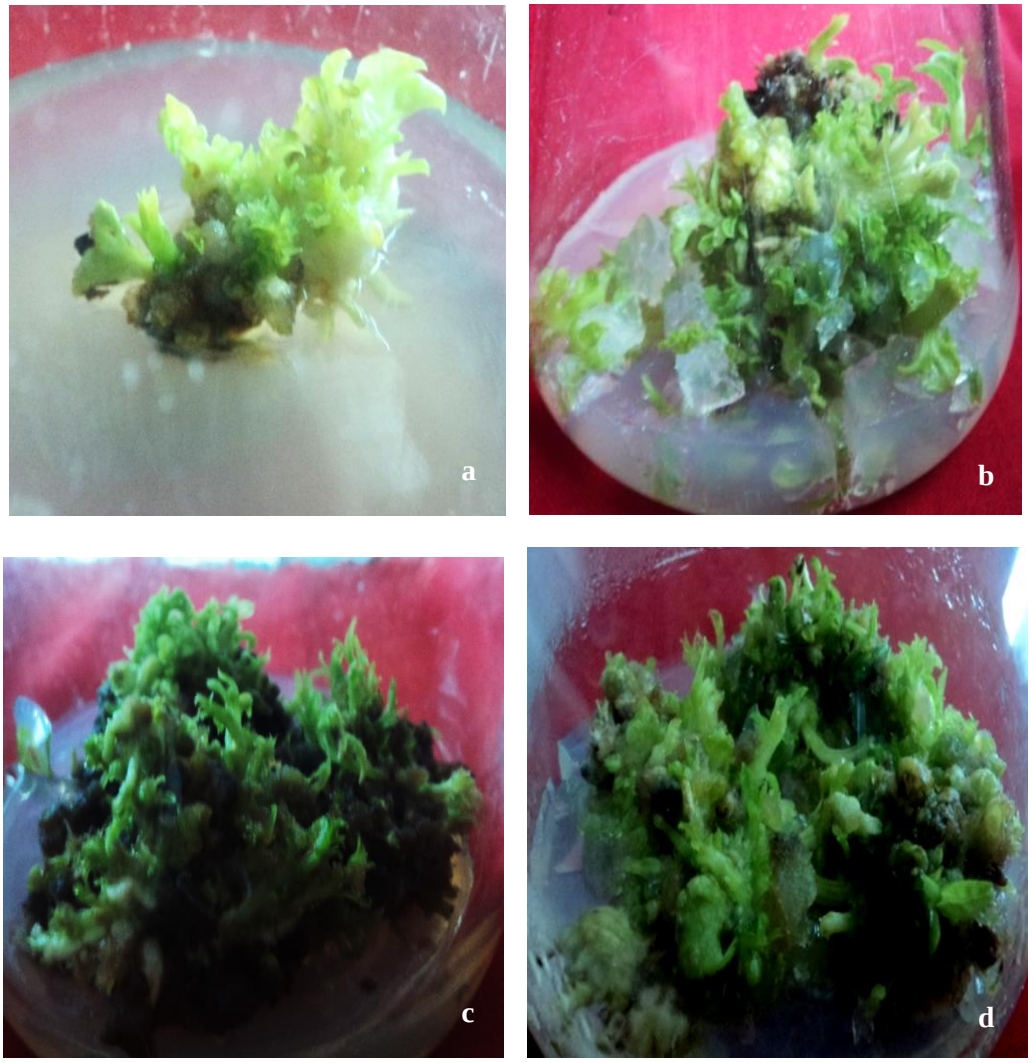

Fig.31: Shoot differentiation in *O.indicum* from morphogenic callus in MS medium fortified with BAP(20µM) + NAA(1µM)

a. Shoot differentiated from morphogenic callus after 8 weeks

b. Increase in shoot number after 12 weeks

c. Shoots differentiated along with callus after 16 weeks

d. Enhancement in shoot number after 24 weeks

Nodal explants

The morphogenic callus was also developed below *in vitro* shoots formed from nodal explants. But compared to cotyledonary node explants developed only shoots with no basal morphogenic callus but in few combinations of two cytokinins differentiated callus at the base of *in vitro* shoots.

4.2.2.4 Synergistic effect of cytokinins

This callus was observed in MS and WPM medium fortified with different combinations of cytokinins.

The MS medium fortified with BAP(20µM) combined with Kn(2µM, 4µM, 8µM),BAP(20 µM) with TDZ(0.1µM, 0.25µM,0.5µM)was subcultured in fresh media. In BAP (20µM) with Kn (2µM) the shoot buds mass increased along with the callus and also new shoots were grown after 8 weeks (first subculture). But after 12 weeks (second subculture) there was increase in shoot buds which failed to elongate into shoots.

The morphogenic callus developed in presence of BAP (20µM) with Kn (4µM)(Fig.32a) when transferred to same medium new shoots proliferated with an average of 4.5 ± 2.0 shoots were formed after 8 weeks (Fig.32b).Whereas increase in concentration to Kn(8µM) induced only shoot buds after 8 weeks (Fig.32c).

In BAP (20µM) with TDZ (0.1µM) many shoot buds regenerated from the base and finally it covered the entire whole explants (Fig.32d).This when transferred to the fresh medium failed to elongate by the end of 8 weeks (Fig.32e). Similarly in BAP (20µM) with TDZ(0.5µM) the differentiating callus developed into few shoots by the end of 8 weeks but failed to show any further growth in subsequent passages.

Morphogenic callus was also transferred to WPM medium fortified with BAP (20µM) with Kn(2µM, 4µM), BAP(16µM) with TDZ(0.5µM), BAP(20µM) with TDZ(0.1µM, 0.2µM).

In WPM medium fortified with BAP (20µM) with Kn (2µM,4µM), BAP(16µM) with TDZ(0.5µM) combinations morphogenic callus failed to grow in terms of shoot formation by the end of 8 weeks. Whereas in BAP (20µM) with TDZ (0.1µM) shoot bud

Fig.32: Indirect organogenesis from nodal explants of *O.indicum* in following combinations

a. Basal morphogenic callus developed in MS+BAP(20µM)+Kn(4µM)after 4 weeks

b. Development of shoots from callus in MS+BAP(20µM)+Kn(4µM) after 8 weeks

c. Differentiation of callus with shoot buds and shoots in MS+BAP(20µM)+Kn(8 µM) after 8 weeks

d. Morphogenic callus in MS+BAP(20µM) +TDZ(0.1µM) after 4 weeks

e. Shoot bud differentiation in MS+BAP(20µM) +TDZ(0.1µM) after 8 weeks

f. Shoot emergence in MS+BAP(20µM) +NAA(0.1 µM) after 4 weeks

g. Development of shoots in MS+BAP(20µM) +NAA(0.5µM) after 8 weeks

h. Multiple shoots from MS+BAP(20µM) +NAA(0.5µM) after 12 weeks

differentiated which slightly grew but by the end of eight weeks they remained as stunted shoots. Therefore this clump was transferred to fresh medium where it failed to grow or differentiate shoot buds after 12 weeks. In medium fortified with BAP (20µM) with TDZ (0.2µM) the morphogenic callus differentiated many shoot buds but only one shoot was able to elongate. By the end of eight weeks this callus failed to survive.

4.2.2.5 Effect of cytokinins with auxins

MS medium fortified with cytokinin and auxin induced morphogenic callus below *in vitro* shoots in combinations like BAP (20µM) with IBA (0.1µM, 1µM), BAP (20µM) with NAA (0.1µM, 5µM).This callus was excised and placed on the respective media for further growth.

After 8 weeks it was observed that in BAP (20µM) with IBA (0.1µM) callus mass differentiated shoot buds which on further subculturing developed into many small shoots by the end of 12 weeks but failed to survive in further passages, similar response was observed in BAP(20µM) with IBA(1µM) combination.

In medium fortified with BAP (20µM) and NAA (0.1µM) shoots arise from the callus and increased in number after 8 weeks (Fig.32f) and in subsequent passages after 12 and 16 weeks the callus mass increased differentiating shoot buds which simultaneously elongated into shoots. The morphogenic callus which formed in presence of BAP (20µM) and NAA (0.5µM) also behaved in the same manner differentiating many shoots after 8 weeks (Fig.32g). These shoots were separated and subcultured further it was observed that there was formation of many shoot buds as well as increase in shoot number upto 11.0 ± 0.6 after 12 weeks(Fig.32h). Subculturing this shoot clumps failed to survive by the end of 16 week.

Therefore indirect organogenesis from the morphogenic callus developed below *in vitro* shoots of nodal explants also had potency to regenerate but the number formed were less compared to cotyledonary node. The maximum of 11.0 ± 0.6 shoot number developed after 12 weeks in BAP (20µM) and NAA(0.5µM).

4.2.3 Rooting of *O.indicum* microshoots

The elongated shoots which developed in presence of different PGR were placed in half and full strength MS and WPM (liquid and static) medium fortified with

IBA(0,2µM,2.5µM,5 µM,10µM) and NAA(0,2µM,2.5µM,5µM,10µM) for rooting(Table 16,17). After 4 weeks it was observed that the medium without auxins failed to induce root in MS and WPM medium except half strength WPM basal liquid medium which evoked 100% response with an average 2.0 ± 0.0 number of roots and root length 2.4 ± 0.0 cm. The roots developed were slightly leathery.

When medium were fortified with different concentrations of IBA and NAA varied results were obtained in terms of root induction. The roots get originated from the base of the stem and they also emerged out from the nodes of the stem. It was observed that in *O.indicum* the liquid medium was effective for root induction producing slender or thick white long roots, but the static media failed to induce roots.

- ***In vitro* rooting in MS medium**

When half strength MS liquid medium was fortified with different concentrations of IBA the roots were induced at IBA (2.5µM and 5µM) concentrations only. But maximum of 100% root induction from the base of shoot was observed at 5 µM with an average of 2.5 ± 0.2 roots with 4.1±1.6 cm root length (Fig.33a). When NAA was added all the concentrations induce roots in liquid medium. At lower concentrations 2.5µM an average of 3.7 ± 1.7 roots were induced. While number of leathery roots were maximum at 5µM (7.0 ± 2.2) in 83% cultures (Fig.33b).With increase in concentration to 10µM there were 100% root induction observed with highest root length reaching to 4.5±0.2 cm and the roots developed were thin with laterals (Fig.33c). In static medium fortified with different concentrations IBA and NAA failed to induce roots except at NAA (2µM) 0.5 ± 0.2 number of roots were developed with 50% response (Table 16).

In full strength medium fortified with different concentrations of IBA poor response was observed in terms of root induction. As all the liquid and static medium failed to induce roots only at 2.5µM, an average of 5.0 ± 2.2 number of roots were induced which was more compared to half strength IBA (5µM). Similarly supplementing NAA in liquid and static medium resulted in poor response in terms of root induction compared to half strength. There were only 1.0±0.6 small roots were induced at 5 µM (Table 16).

Therefore in half strength MS medium fortified with NAA was effective for root induction.

Table 16. Effect of half and full strength MS medium fortified with IBA and NAA on root induction of *O.indicum* after 4 weeks

Medium	Auxin (µM)		% Response	Number of roots*	Root Length* (cms)	% Response	Number of roots*	Root Length* (cms)
			Liquid			Static		
Half strength	0		0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	IBA	**NAA**						
	2	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	2.5	-	50	0.5±0.2^{ab}	2.5±1.1^{bc}	0	0.0±0.0^{a}	0.0±0.0^{a}
	5	-	100	2.5±0.2^{bc}	4.1±1.6^{cd}	0	0.0±0.0^{a}	0.0±0.0^{a}
	10	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	2	33	1.0±0.6^{ab}	2.7±1.7^{c}	50	0.5±0.2^{ab}	2.0±0.9^{abc}
	-	2.5	67	3.7±1.7^{c}	0.5±0.2^{ab}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	**5**	**83**	**7.0±2.2^{d}**	2.0±0.6^{abc}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	10	100	2.0±0.4^{abc}	4.5±0.2^{d}	0	0.0±0.0^{a}	0.0±0.0^{a}
Full strength	0		0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	IBA	**NAA**						
	2	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	2.5	-	50	5.0±2.2^{b}	1.3±0.6^{b}	0	0.0±0.0^{a}	0.0±0.0^{a}
	5	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	10	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	2	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	2.5	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	5	50	1.0±0.6^{a}	0.8±0.4^{b}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	10	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

- ***In vitro* rooting in WPM medium**

The *in vitro* shoots were also placed in WPM medium to evaluate its effect on root induction. Similar to MS in WPM medium also liquid medium was effective for root induction.

In half strength liquid medium without PGRs 100 % roots were induced with an average of 2.0 ± 0.0 in number after 4 weeks. When medium was fortified with auxins it was observed that the liquid medium fortified with IBA resulted in 100 % response at 2, 5 and 10µM.

Fig. 33: *In vitro* root induction in *O.indicum* after 4 weeks

a. Roots induced from the base of shoot in ½ MS liquid+ IBA(5µM)
b. Many leathery roots developed in ½ MS liquid +NAA (5µM)
c. Single thin root with fine laterals in ½ MS liquid +NAA(10µM)
d. Leathery roots induced at base of shoot in ½ WPM liquid+IBA(10µM)
e. Small thick roots developed in ½ WPM static+ IBA(10µM)
f. Thin white smooth roots developed from node of stem in WPM liquid + IBA (10µM)
g. Long leathery roots developed in MS liquid +NAA (2.5µM)

In which highest root length (3.3 ± 0.0cm) was observed at 5 µM and maximum number was obtained at 10µM (7.0 ± 1.3) forming leathery roots at the base of stem (Fig.33d). When medium was fortified with NAA the lower concentration at 2µM induced 100% leathery roots with an average of 2.5 ± 0.2 number and 2.2 ± 0.2 cm root length. Whereas with increase in concentration the percent response and root number decreased. All the static medium fortified with IBA and NAA failed to induce roots except IBA (10µM) which resulted in 50% response with small thick roots (Fig.33e) (Table 17).

Table 17. Effect of half and full strength WPM medium fortified with IBA and NAA on root induction of *O.indicum* after 4 weeks

Medium	Auxins (µM)		% Response	Number of roots	Root Length (cms)	% Response	Number of roots	Root Length (cms)
			Liquid			Static		
Half strength	0		100	2.0±0.0^{a}	2.4±0.0^{c}	0	0.0±0.0^{a}	0.0±0.0^{a}
	IBA	**NAA**						
	2	-	100	4.5±1.6^{b}	0.4±0.04^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	2.5	-	50	4.5±2.0^{b}	0.5±0.3^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	5	-	100	1.0±0.0^{a}	3.3±0.0^{c}	0	0.0±0.0^{a}	0.0±0.0^{a}
	10	-	**100**	**7.0±1.3^{c}**	0.7±0.3^{b}	50	1.5±0.7^{a}	0.4±0.2^{a}
	-	2	100	2.5±0.2^{ab}	2.2±0.2^{bc}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	2.5	50	0.5±0.2^{a}	0.5±0.2^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	5	83	2.2±0.7^{ab}	1.6±0.4^{b}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	10	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
Full strength			Liquid			Static		
	0		0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	IBA	**NAA**						
	2	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	2.5	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	5	-	0	0.0±0.0^{a}	0.0±0.0^{a}	0	0.0±0.0^{a}	0.0±0.0^{a}
	10	-	100	3.5±0.2^{b}	3.2±0.1^{e}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	2	66	3.3±1.1^{b}	0.6±0.0^{ab}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	2.5	100	5.7±0.6^{c}	1.9±0.5^{cd}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	5	100	5.5±0.2^{c}	1.5±0.2^{bc}	0	0.0±0.0^{a}	0.0±0.0^{a}
	-	10	83	4.7±1.2^{bc}	2.5±0.8^{de}	33	0.3±0.2^{a}	0.7±0.4^{ab}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

In comparison to half strength, the full strength WPM liquid medium fortified with IBA failed to induce roots at 2,2.5,5µM, except at 10µM 100% smooth slightly thin roots arise from node of stem (Fig.33f) were observed with an average of 3.5 ± 0.2 number and 3.2 ± 0.1 cm in length which was highest among all the concentrations. When full strength medium was fortified with NAA, at 2.5µM and 5µM, 100 % roots were formed which were thick and leathery of which NAA at 2.5µM resulted in maximum (5.7 ± 0.6) number of roots (Fig.33g). As similar to MS medium all the static media failed to induce roots except at NAA (10µM) which evoked only 33% response (Table 17).

Overall results revealed that although both MS and WPM medium induced roots but half strength WPM medium fortified with IBA and NAA proved to be effective compared to MS. Also liquid medium was efficient in terms of formations of roots as static media obtained poor response. Out of all in the MS medium fortified with NAA (5µM) maximum 7.0 ± 2.2 roots were obtained in 83% cultures while in ½ WPM medium fortified with IBA (10µM) 7.0 ± 1.3 roots were obtained in 100% cultures.

4.2.4 Hardening of *O.indicum* plantlets

The *in vitro* plantlets of *O. indicum* were transferred to different natural planting substrates for hardening so as to change their mode of nutrition from heterotrophic to autotrophic. It was carried out in two ways:

1. Initially the plantlets were placed in culture room and then transferred to greenhouse
2. The plantlets were directly transferred to greenhouse

4.2.4.1 Under lab conditions

The *in vitro* plantlets were placed in following sterilised planting substrates:

- Cocopeat,
- Cocopeat: Sand(1:1)
- Cocopeat: Sand:Soil(1:1:1)
- Sand: Soil (1:1:1)

Observation after 4 weeks it was observed that only in cocopeat 50% of them were able to survive whereas in other substrates there was mortality.

Fig.34: Hardening of *O.indicum* plants in Cocopeat substrate under lab conditions

a. Emergence of new leaves at apices within two weeks of transfered to cocopeat substrate

b. New roots developed by the end of four weeks

c. Plantlets transferred to polybags

New leaves were observed to emerge at the shoot tips after two weeks of transfer (Fig.34a) and new roots were formed by the end of 4 weeks (Fig.34b).These plantlets were transferred to polybags containing soil and placed in greenhouse for further growth. But all the plantlets perished within two weeks of transfer.

4.2.4.2 Under greenhouse conditions

The *in vitro* plants were placed in following different substrates without sterilising them.

- Cocopeat
- Sand
- Soil
- Sand: Soil (1:1)
- Cocopeat: Sand (1:1)
- Cocopeat: Soil (1:1)
- Cocopeat: Sand: Soil (1:1:1).

Prior to placing the plants in thermocol cups in greenhouse the initial data for root length, shoot length and plant height was recorded for each substrate. The percent survival, increase in shoot and root length as well as plant height with new leaf formation was also recorded in each substrate after 4 weeks. It was observed that all the substrates varied in their response.

Cocopeat

In vitro plantlets (Fig.35a) when transferred in cocopeat substrate (Fig.35b) and only 17% plants survived by the end of four weeks(Fig.36).In these plants there was formation of new leaves at the apices within two weeks(Fig.35c) of transfer which increased in length at the end of four weeks(Fig.35d) (Fig.37). There was an increase in shoot and root length and the initial plant height was 2.0 ± 0.3cms which reached to 13.3 ± 0.0cms after 4 weeks (Fig 35e) (Fig.38). After these plants were transferred to plastic pot filled with soil and covered with polythene bags for 4-5 days and later on removed. But it failed to show any further growth.

Fig.35: Hardening of *O.indicum* plants in Cocopeat substrate

a. *In vitro* plantlet
b. Plantlet placed in cocopeat substrate
c. New leaves emerged at the apex after two weeks of transfer
d. Increased in size of leaves by the end of four weeks
e. Plant showing growth in terms of increase in shoot and root length
f. Plant transferred to normal soil in plastic pots for further growth

Sand

The rooted plantlets were also transferred to thermocol cup filled with sand (Fig.39a,b) and placed in greenhouse. As compared to cocopeat substrate the percent survival increased 67% in sand (Fig.36). After transfer there was emergence of new leaves within two weeks which increased in number and length by the end four weeks (Plate39b, c). The leaf length reached upto (2.6±0.4cms) (Fig.37) which indicates the suitability of the substrate for growth. There was an increase in shoot length to 6.4±0.5cms and root length (5.0±1.2cms) reaching plant height to 12.3±0.3 cm (Fig.38; Fig.39d).

This plants were transferred to plastic pots containing soil for further growth. They were covered with polyhthene bags for 4-5 days to ensure that there is less water loss and retains

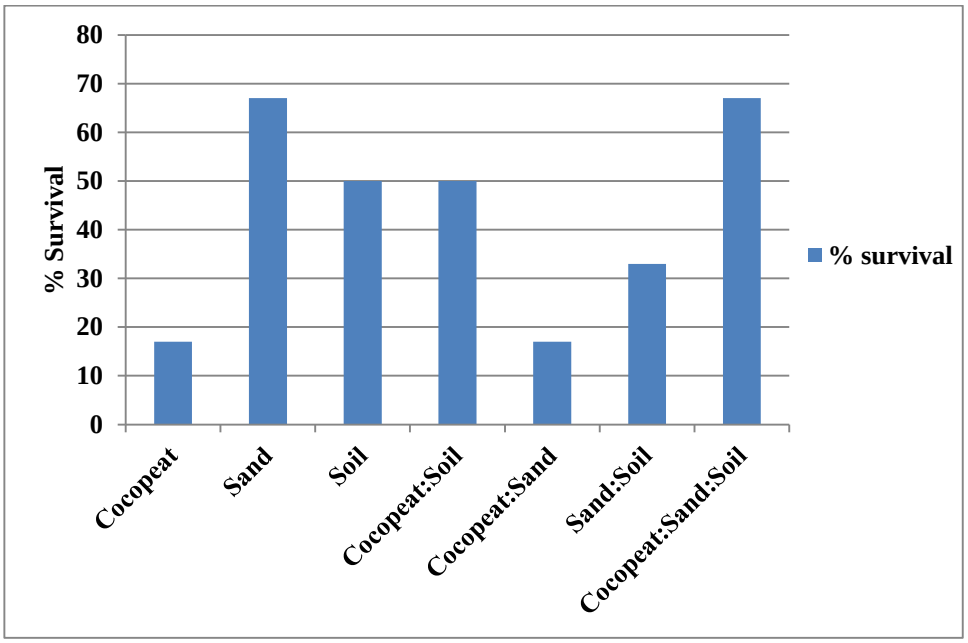

Fig.36: Percent survival in different substrates after four weeks of transfer of *O.indicum* plants to greenhouse

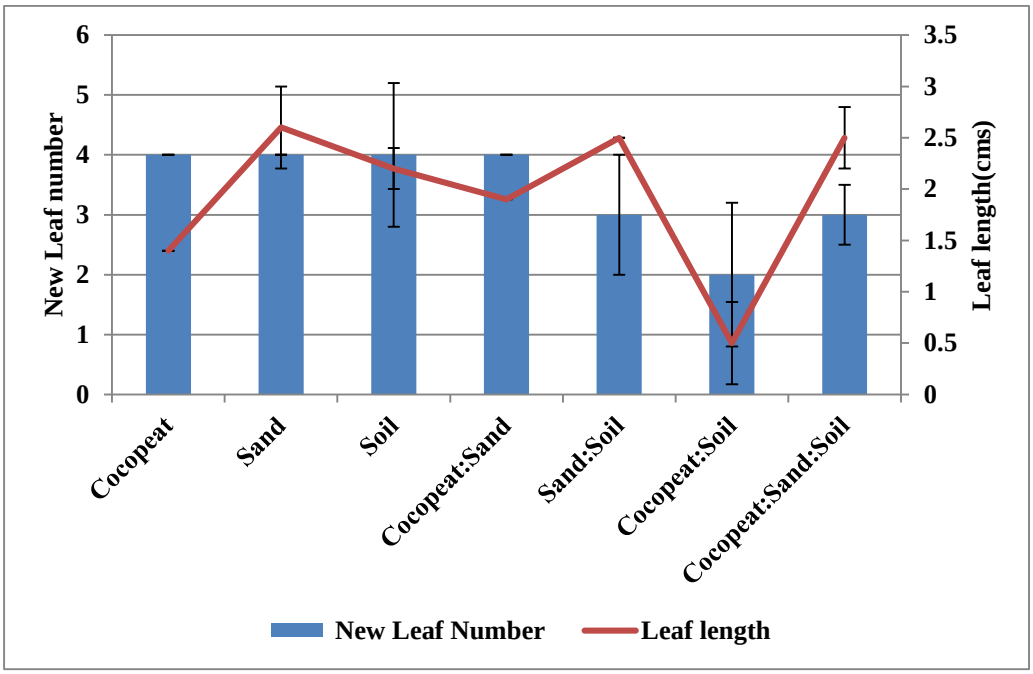

Fig.37: Leaf number and leaf length of *O.indicum* plants in different substrates after 4 weeks

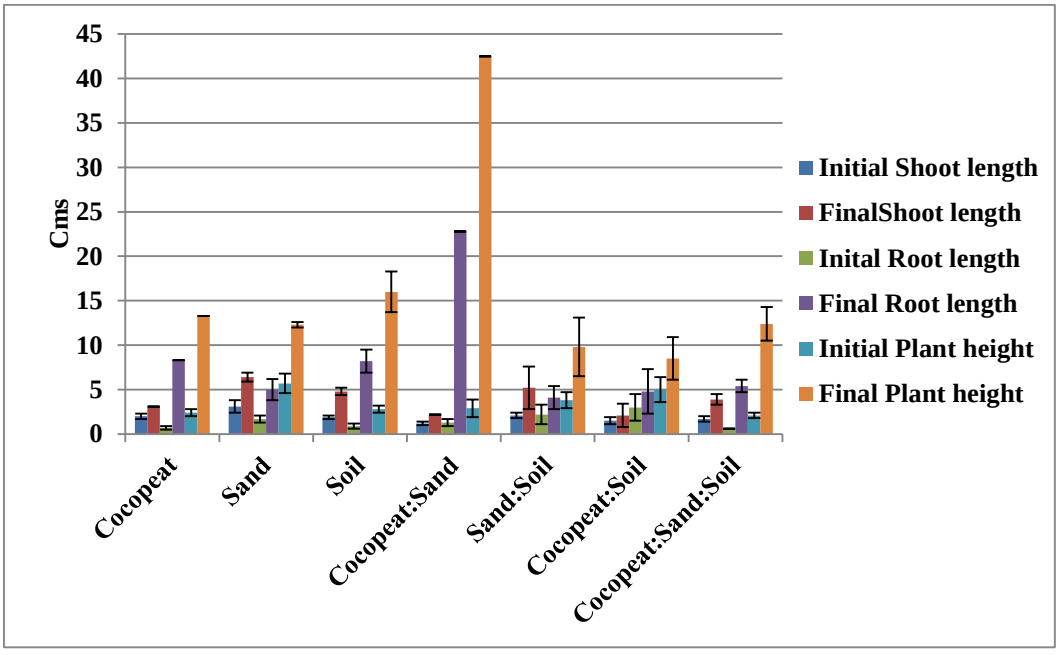

Fig.38:Initial and final shoot, root length and plant height in different substrates in *O.indicum*

Fig.39: Hardening of *O.indicum* plants in Sand substrate

a. *In vitro* plantlet
b. Emergence of new leaves after two weeks
c. Leaves increased in size
d. Plant growth after four weeks before being transfer to plastic pots
e. Plant increase in height with leaves enlarged in size
f. Plant growth after eight weeks with development of new secondary roots
g. Plant transferred to garden pot for normal growth

humidity. After that the polythene bags was removed and plants were allowed to grow open in greenhouse. After eight weeks all the plants transferred in plastic pots survived and obtained growth in terms of increase in shoot length(8.5 ± 0.8cms), root length (7.9 ± 3.3cms) and plant height(17.1 ± 4.1cms) (Fig.39e,f;Fig.40). The secondary root system that well developed was healthy and extensive with laterals and the leaf changed from thin membranous to thick coraceous. These well developed plants were transferred to garden pots. These pots were initially kept for four weeks in greenhouse and then placed (Fig.39g) in botanical garden under sunlight in the 16th week. These growth of the plant was slow initially but after 6 months the plant height (above the soil) increased in all the plants (Fig.41). They were regularly watered and by the end of 10 months they grow in strong plants as the stem showed formation of bark became thick and woody. The plant height and girth of the stem increased at each period in all the plants by end of 12 months (Fig.41) Hence the plants were growing normally and healthily into a one year old and they continued to grow independently in the garden (Fig.42a-d).

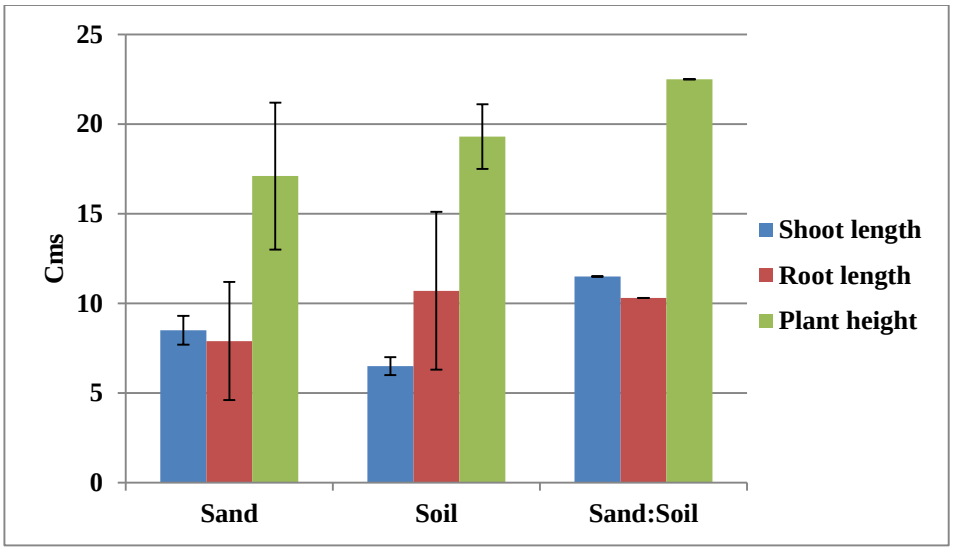

Fig. 40: Growth in *O.indicum* plants in Sand, Soil and Sand:Soil substrate after 8 weeks

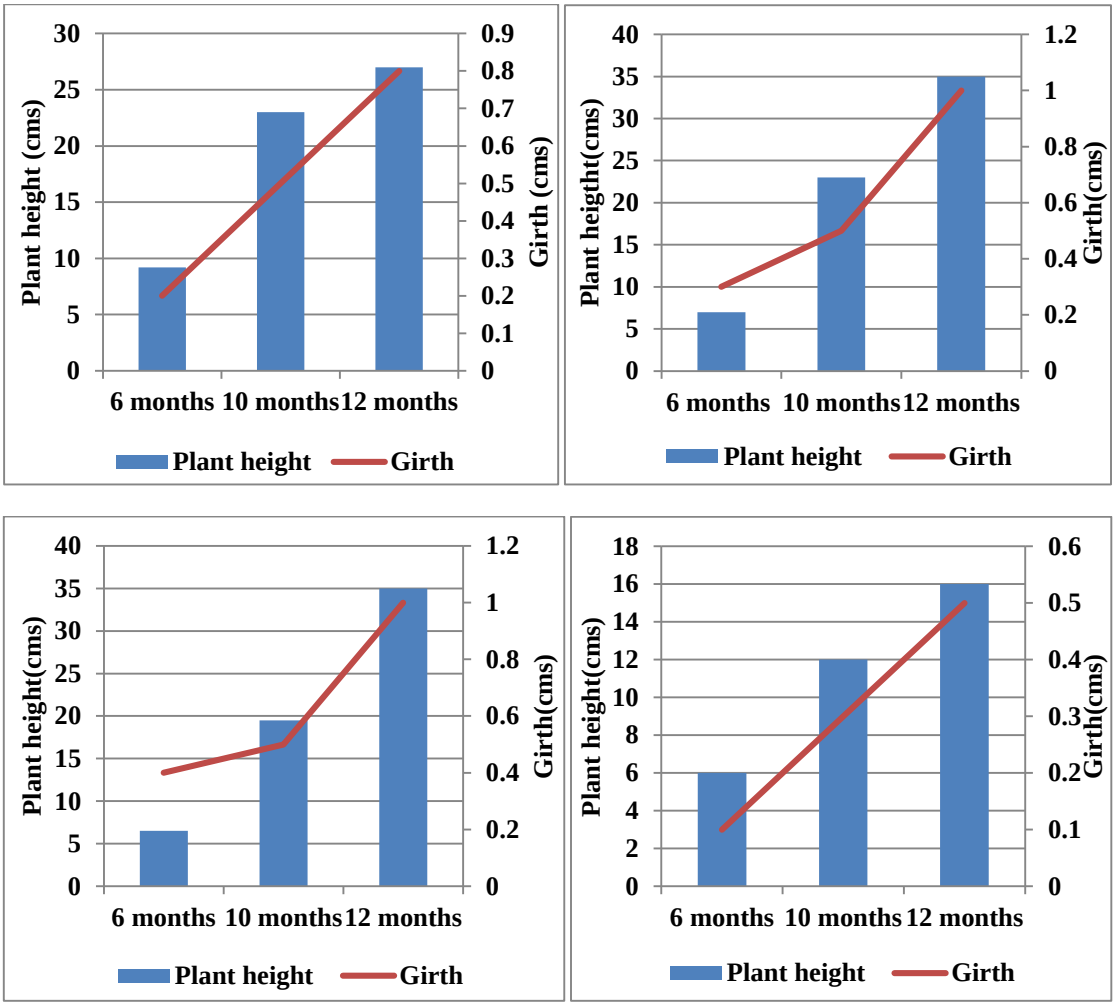

Fig. 41:Plant height and girth in *O.indicum* (four) plants after transfer to garden

Soil

Similarly the *in vitro* plantlets (Fig.43a) were transferred to soil substrate for hardening. In this substrate 50 % of the plants survived after four weeks (Fig.36).

As recorded for the previous substrates there was emergence of new leaves at the apices within 15 days and increased in size (Fig.37; Fig.43b,c). By the end of one month growth in terms of increase in shoot (4.8 ± 0.4cms) and root length (8.2 ± 1.3cms) as well as plant height (16.0 ± 2.3cms) was observed (Fig.38).The plant exhibited an extensive root system (Fig.43d) and they were transferred to plastic pots filled with soil for their further

Fig.42: Tissue culture plants of *O.indicum* grown in botanical garden
a. 6 months old plants
b.and c. 10 months old plants
d. One year old healthy plants

development. The growth was observed in plastic pots there was an increase in shoot length to 6.5 ± 0.5cm,root length to 10.7 ± 4.4cms and plant height to 19.3 ± 1.8cms by end of 8 weeks (Fig.40;Fig.43e,f). The girth or thickness of stem and roots also increased. The leaf texture also gets changed to rough and thick. Thus the plants were transferred to garden pots and kept in greenhouse (Fig.43g) for further growth.

The individual substrates played a vital role in hardening the *in vitro* plantlets and sand was a suitable substrate for overall growth and development followed by soil. In cocopeat the plants were able to survive only under greenhouse conditions. Thus further studies for hardening were carried out with combination of substrates in 1:1 ratio. This was done to understand whether a combinations of substrates could play a synergistic role in improving growth as compared to individual substrate.

Cocopeat: Sand

When the plantlets were transferred to cocopeat in combination with sand (1:1) substrate (Fig.44a,b) it obtained poor rate of survival (Fig.36). Similar to other substrates there was emergence of new leaves but were smaller in length with an average of 1.9 ± 0.0cms (Fig.37;Fig.44c).The plants obtained growth in terms of increase in root length only which was 42.5 ± 0.0cms but the shoot remain stunted with only slight increase in length ie. 2.2 ± 0.0 cm from the initial (1.2 ± 0.2cms) (Fig.38). The root length and plant height was highest in this substrate compared to other (Fig.44d,e). As done in each substrate the plant having growth in this substrate was also transferred to plastic pot filled with soil but it failed to show any further growth (Fig.44f).

Sand:Soil

The sand and soil were also used in combination for hardening and the *in vitro* plants were transferred in thermocol cups for hardening (Fig.45a,b).The rate of survival of plants in this was less but comparatively more(33%) than cocopeat: sand(Fig.36). There was emergence of new leaves which were healthy (Fig.45c) and compared to cocopeat:sand larger in length 2.5 ± 0.0cms(Fig.37). There was an increase in plant height (9.8 ± 3.3cms), shoot (5.2 ± 2.4cms) and root length (4.1 ± 1.3cms) but was less as compared to individual sand substrate (Fig.38) (Fig.45d).Hence these plants were also transferred in plastic pot filled with soil and by the end of eight weeks the plant (Fig.45e).

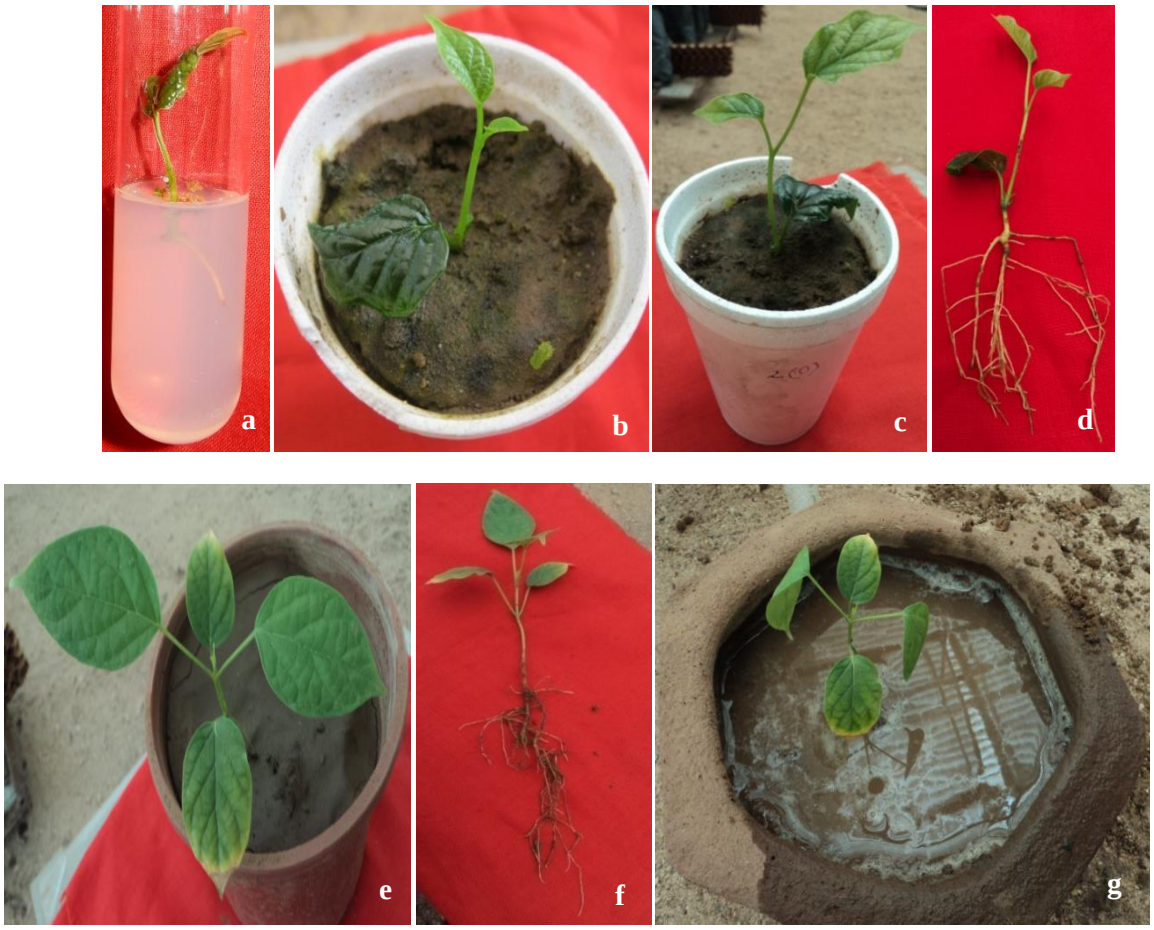

Fig. 43: Hardening of *O.indicum* plants in Soil substrate

a. *In vitro* plantlet
b. and c. New leaves emerged after two weeks and increased in size by the end of four weeks
d. Plant growth after four weeks with extensive root system
e. and f. Plant growth in plastic pots filled with soil after 8 weeks
g. Plant transfered to garden pot

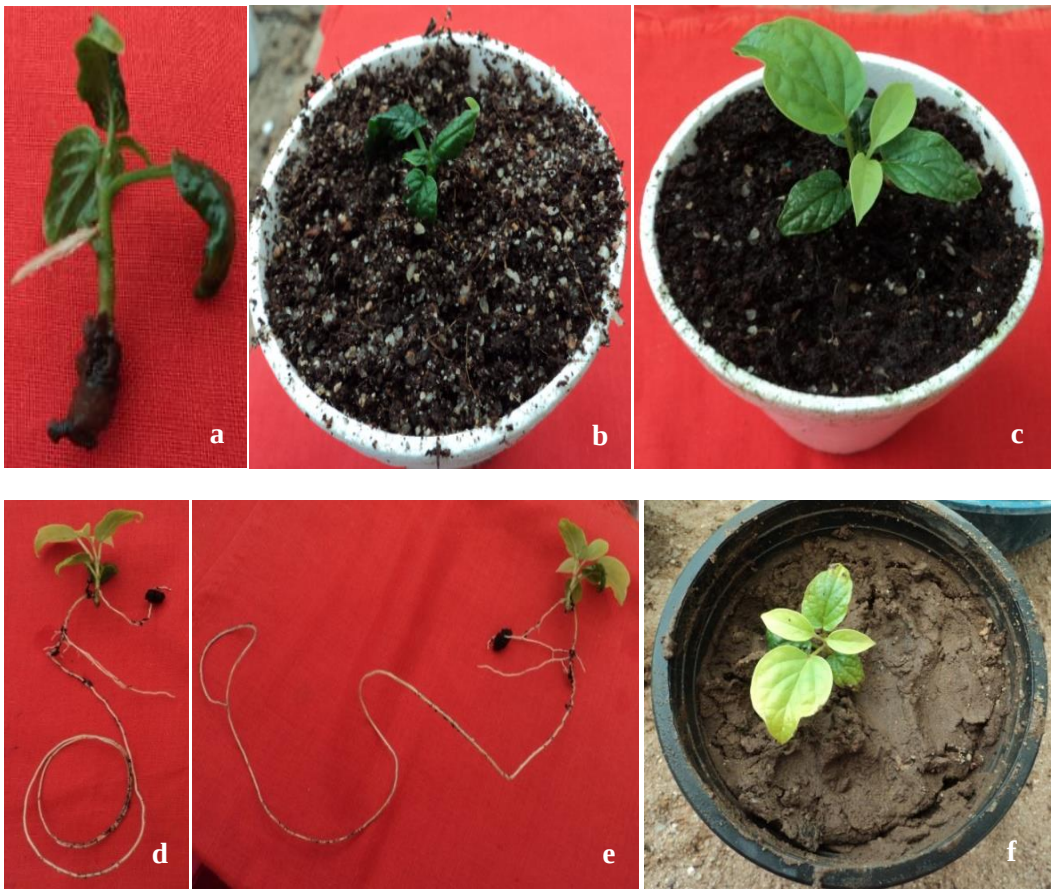

Fig. 44: Hardening of *O.indicum* plants in Cocopeat:Sand substrate

a. Rooted plantlet
b. Plantlet transfered to cocopeat:sand substrate
c. New leaves emerged at the apex and increased after 4 weeks
d. and e. Plant growth with very long root developed after 4 weeks
f. Plant transferred to plastic pot filled with soil

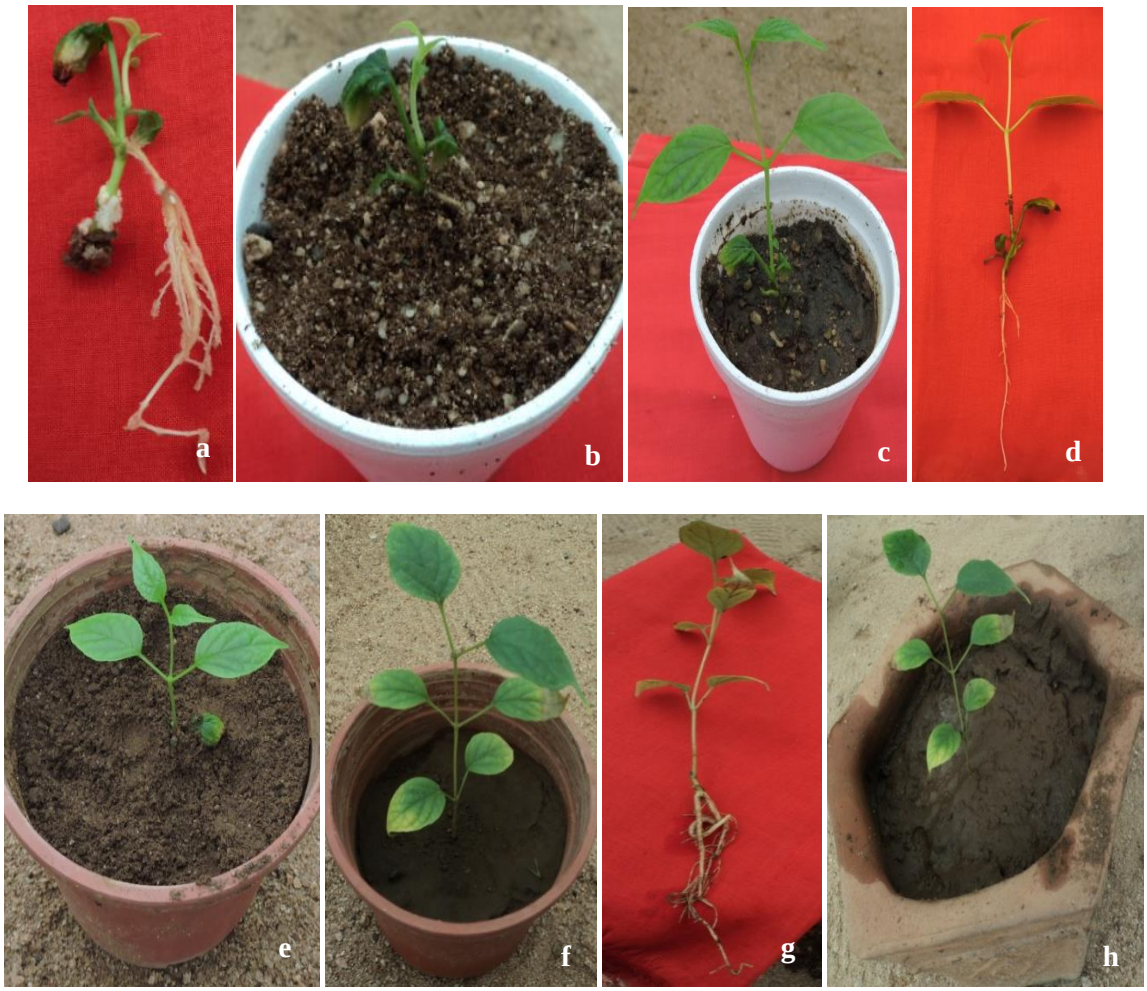

Fig.45: Hardening of *O.indicum* plants in Sand:Soil substrate

a. *In vitro* plantlet
b. Plantlet placed in Sand:Soil substrate
c. New leaves grown at the apex and increased in length after 4 weeks
d. Plant showed increase in shoot and root length after 4 weeks
e. Plant transferred to plastic pot filled with soil
f. and g. Plant growth with increased in height after 8 weeks
j. Plant transfered to garden pot for further growth

obtained healthy growth in terms of increase in plant height, shoot and root length (Fig.40; Fig.45f, g)and the roots also gets changed from thin to hard and thick. The girth of stem also increased and the leaf size enlarged and become rough and thick in texture. Hence the plants were transferred to garden pot (Fig.45j) and kept in greenhouse for their further growth but there was not much improvement seen in growth and the plant ceases to grow within 20 days.

Cocopeat:Soil

Another combination of substrate used was cocopeat:soil(1:1) and the plantlets were transferred to the same for hardening. Out of the different plantlets 50 % of them survived by the end of four weeks (Fig.36). Emergence of new leaves took place but their length was very less with only 0.5 ± 0.4 cms(Fig.37).As similar to other substrates in this also the plant showed increase in shoot (2.1 ± 1.3cms) and root length(4.8 ± 2.5cms), plant height(8.5 ± 2.4cms) but was least among all the other(Fig.38). For improvement in growth the plants were transferred to plastic pots filled with soil but it failed to survive within a week.

Cocopeat:Sand:Soil

As all the three individual substrate cocopeat,soil and sand were able to induce growth in the transferred plantlets the combination of all the three cocopeat:sand:soil (1:1:1 ratio) was also tried for hardening, hence the plantlets were transferred to the same (Fig.46a).In this substrate out of the different plantlets transferred 67% got survived by the end of four weeks(Fig.36). This proved to be better compared to cocopeat:soil, cocopeat:sand and sand:soil combination as the new leaves emerged were healthy (Fig.46b,c) with 2.5 ± 0.3 cms in length(Fig.37) and also there was an increase in root and shoot length and plant height, reached to 12.4 ± 1.9 cms in length by the end of 4 weeks(Fig.46d) (Fig.38).Similar to other these plants were also transferred to plastic pots filled with soil (Fig.46e) but their growth ceased within two weeks and finally resulted in mortality.

From the above results it can be concluded that sand individually was able to allow a healthy growth of *in vitro* plantlets of *O.indicum*. These plants when transferred to garden soil slowly hardened under natural conditions and are growing in botanical garden.

Fig.46: Hardening of *O.indicum* plants in Cocopeat:Sand:Soil substrate

a. Rooted plantlet
b. Small new leaves emerged at the apex after two weeks
c. Leaves increased in size after four weeks
d. Plant showing increased in height in terms of shoot and root length after 4 weeks.
e. Plant transferred to plastic pots filled with soil

It was concluded that in *O.indicum*

- The cotyledonary leaf and hypocotyl explants failed to establish shoot cultures in MS and WPM medium fortified with the cytokinins tried. Cotyledonary node and nodal explants revealed their potency to regenerate shoot in presence of individual cytokinins and synergistic combinations of PGRs.The nodal explants was proved to be better explants in comparison to cotyledonary node as it induced healthy shoots.
- Although both the media helped in shoot and root induction but WPM medium was the effective in terms of overall performance and BAP was the suitable cytokinin among all the cytokinins used
- Indirect shoot formation from morphogenic callus was observed in both the explants but from cotyledonary node explants the number was highest in MS medium fortified with BAP (4µM) with GA_3(1µM) resulted in 19.3 ± 2.4 shoots.
- Half and full strength medium (MS and WPM) fortified with IBA and NAA induced roots but IBA proved to be effective auxin in terms of root induction.
- Hardening in sand substrate was optimum for establishment of *O.indicum* plants under greenhouse. This were later transferred to soil and placed under natural sunlight.

4.3 Section III: Synthetic seed studies

Synthetic seed preparation was another objective which was carried out to study whether the *in vitro* nodes explants were able to retain the potency to develop into shoots after storage. First of all gel matrix was standardised for both the species in order to select best matrix for synseed preparation.

4.3.1 Effect of different concentration of sodium alginate and calcium chloride on formation of synthetic seed in both the species

A preliminary experiment was carried out to optimize matrix for shape and texture of synseeds. The *in vitro* nodes were encapsulated in different concentrations of sodium alginate (2, 3 and 4%) and allowed to incubate for 30 minutes in various calcium chloride (50, 75 and 100mM) concentrations. After incubation the encapsulated *in vitro* nodes were removed and evaluated for the shape and texture. This encapsulated *in vitro* nodes were designated as synseeds.

It was observed that the synseeds varied in shape and texture according to sodium alginate and calcium chloride concentrations. Lower concentration of sodium alginate (2%) at all concentrations of calcium chloride(50 mM,75 mM and 100mM) resulted in forming synthetic seed which were irregular in shape with very soft texture(Table.18) (Fig.47a). Increasing sodium alginate to 3% concentration resulted in oval and soft synseeds at 50 mM of calcium chloride. Increase in $CaCl_2$ to 75mM formed round and firm synseeds (Fig.47b) and to 100 mM solution resulted in oval and firm synseeds. Further increase in sodium alginate to 4% resulted in tailed synseeds for all the concentrations of $CaCl_2$ solution and these were with hard texture (Fig.47c)(Table18).

Table 18. Effect of different concentration of sodium alginate and calcium chloride on synthetic seed formation

Sodium alginate concentration	Calcium chloride concentration (mM)	Shape	Texture
2%	50	Irregular and tailed	Fragile
	75	Irregular	Very Soft
	100	Irregular and tailed	Very Soft
3%	50	Oval	Soft
	75	Round	Firm
	100	Oval	Soft
4%	50	Round and tailed	Slightly hard
	75	Tailed	Hard
	100	Tailed	Hard

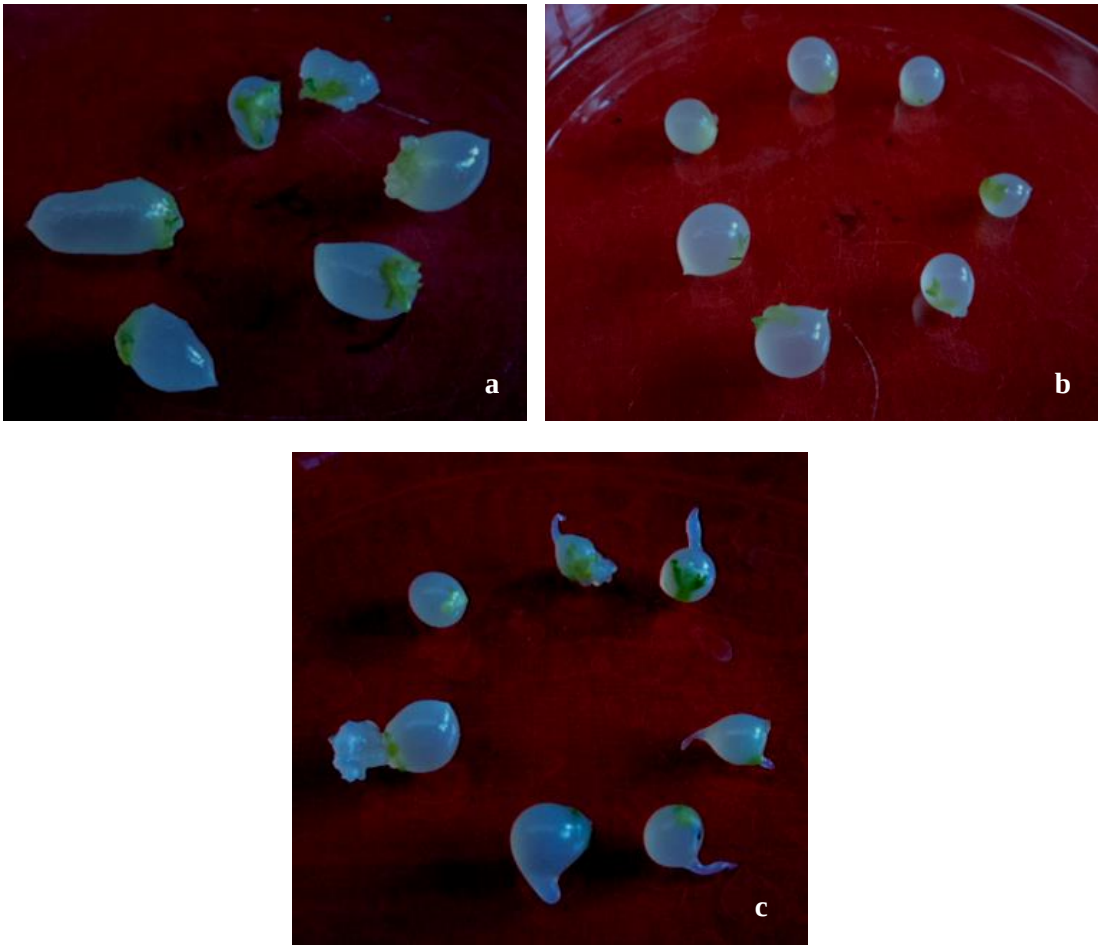

Fig.47: Effect of sodium alginate and calcium chloride on synseed formation

a.Formation of irregular and soft synseeds in 2% sodium alginate with 75mM $CaCl_2$ solution

b.Round and firm synseeds were formed in 3% sodium alginate with 75mM $CaCl_2$ solution

c.Tailed and hard synseeds formed in 4% sodium alginate with 75mM $CaCl_2$ solution

Thus from the above results it was concluded that synseeds which were prepared in 3% sodium alginate and incubated in 75 mM $CaCl_2$ were round with a firm texture.Hence the same was used for further studies on synseeds in both the species(*O.indicum* and *S.suaveolens*).

The synthetic seed studies were carried out in *O.indicum* as follows:

4.3.2 Effect of various encapsulation matrices on synseed germination in regenerative media with different storage period in *O.indicum*

In *O.indicum* the synseeds contained the *in vitro* nodes as explants which were encapsulated in different matrices of half and full strength WPM medium. As WPM medium fortified with PGRs induced an optimised response in regeneration of *O.indicum* the same was utilised for matrix and regenerative medium. In the studies combination of matrices and regenerative media were tried with different storage period to evaluate their effect on germination of synseeds. The WPM basal medium (half and full strength) utilised for encapsulation matrix and regenerative media served as control. The other combination of matrices and regenerative media were supplemented with different PGRs and were compared with control for synseeds germination. The *in vitro* nodes were encapsulated in 3 % sodium alginate concentration mixed with half or full strength WPM medium without or with PGRs and dropped in $CaCl_2$ aqueous solution. These synseeds after incubation of 30 minutes were transferred immediately into various media which was considered as 0 day. While the other synseeds developed in half or full strength without or with PGRs were stored at 4 ºC in petridish containing filter paper with half or full strength WPM liquid media. These synseeds were harvested at regular time intervals (7, 15 and 30 days) and placed on respective regenerative media. After placing them on various regenerative media at 0 day or after storage their regeneration capacity for formation of shoot with roots/shoots was evaluated after 4 weeks.

The different half strength and full strength matrices (WPM medium, WPM+BAP(16µM) and WPM+BAP (16µM) +IBA (0.1µM) and their regenerative medium are discussed below:

4.3.2.1 Effect of half and full strength WPM medium matrix on synseed germination

The *in vitro* nodes were encapsulated in **half strength** basal medium matrix were placed in WPM basal medium, WPM medium fortified with PGRs ie. BAP(16µM)+ IAA(1µM),

WPM+GA_3 (10µM) and one of the organic supplement ie. Coconut water (10%). The observation for % shoot emergence and % shoot and root emergence in different regenerative medium with respect to storage period were recorded after 4 weeks.

In basal regenerative medium a 50% synseeds containing *in vitro* nodes were able to germinated and formed only shoots with an average number (1.0 ± 0.4) on 0 day whereas storing the synseeds resulted in loss of morphogenic response as they failed to form shoot or shoot and root(Table 19).

In the next regenerative medium which was fortified with BAP (16µM) with IAA(1µM) only 17% synseeds germinate and formed 0.2 ± 0.2 shoots after 7 days of storage. When the cytokinins were replaced with GA_3 (10µM) in medium the synseeds responded as there was formation of shoots within 4 weeks. The response for % germination was maximum(67%) with an average of 1.2 ± 0.5 shoots at 0day (Fig.48a)and it decreased to 33% forming 0.3 ± 0.2 shoots for 7 days of storage. But after 15 days of storage the percent germination in terms of (0.5 ± 0.2) shoots emergence reached to 50%. Another regenerative medium which was tried for germination was fortified with only coconut water (10%) as an organic supplement. In this medium there was emergence of 0.2 ± 0.2 shoots after 4 weeks of transfer at 0day and after storage of 7 days the % germination increased to 33% in terms of 0.3 ± 0.2 shoots emergence but further in increase in storage period the efficiency decreased (Table 19).

In **full strength WPM** basal regenerative medium the synseeds failed to respond for all the storage period (Table 19).

In the second regenerative medium which was fortified with BAP(16µM) with IAA(1µM) helped in inducing 0.3 ± 0.2 shoots only at 0 day. Increase in the duration of storage period to 7 days increased the germination of synseeds to 50 % with an average of 0.5 ± 0.2 shoots (Fig.48b). Incorporation of GA_3 (10µM) in the medium induced response in all the synseeds for different time period with varied shoot number (Table 19). At 0 day 50% synseeds germinated into 1.0 ± 0.4 shoots (Fig.48c) and after 15 days of storage 50% synseeds were able to emerged as shoots only (Fig.48d),which was slightly more (0.8 ± 0.4) compared to half strength.

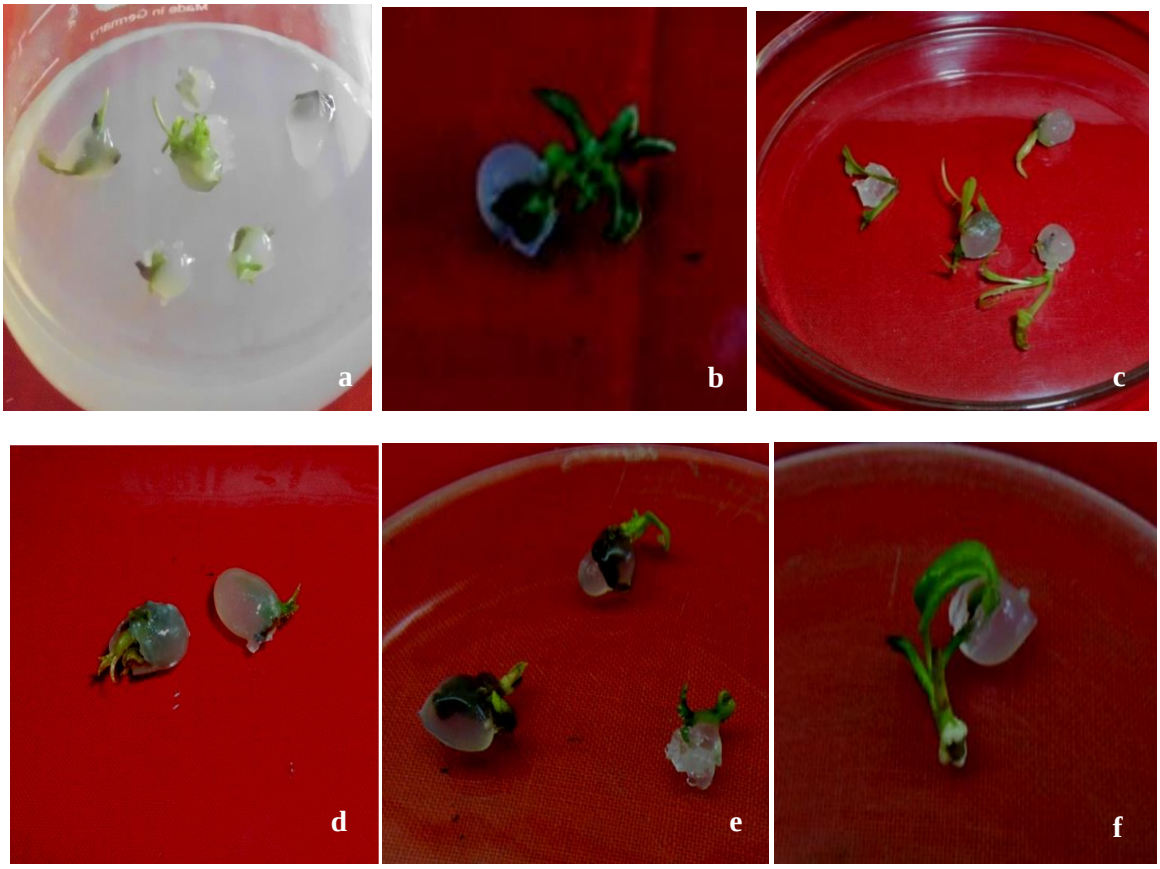

Fig.48: Germination of *in vitro* nodes encapsulated in half and full WPM medium matrix in different regenerative medium in *O.indicum* after 4 weeks

a. Emergence of shoots from synseeds in ½ WPM+GA_3(10µM) regenerative medium at 0 day
b. Shoot emergence in WPM+BAP(16µM)+IAA(1µM) regenerative medium transferred after 7 days of storage
c. Shoots emerged from synseeds placed in WPM+ GA_3 (10µM) regenerative medium at 0 day
d. Stunted shoots sprouted from synseeds after 15 days of storage placed in WPM + GA_3 (10 µM)
e.Shoot emergence from synseeds in WPM+ coconut water (10%) regenerative medium after 7 days of storage
f. Germination of synseed into single shoot in WPM +coconut water (10%) after 15 days of storage

Table 19. Effect of half and full strength WPM medium matrix on synseed germination in *O.indicum* after 4 weeks

	Half strength WPM matrix		
RM	**Storage days**	**% Shoot emergence**	**Number of shoots***
Half strength			
BM	0	50	1.0 ± 0.4^{bc}
	7	0	0.0 ± 0.0^{a}
	15	0	0.0 ± 0.0^{a}
	30	0	0.0 ± 0.0^{a}
BAP16(µM) + IAA1(µM)	0	0	0.0 ± 0.0^{a}
	7	17	0.2 ± 0.2^{a}
	15	0	0.0 ± 0.0^{a}
	30	0	0.0 ± 0.0^{a}
GA_3 (10 µM)	0	67	1.2 ± 0.5^{c}
	7	33	0.3 ± 0.2^{a}
	15	50	0.5 ± 0.2^{ab}
	30	0	0.0 ± 0.0^{a}
CW(10%)	0	17	0.2 ± 0.2^{a}
	7	33	0.3 ± 0.2^{a}
	15	17	0.2 ± 0.2^{a}
	30	0	0.0 ± 0.0^{a}
	Full strength WPM matrix		
Full strength			
BM	0	0	0.0 ± 0.0^{a}
	7	0	0.0 ± 0.0^{a}
	15	0	0.0 ± 0.0^{a}
	30	0	0.0 ± 0.0^{a}
BAP16(µM) +IAA(1µM)	0	33	0.3 ± 0.2^{ab}
	7	50	0.5 ± 0.2^{abc}
	15	0	0.0 ± 0.0^{a}
	30	0	0.0 ± 0.0^{a}
GA_3 (10µM)	0	50	1.0 ± 0.4^{c}
	7	50	0.8 ± 0.4^{bc}
	15	50	0.8 ± 0.4^{bc}
	30	17	0.2 ± 0.2^{a}
CW (10%)	0	0	0.0 ± 0.0^{a}
	7	50	0.5 ± 0.2^{a}
	15	17	0.2 ± 0.2^{a}
	30	0	0.0 ± 0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test

In this medium the synseeds retain the regenerating ability after 30 days of storage with only 17% response. Similar response was observed when medium was supplemented with coconut water as there was 50% germination into 0.5 ± 0.2 shoots after 7 days of storage (Fig.48e)

which decreased to 17% response in terms of 0.2 ± 0.2 shoot emergence after 15 days of storage (Fig.48 f) (Table 19).

Therefore in basal medium matrix synseeds germinated and only shoot formation was observed but only in terms of shoot formation. But compared to half strength matrix there was an improved response in terms of percent germination when *in vitro* nodes were encapsulated in full strength matrix. None of the regenerative media were able to induce the *in vitro* nodes to develop into plantlets therefore another matrix fortified with PGR was tried in the further studies.

4.3.2.2 Effect of half strength and full strength WPM medium matrix fortified with BAP (16µM) on synseed germination

The second matrix tried for encapsulating *in vitro* nodes was medium fortified with BAP (16 µM).These synseeds were then transferred to various regenerative media for germination.

It was observed that the synseeds when transferred to half strength basal regenerative medium at 0 day failed to germinate after 4 weeks. But with increase in duration of storage period till 15 days the percent germination increased to 33% in terms of 0.3 ± 0.2 shoot formation (Table.20).

When the regenerative medium was fortified with two cytokinins ie.BAP (16µM) and Kn(4 µM) the synseeds remained viable till 15 days of storage. The germination response was observed in 33% synseeds after 7 days of storage as shoots (0.3 ± 0.2) emerged breaking the matrix but these shoots failed to elongate and remained stunted. At 15 days storage the percent decreased to 17% and resulted in a similar morphogenic response. Incorporation of individual auxin ie. NAA (5µM) in the medium resulted in emergence of shoot and root. When the synseeds were transferred at 0 day 33% germination was observed where shoots (0.3 ± 0.2) and roots (0.8 ± 0.5) emerged by breaking the matrix after 4 weeks but the plantlets remain stunted. After storage period of 7 days 33% synseeds germinated and formed only shoots and 33% formed shoot and root making a total of 0.6 ± 0.4 shoots and 0.6 ± 0.5 roots. After 15 days of storage the germination reached to 50% where the synseeds broke the matrix forming only shoots which remain stunted (Fig.49a) while 17 % of them developed

Table 20. Effect of half strength and full strength WPM medium matrix fortified with BAP(16µM) on synseed germination in *O.indicum* after 4 weeks

	1/2 WPM +BAP(16 µM) matrix				
RM	**Storage days**	**%Shoot emergence**	**% Shoot and Root emergence**	**Number of shoots***	**Number of roots***
Half strength					
BM	0	0	0	0.0±0.0[a]	0.0±0.0[a]
	7	17	0	0.2±0.2[a]	0.0±0.0[a]
	15	33	0	0.3±0.2[a]	0.0±0.0[a]
	30	0	0	0.0±0.0[a]	0.0±0.0[a]
BAP(16 µM) + Kn(4 µM)	0	33	0	0.3±0.2[a]	0.0±0.0[a]
	7	33	0	0.3±0.2[a]	0.0±0.0[a]
	15	17	0	0.2±0.2[a]	0.0±0.0[a]
	30	0	0	0.0±0.0[a]	0.0±0.0[a]
NAA(5 µM)	0	0	33	0.3±0.2[a]	0.8±0.5[b]
	7	33	33	0.6±0.4[a]	0.6±0.5[b]
	15	50	17	0.6±0.4[a]	0.2±0.2[ab]
	30	0	0	0.0±0.0[a]	0.0±0.0 [a]
	WPM+BAP(16 µM) matrix				
Full strength					
BM	0	33	0	0.3±0.2[ab]	0.0±0.0[a]
	7	17	0	0.2±0.2[ab]	0.0±0.0[a]
	15	0	0	0.0±0.0[a]	0.0±0.0[a]
	30	0	0	0.0±0.0[a]	0.0±0.0[a]
BAP(16 µM) +Kn(4 µM)	0	33	0	0.3±0.2[ab]	0.0±0.0[a]
	7	50	0	0.5±0.2[b]	0.0±0.0[a]
	15	0	0	0.0±0.0[a]	0.0±0.0[a]
	30	0	0	0.0±0.0[a]	0.0±0.0[a]
NAA(5 µM)	0	0	0	0.0±0.0[a]	0.0±0.0[a]
	7	0	0	0.0±0.0[a]	0.0±0.0[a]
	15	0	0	0.0±0.0[a]	0.0±0.0[a]
	30	0	0	0.0±0.0[a]	0.0±0.0[a]

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

into shoot and root which reached to total of 0.6 ± 0.4 shoots and 0.2 ± 0.2 roots (Table 20).

As coconut water (10%) was effective for elongation in regeneration studies therefore the stunted shoots and roots developed from synseeds in medium with NAA (5µM) were transferred to the same. It was observed that shoot slightly elongated while it helped in increasing root length.

The synseeds prepared in full strength BAP (16µM) matrix when transferred to basal medium at 0 day 33% germination was observed in terms of shoots (0.3 ± 0.2), but the frequency in percent germination decreased with each storage period. When medium was supplemented with BAP(16µM) and Kn(4µM) at 0 day stunted shoots with small amount of callus was formed (Fig. 49b).The response increased to 50% after 7 days of storage(Fig.49c).But the synseeds lost its viability after 15 and 30 days of storage. In comparison to half strength full strength WPM medium fortified with NAA(5µM) failed to evoke any response in synseeds (Table 20).The synseeds which germinated into stunted shoots were transferred to medium with coconut water(10%) for elongation. It was observed that the stunted shoots with callus developed in medium fortified with BAP(16µM) with Kn(4µM) get elongated into long shoots(Fig. 49d).Hence this matrix was able to helped in germination of synseeds in terms of shoot and root only in half strength regenerative medium.

4.3.2.3 Effect of half and full strength WPM medium matrix fortified with BAP(16µM) +IBA(0.1µM) on synseed germination

Another matrix utilised for synseed preparation was medium fortified with BAP (16µM**)** and IBA (0.1µM). The *in vitro* nodes encapsulated in this matrix were also transferred to basal regenerative medium at 0day where only 33% responded and formed only shoots (0.3 ± 0.2) after 4 weeks. With increase in storage period to 7 days synseeds responded in a similar way (33%) but this response decreased to 17% after 15 days of storage. When medium was fortified with NAA (5µM) there was shoot and root emergence from synseeds transferred at 0 day and different storage period (7 and 15 day). The response was nearly same for 7 and 15 days of storage ie. in 33% synseed 0.3 ± 0.2 shoots emerged but they remained stunted even after 4 weeks(Fig.50a,b) while the number of roots were slightly more (0.8±0.5) after 7 days of storage (Table 21).

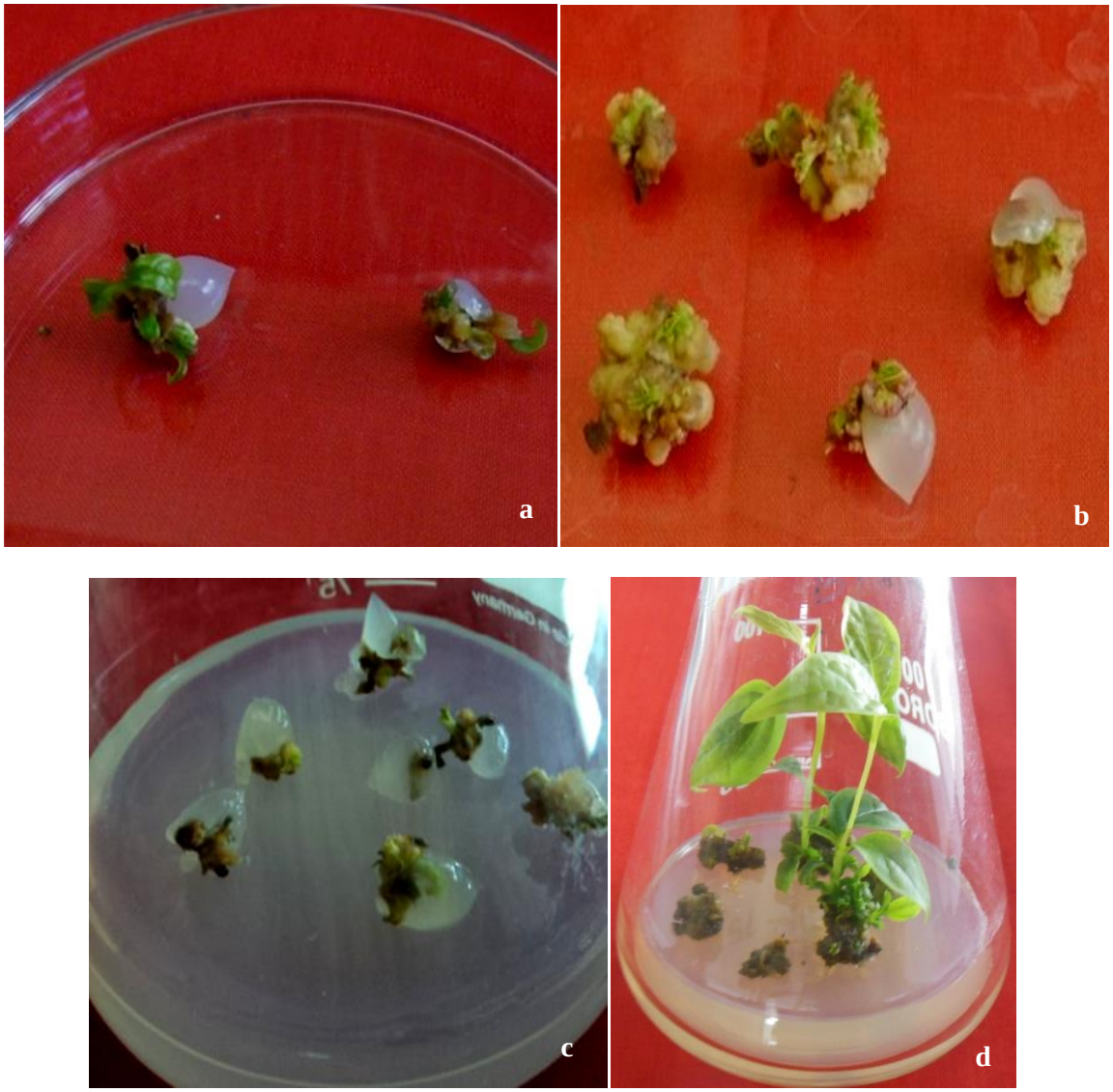

Fig.49: Germination of *in vitro* nodes encapsulated in half and full WPM + BAP(16µM) matrix in different regenerative medium in *O.indicum* after 4 weeks

a. **Stunted shoot emerged from synseeds placed after 15 days of storage in ½ WPM + NAA(5 µM) regenerative medium**
b. **Stunted shoots with callus developed from synseeds transferred at 0 day in full strength WPM+BAP(16µM)+Kn(4µM) regenerative medium**
c. **Formation of stunted shoots from synseeds in WPM+BAP(16µM)+Kn(4µM) regenerative medium after 7 days of storage**
d. **Elongation of stunted shoots after transferring to WPM+coconut water (10%)**

The *in vitro* nodes encapsulated in full strength medium matrix fortified with BAP (16µM) and IBA(0.1µM) when transferred to basal regenerative medium at 0day showed similar response as in half strength There was 33% germination of synseeds in terms of 0.3 ± 0.2 shoots after 4 weeks. Which remained same even after 7 days of storage (33%) while increased storage period decreased the germination frequency (Table 21). The synseeds after transferring to WPM fortified with NAA(5µM) medium at 0 day also resulted in 33% germination into 0.3 ± 0.2 shoots after 4 weeks.

Table 21. Effect of half and full strength WPM medium matrix fortified with BAP (16 µM)+IBA(0.1µM) on synseed germination in *O.indicum* after 4 weeks

	1/2 WPM +BAP(16µM)+IBA(0.1µM) matrix				
RM	**Storage days**	**%Shoot emergence**	**% Shoot and Root emergence**	**Number of shoots***	**Number of roots***
Half strength					
BM	0	33	0	0.3±0.2^{a}	0.0±0.0^{a}
	7	33	0	0.3±0.2^{a}	0.0±0.0^{a}
	15	17	0	0.2±0.2^{a}	0.0±0.0^{a}
	30	0	0	0.0±0.0^{a}	0.0±0.0^{a}
NAA(5µM)	0	17	17	0.2±0.2^{a}	0.7±0.7^{a}
	7	0	33	0.3±0.2^{a}	0.8±0.5^{a}
	15	0	33	0.3±0.2^{a}	0.7±0.4^{a}
	30	0	0	0.0±0.0^{a}	0.0±0.0^{a}
	WPM+BAP(16µM)+IBA(0.1µM) matrix				
Full strength					
BM	0	33	0	0.3±0.2^{a}	0.0±0.0^{a}
	7	33	0	0.3±0.2^{a}	0.0±0.0^{a}
	15	0	0	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0.0±0.0^{a}	0.0±0.0^{a}
NAA(5µM)	0	33	0	0.3±0.2^{a}	0.0±0.0^{a}
	7	0	33	0.3±0.2^{a}	0.7±0.4^{b}
	15	33	0	0.3±0.2^{a}	0.0±0.0^{a}
	30	0	0	0.0±0.0^{a}	0.0±0.0^{a}

*Values represents mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤0.05 according to Duncan's Multiple range test

Fig.50: Germination of synseeds encapsulated in half and full strength WPM+BAP(16µM)+IBA(0.1µM) matrix in different regenerative media in *O.indicum* after 4 weeks

a. Germination of synseeds into stunted shoot and root in ½ WPM+NAA(5µM) medium when placed after 7 days of storage
b. Emergence of shoot and root from synseeds placed after 15 days of storage in ½ WPM+NAA(5µM) regenerative medium
c. 7 day stored synseeds germinated into single shoot and root in WPM+NAA(5 µM) regenerative medium

But after 7 days of storage 33% synseeds germinated in terms of single shoots with root (Fig.50c) with an average of 0.3 ± 0.2 shoots and 0.7 ± 0.4 roots. The synseeds after 15 days of storage again germinated into shoots only with 33% percent response (Table 21).

Therefore in this matrix both half and full strength medium was able to induce the synseeds to germinate and form shoot and roots.

Hence it can be concluded that:

- The maximum germination in terms of shoot emergence (50%) with an average of 0.8 ± 0.4 was observed from *in vitro* nodes encapsulated in full strength basal medium matrix when placed in regenerative medium fortified with GA_3 (10µM) after 15 days of storage. In this medium only the synseeds remain viable upto 30days.
- The maximum percent germination into shoot and root (33%) was obtained when in vitro nodes were encapsulated in half strength BAP(16µM)+IBA(0.1µM) matrix and transferred to NAA (5µM) regenerative medium after 15 days of storage. It resulted in an average of 0.3 ± 0.2 shoots and 0.7 ± 0.4 roots.
- Therefore in *O.indicum* the half strength and full strength medium helped in germination of synseeds but half strength medium containing 1% sucrose was able to germinate the synseeds into shoot and root while full strength medium having 3% sucrose germinated synseeds into shoots only.
- The germination frequency of synseeds was mostly observed after 15 days of storage and it decreased after 30 days of storage in most of the regenerative media.

4.3.3 Effect of substrates on synseeds germination

The matrix and regenerative medium which were suitable for synseed germination from the substrate which was utilised for storage ie half and full strength WPM basal medium placed in petridish containing filter paper were selected and the same were taken for agar (static) substrate. Since the half strength WPM medium fortified with BAP (16µM) +IBA(0.1µM) matrix and NAA(5µM) regenerative medium proved to effective for retaining the regenerative ability of synseeds into shoot and root after 15 days of storage ,hence the same was selected for synseed germination. The *in vitro* nodes encapsulated in half strength WPM

matrix were stored in petridish containing half/full strength static basal WPM medium respectively. The synseeds were stored at 4 ºC and harvested after 7, 15 and 30 days storage and observations were recorded after 4 weeks of transfer to each time interval.

4.3.3.1 Comparison of filter paper and agar substrate on synseeds germination of *O.indicum*

The encapsulated *in vitro* nodes after 7 days of storage in static substrate when transferred to NAA(5µM) regenerative medium resulted in 17 % synseeds germination into shoots. The response was similar ie. 17% synseeds germinated into single shoot when transferred to medium after 15 days of storage. With increase in storage period to 30 days the viability decreased(Fig.51) as similar to filter paper substrate Therefore in comparison to filter paper the germination frequency was less when *in vitro* nodes encapsulated in this matrix were stored in static substrate.

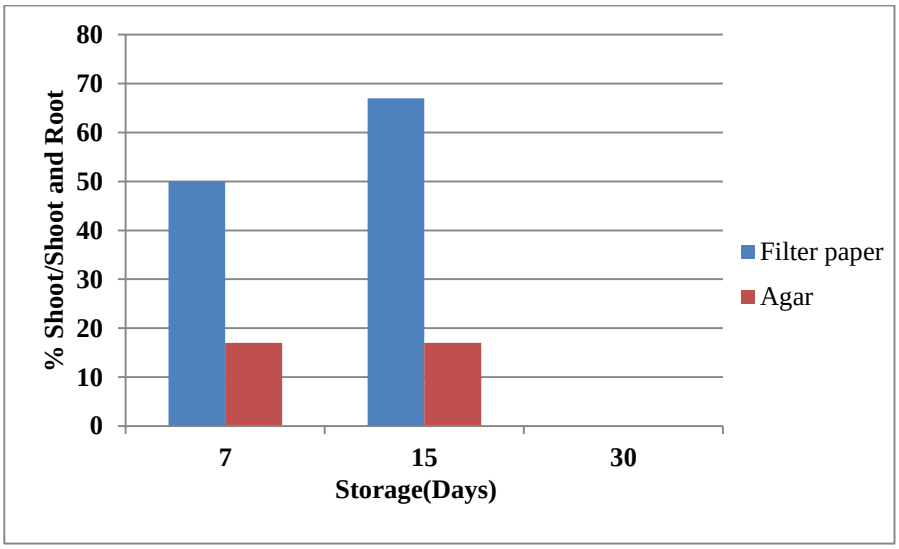

Fig. 51: Effect of agar substrate in ½ WPM+BAP(16µM)+IBA(0.1 µM) matrix and ½ WPM+NAA(5µM) regenerative medium in *O.indicum* after 4 weeks

Hence it can be concluded that the filter paper substrate proved to be effective for retaining the regenerative ability of synseeds compared to static substrate in *O.indicum*.

4.4 *Stereospermum suaveolens*

This was the second species in which similar studies were carried out.

4.4.1 Section I: Seed germination studies

Germination studies were carried out in *S.suaveolens* seeds as this plant has low percent of seed germination. The seeds were placed in different substrates to find the optimised one which can develop a large number of seedlings. The % germination in different substrates and germination parameters were studied.

4.4.1.1 Germination of *S.suaveolens* in different substrates

Similar to *O.indicum* in this species also the seeds soaked overnight in water were germinated in substrates under natural conditions (sand and soil) and aseptic conditions (cocopeat, cocopeat:soil, cocopeat:sand, filter paper, MS and WPM basal medium).Observations on seed germination in different planting substrates was recorded after 4 weeks (Fig.52).

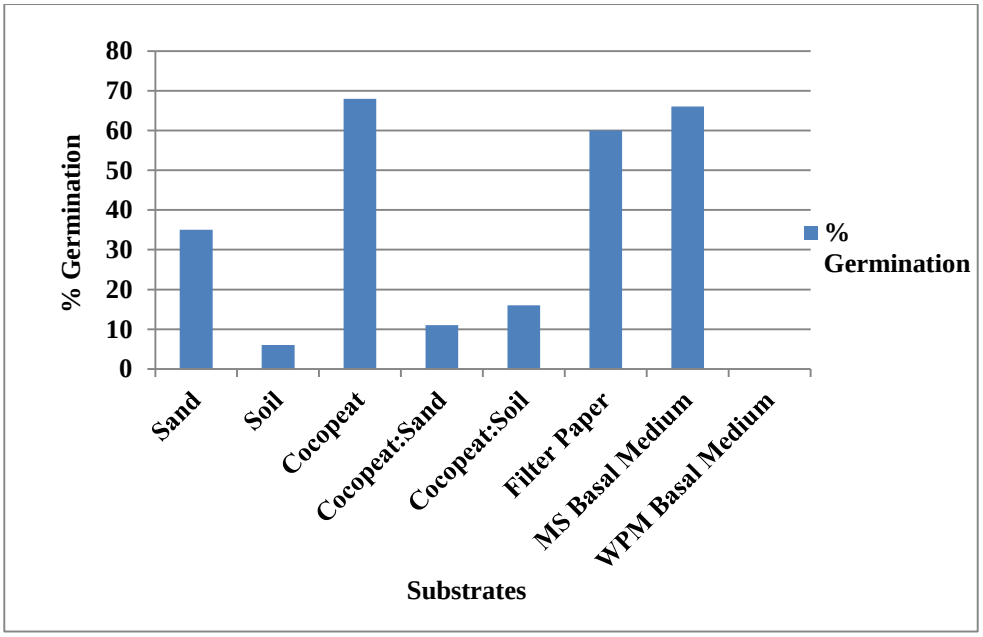

Fig. 52: Germination of *S.suaveolens* seeds in different substrates

- **Under natural conditions:** Sand and soil substrate were utilised for seed germination under natural conditions

  - **Sand:** The individual sand substrate resulted in only 35 % seed germination.

- **Soil:** Whereas in soil substrate poor germination was observed as least percent of seeds were germinated (6%).

➢ **Under lab conditions:** Different substrates were placed under lab conditions for germinating seeds.

- **Cocopeat:** This substrate recorded maximum seed germination (68%) (Fig.52) with healthy development of seedlings with fully expanded cotyledonary leaves (Fig.53a).
- **Cocopeat:sand (1:1) :** Cocopeat was combined with sand in ratio of 1:1 and observations revealed that only 11% of seeds germinated after 4 weeks.
- **Cocopeat:soil (1:1) :** In this combination cocopeat was added with soil(1:1) but this mixture of substrate also failed to improve the % germination (16 %).
- **MS and WPM basal medium:** Seeds were germinated aseptically in MS and WPM basal medium with sucrose (3%), MS medium resulted in 66% germination of seeds whereas in WPM medium seeds failed to germinate.
- **Filter paper:** The seeds inoculated in filter paper containing petridish resulted in 60 % germination of seeds but cotyledonary leaves got curled due to less surface area(Fig.53b).

Thus from the above experiment it was clear that for *S.suaveolens* seeds under natural conditions ie. soil substrate is poor in terms of percent and behaviour of seedlings. Out of all the cocopeat substrate proved to be optimum substrate in terms of percent germination which developed healthy seedlings within 3 weeks (Fig.53c).Also the seeds soaked overnight in water was sufficient for germination of seeds.

4.4.1.2 Germination parameters

The germination parameters in different substrates were calculated to find out the suitable substrate which develops the seedlings faster for that germination rate was calculated. It was observed that germination rate of seeds was highest in cocopeat (5.4) as seedling emerged within 10 days and all seedlings developed by the end of third week(Fig.54). Whereas in filter paper and MS medium the germination rate was lower compared to cocopeat as the seedlings emerged within 12 days and developed by the end of fourth week. In comparison the substrates like cocopeat:soil, cocopeat:sand, sand, soil the germination rate was poor and the growth of seedling was also slow, as it took more than 15 days for seed emergence and with less number of seedlings developed after a month (Fig.54).

Fig.53: Seed germination in *S.suaveolens*

a. Development of seedlings in cocopeat after 4 weeks
b. Germination of seeds in Filter paper with leaves getting curled
c. Healthy seedlings of *S.suaveolens*

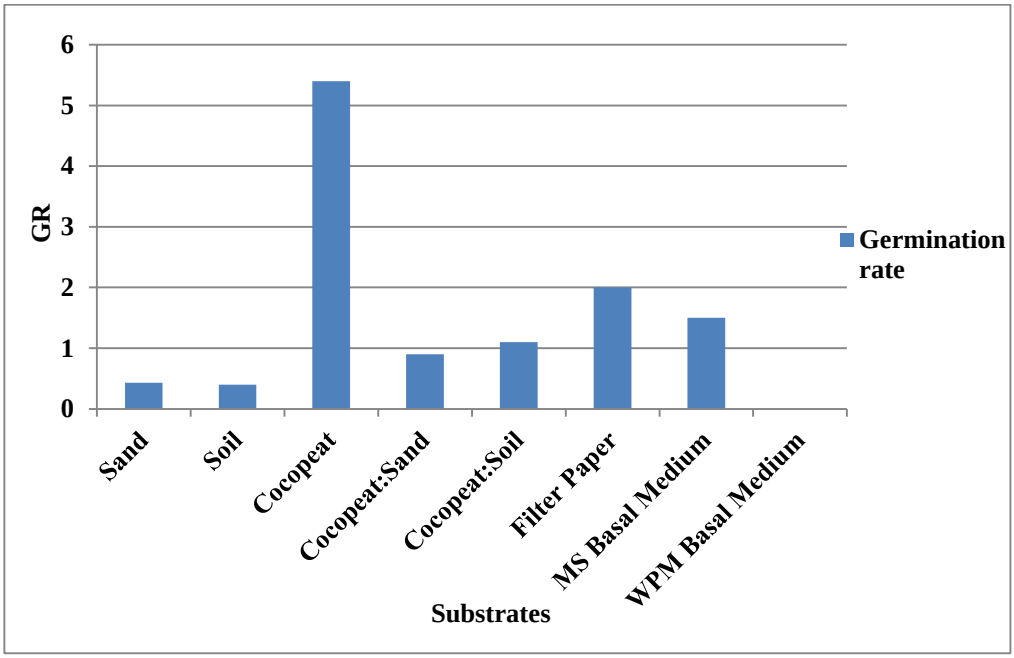

Fig.54: Germination rate (GR) of *S. suaveolens* seeds in different planting substrates

Another parameter was mean daily germination in which the day from the seeds were inoculated every day they were checked for seedling emergence and recorded and after one month the mean daily germination (MDG) was calculated in all substrates.

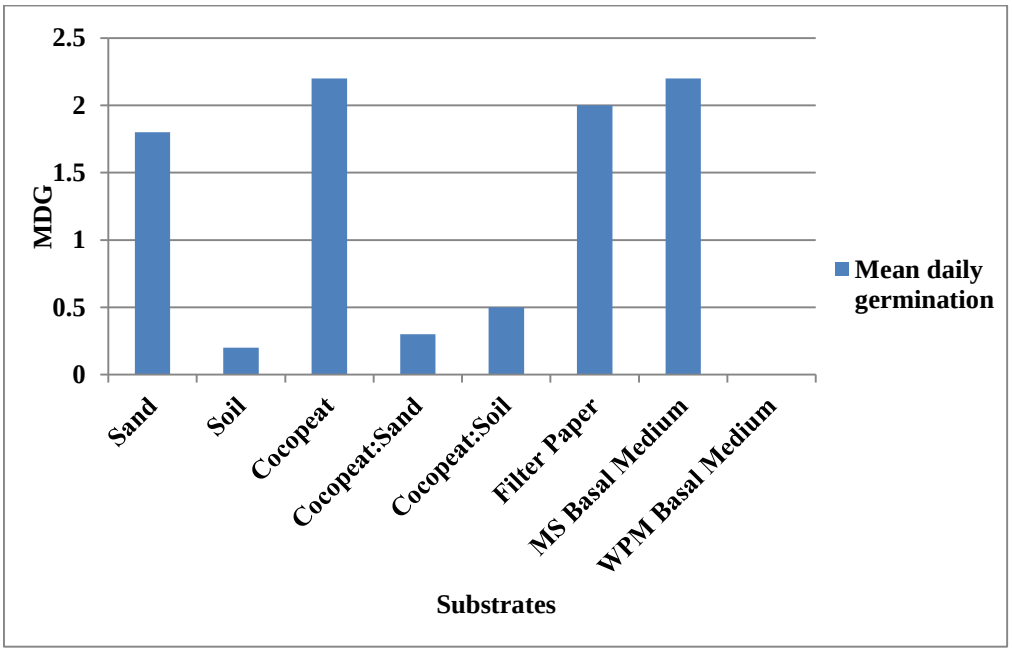

Fig.55: Mean daily germination (MDG) of *S.suaveolens* seeds in different planting substrates

It was observed that MDG had the same value (2.2) for both coco peat and MS medium, followed by filter paper and sand. The other substrates, like mixture of cocopeat with sand and soil had lower MDG (Fig. 55) but the soil substrate was having the least MDG of all the substrates (Fig.55).

The germination index is another important parameter for seed germination and hence was also calculated after one month. It was observed maximum GI was obtained in MS medium (9.9) and was nearly same in cocopeat (8.9) and was followed by filter paper. The least GI was observed in cocopeat:sand and soil (Fig. 56).

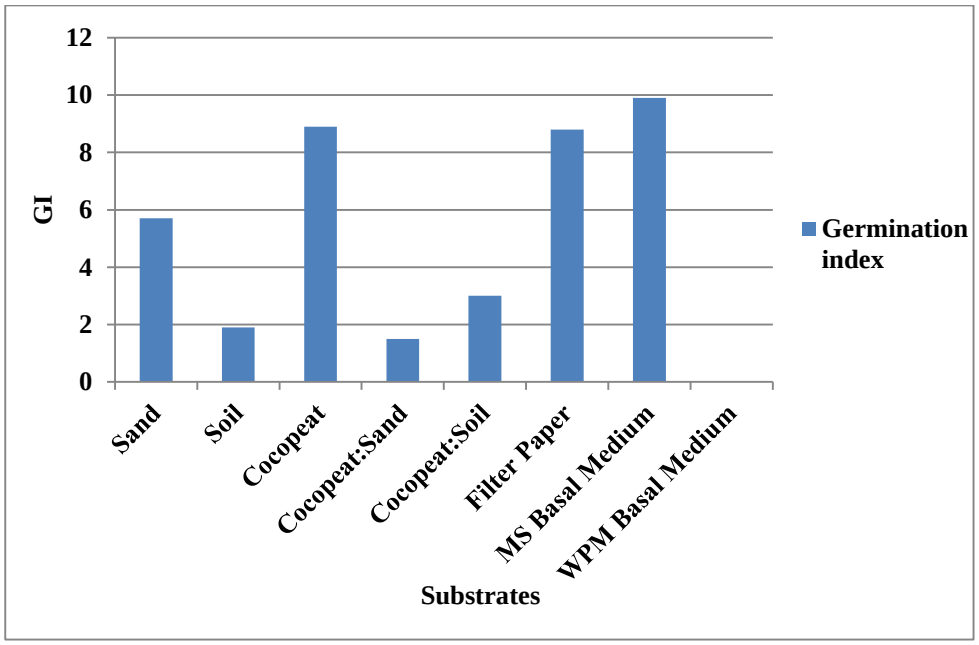

Fig.56: Germination index (GI) of *S. suaveolens* seeds in different planting substrates

From the above experiment it is clear that cocopeat resulted in highest germination rate and maximum mean daily germination and germination index was observed in cocopeat and MS medium followed by filter paper substrate.Thus overall results depict that cocopeat was the ideal substrate for *S.suaveolens* seeds having maximum percent and speed of germination.

Hence similar to *O.indicum* the seeds of *S.suaveolens* were germinated in cocopeat substrate for generating large number of seedling explants which were utilised for regeneration studies.

4.4.2 Section II: Regeneration studies

To regenerate the *S.suaveolens* species rapidly the selection of suitable explants was done. Similar to *O.indicum* the explants used were seedling (cotyledonary leaf, cotyledonary node and hypocotyl) and nodal which were placed on MS and WPM medium fortified without or with PGRs.

The regeneration studies was divided into four stages:

- Establishment of shoot cultures
- Multiplication of shoots
- *In vitro* rooting of microshoots
- Hardening of plantlets

4.4.2.1 Establishing and multiplication of shoots

In *S. suaveolens* all the four different explants were utilised for evaluating their regeneration capacities and establishing cultures. The explants were placed in MS and WPM medium fortified with individual cytokinins BAP (2-30µM), Kn(2-30µM) and TDZ(0.1-2µM) and the explants which regenerated into shoots were selected for further experiments.

4.4.2.1.2 Effect of individual cytokinins on shoot induction in MS and WPM medium from cotyledonary leaf explants

The cotyledonary leaves (1x1cm) were excised from seedlings and placed horizontally on MS medium with the lower surface touching the medium. It was observed that the leaves started swelling within two weeks and in both the media there was formation of friable callus (creamish white in color) from the cut ends by the end of 4 weeks at different concentrations of BAP. In the lower concentrations of BAP (2µM, 4µM, BAP8µM and 16µM) of MS medium there was only swelling with slight callus induction (Fig.57a,b).The higher concentrations of BAP (20µM to 30µM) there was induction of profuse friable callus(Fig.57c,d). Similar observations were recorded in presence of Kn where the lower concentration 2µM to 8µM induced only swelling only while higher concentration (20µM, 25µM and 30µM) resulted in formation of callus. Incorporating TDZ in the medium also resulted in callus induction.

In WPM medium fortified with lower concentrations of BAP (2µM,4µM,8µM) induced response in terms of swelling and callus induction. All the Kn concentrations (2µM -25 µM) induced swelling of explants except Kn(4µM) where callus induction was obtained and the 30 µM failed to respond. The callus was transferred to fresh medium to see whether it gets differentiated into shoots but it failed to regenerate into shoots. Therefore this explant failed to regenerate in terms of shoot in the presence of individual cytokinins.

4.4.2.1.3 Effect of individual cytokinins on shoot induction in MS and WPM medium from hypocotyl explants

The hypocotyl (1 cm) were also placed horizontally in medium fortified with individual cytokinins. The explant started swelling within two weeks and after 4 weeks it was observed that slight callus was induced at both the cut ends. In MS medium fortified with BAP (2µM) only shoot buds were induced (Fig.57e) which failed to proliferate into shoot, while in at 4 µM there was only swelling of explants took place. The explants failed to respond in the other concentrations (8µM,16µM,20µM ,25µM and 30µM) .When Kn was added, in the 2µM ,8µM , and 20µM to 30µM concentrations there was formation of callus except Kn at 4µM healthy shoots were formed in only 20% cultures(Fig.57 f,g) whereas all the TDZ concentrations failed to respond.

The WPM medium also failed to respond in terms of shoot formation. In medium fortified with BAP (4µM), (8µM), there was only swelling of explants. Similar response was observed in lower concentrations of Kn. While in rest all the concentrations of BAP and Kn the explant failed to respond. Similar to MS the explants in all the TDZ concentrations failed to respond in WPM medium.

As both the explants (cotyledonary leaf and hypocotyl) failed to regenerate into shoot in the presence of individual cytokinins these were omitted in further studies.

4.4.2.1.4 Effect of individual cytokinins on shoot induction in MS and WPM medium from cotyledonary node explants

The cotyledonary node (1.5 cm) was placed vertically on MS and WPM medium fortified BAP (2-30µM), Kn(2-30µM) and TDZ(0.1-2µM) and their effect on shoot induction was evaluated.

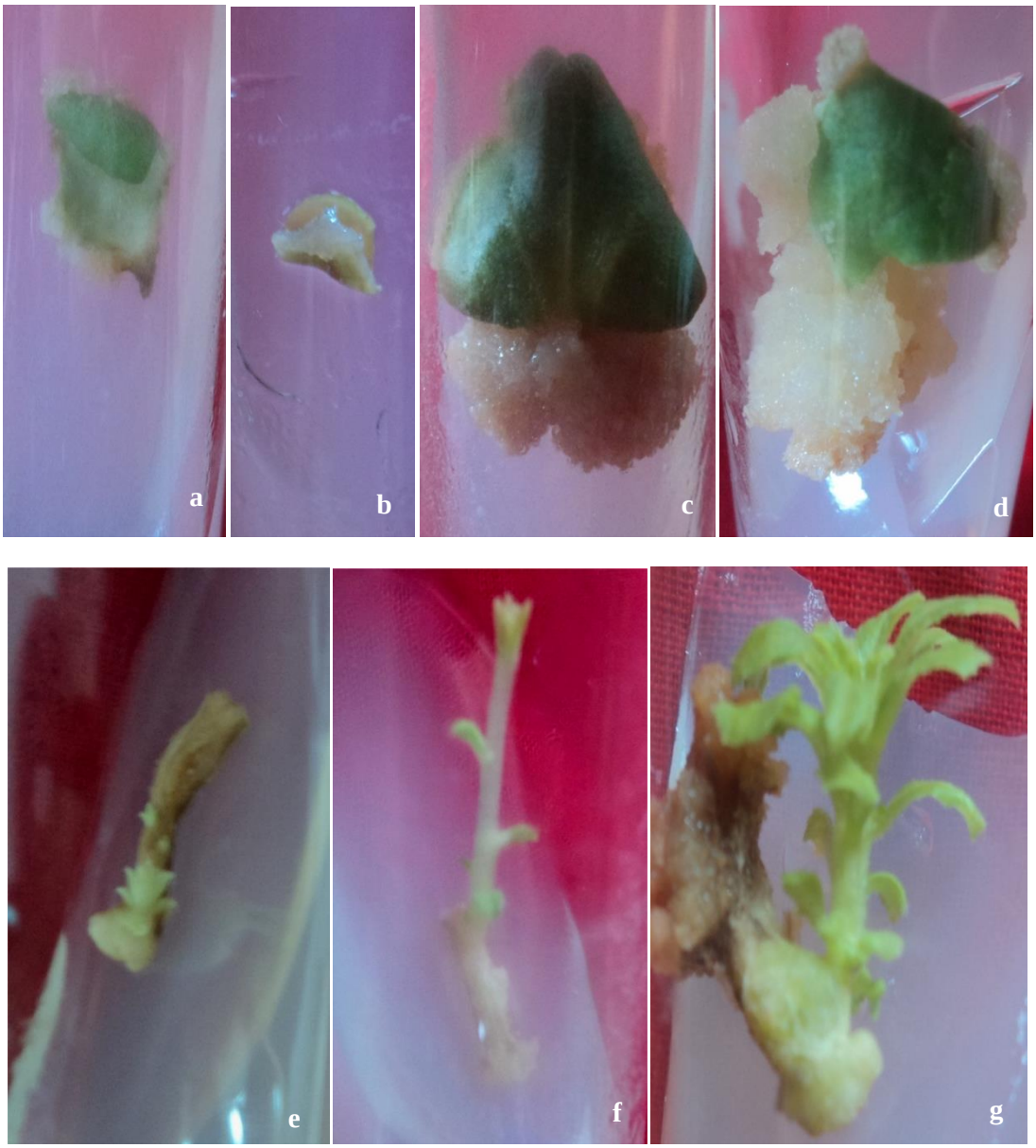

Fig.57: Morphogenic response of cotyledonary leaf and hypocotyl explants of *S.suaveolens* in presence of individual cytokinins after 4 weeks

a. Swelling and induction of callus in MS+BAP(2µM)
b. Callus induced at cut ends from cotyledonary leaf explant in MS+BAP(4 µM)
c. Fibrous callus induced from cotyledonary leaf explant in MS+BAP(20µM)
d. Profuse fibrous callus from cut ends of cotyledonary leaf explant in MS+BAP(30µM)
e. Shoot bud induction from hypocotyl explant in MS+BAP(2µM)
f. and g. Shoot emergence from hypocotyl explant in MS+Kn(4µM)

The explants in **MS medium** without PGRs formed shoots in 50% cultures. When medium was supplemented with different concentrations of BAP, the lower concentrations (2µM,4µM and 8µM) resulted in formation of single shoot in 50 to 67% cultures. BAP at 20 µM also induced a 67 % response in cultures in which 1.2 ± 0.6 number of shoots with an average of 2.7 ± 0.8 nodes were formed (Fig.58a).

Table 22. Effect of individual cyokinins on shoot induction from cotyledonary node explants of *S.suaveolens* after 4 weeks

Cytokinin (µM)			MS medium			WPM medium		
BAP	**Kn**	**TDZ**	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
0	0	0	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}	50	0.5 ± 0.3^{abc}	0.5 ± 0.3^{a}
2	-	-	50	0.7 ± 0.3^{ab}	0.8 ± 0.5^{abcd}	90	0.8 ± 0.1^{bc}	2.7 ± 0.7^{b}
4	-	-	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}	100	1.2 ± 0.2^{c}	2.3 ± 0.4^{b}
8	-	-	67	0.7 ± 0.2^{ab}	1.0 ± 0.4^{bcd}	**100**	$\mathbf{1.2 \pm 0.2^{c}}$	$\mathbf{2.4 \pm 0.4^{b}}$
16	-	-	16	0.2 ± 0.2^{a}	0.7 ± 0.7^{abc}	71	0.4 ± 0.2^{ab}	1.3 ± 0.6^{ab}
20	-	-	**67**	$\mathbf{1.2 \pm 0.6^{b}}$	$\mathbf{2.7 \pm 0.8^{e}}$	50	0.6 ± 0.4^{abc}	0.5 ± 0.2^{a}
25	-	-	33	0.3 ± 0.2^{a}	0.7 ± 0.5^{abc}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	2	-	50	0.5 ± 0.2^{a}	1.0 ± 0.5^{bcd}	100	1.0 ± 0.0^{bc}	3.2 ± 0.3^{c}
-	4	-	67	0.7 ± 0.2^{a}	0.7 ± 0.2^{abc}	100	1.0 ± 0.0^{bc}	2.4 ± 0.4^{bc}
-	8	-	67	0.7 ± 0.2^{a}	1.5 ± 0.7^{bcde}	80	0.8 ± 0.2^{ab}	2.0 ± 0.5^{c}
-	16	-	16	0.2 ± 0.2^{a}	0.2 ± 0.2^{a}	50	0.5 ± 0.1^{a}	1.5 ± 0.5^{ab}
-	20	-	16	0.2 ± 0.2^{a}	0.2 ± 0.2^{a}	83	0.8 ± 0.1^{ab}	2.7 ± 0.7^{bc}
-	25	-	16	0.2 ± 0.2^{a}	0.8 ± 0.8^{abcd}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.1	67	0.7 ± 0.2^{a}	1.7 ± 0.6^{cde}	83	0.7 ± 0.2^{a}	2.2 ± 0.7^{b}
-	-	0.2	83	0.8 ± 0.2^{a}	2.2 ± 0.5^{de}	50	0.5 ± 0.2^{a}	1.5 ± 0.7^{ab}
-	-	0.25	50	0.5 ± 0.2^{a}	0.8 ± 0.5^{abcd}	66	0.7 ± 0.2^{a}	2.3 ± 0.8^{b}
-	-	0.5	67	0.7 ± 0.2^{a}	1.2 ± 0.5^{bcd}	40	0.4 ± 0.2^{a}	0.6 ± 0.3^{ab}
-	-	1	67	0.7 ± 0.2^{a}	0.7 ± 0.2^{abc}	40	0.4 ± 0.2^{a}	0.5 ± 0.2^{a}
-	-	2	33	0.3 ± 0.2^{a}	0.3 ± 0.2^{ab}	50	0.5 ± 0.2^{a}	0.5 ± 0.2^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test.

When Kn was added in medium the lower concentrations were comparatively effective. A maximum of 67 % cultures responded with formation of 0.7 ± 0.2 shoots at 4 and 8 µM, and at 8 µM maximum number of nodes (1.5 ± 0.7) were formed(Fig.58b). The higher concentrations of Kn (16µM, 20µM and 25µM) resulted in poor response as only 16 % cultures responded in MS medium. Replacing Kn with TDZ induced morphogenic response which was better in terms of percent response as compared to BAP and Kn. The highest percent response (83%) was obtained at 0.2µM with 0.8 ± 0.2 shoots and 2.2 ± 0.5 number of nodes and these single shoot were healthy (Fig.58c) (Table 22).

In **WPM** basal **medium** 50 % response was obtained which was similar to MS medium. WPM medium was also fortified with different concentrations of individual cytokinin to study its effect on cotyledonary node explant. Incorporating BAP the lower concentrations proved to be effective in terms of shoot induction compared to MS medium resulting in 90% to 100% at 2µM, 4µM and 8µM (Fig.58d) and these shoots were long having many nodes (Table 22). At 16µM and 20µM the shoots which developed were weak and associated with basal callus. When medium was supplemented with Kn, the lower concentrations (2µM, 4µM and 8µM) resulted in maximum percent response where at 2 µM and at 4 µM there was 100 % response with formation of 1.0 ± 0.0 shoots (Fig.58e,f) while the number of nodes (3.2 ± 0.3) were maximum at 2µM. Incorporating TDZ in the medium resulted in nearly same response but lower concentrations were effective in terms of morphogenic response. At 0.1µM the explant responded in 83 % cultures with an average of 0.7 ± 0.2 shoots while at 0.25µM resulted in maximum number of nodes 2.3 ± 0.8(Table 22).

All the cytokinins were effective in terms of shoot induction in this species, but BAP proved to be better in terms of shoot number, Kn in terms of forming elongated shoots with many nodes and TDZ was also able to induce morphogenic response at all concentrations.

The further multiplication of shoots was achieved utilizing the *in vitro* nodes obtained from *in vitro* shoots developed from the cotyledonary node explants.

4.4.2.1.4.1 Multiplication of shoots from *in vitro* nodes in presence of individual cytokinins

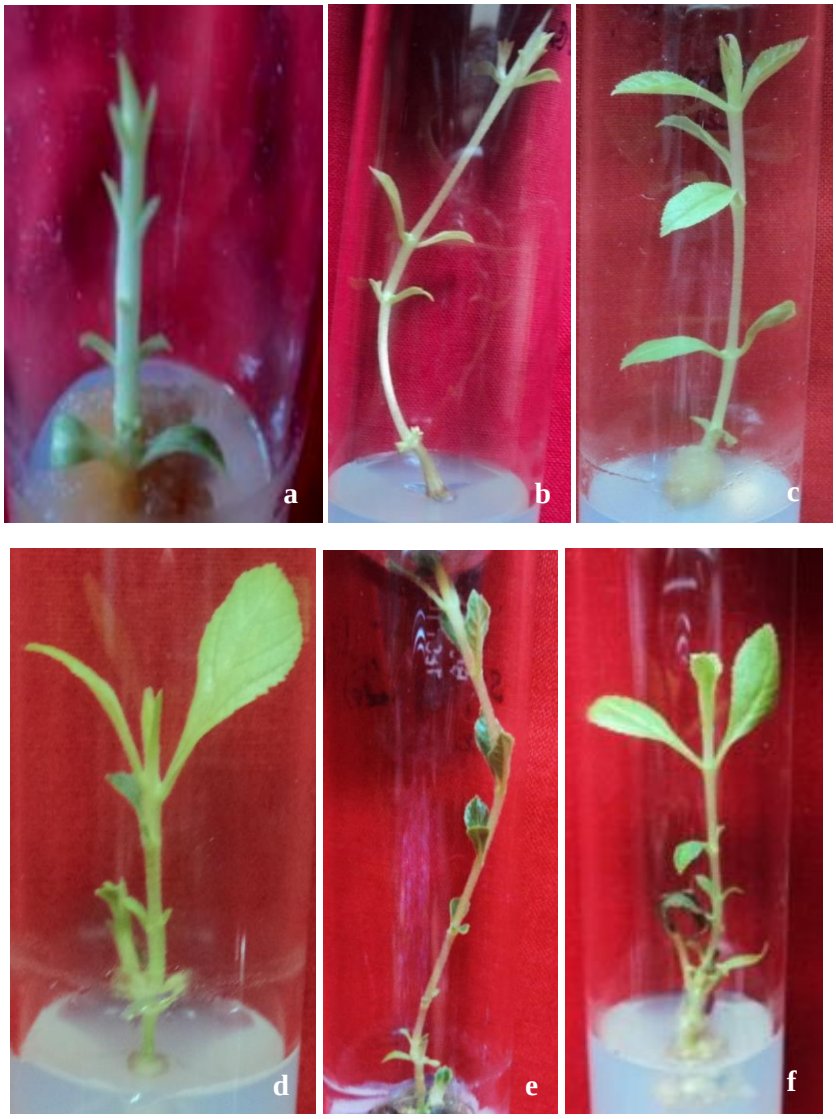

Fig.58: Shoot formation from cotyledonary node explants of *S.suaveolens* in presence of individual cytokinins after 4 weeks

a. Single shoot developed in MS+BAP (20µM)
b. Healthy long shoots formed in MS+ Kn(8µM)
c. Single shoot developed with healthy leaves in MS+TDZ(0.2µM)
d. Development of small shoots in WPM+BAP (8µM)
e. Elongated shoot formed in WPM +Kn(2µM)
f. Single shoot developed in WPM +Kn(4µM)

Single *in vitro* nodes were placed in MS and WPM medium fortified with those concentrations of BAP/ Kn and TDZ which induced response.

In MS medium the *in vitro* nodes from cotyledonary node were placed in the respective concentrations of BAP (2µM, 4µM, 8µM and 20µM), Kn (2µM, 4µM, 8µM) and TDZ(0.1 µM,0.2µM).

In MS medium out of all the concentrations shoots were formed only in presence of BAP (8µM), Kn (8µM) and TDZ (0.1µM, TDZ0.2µM) concentrations after 8 weeks .The other concentrations failed to develop shoots after 8 weeks. In BAP (8µM) single shoot was formed (Fig.59) in 17 % of cultures and in Kn (8µM) an average of 1.5 ± 0.5 shoots were formed (Fig.60a). In TDZ at 0.1µM also one or two shoots were formed with an average of 1.3 ± 0.2 shoots in 100% cultures but the shoots formed was very healthy having large leaves(Fig.60b), the % response decreased to 83% at 0.2µM (Fig.60c).

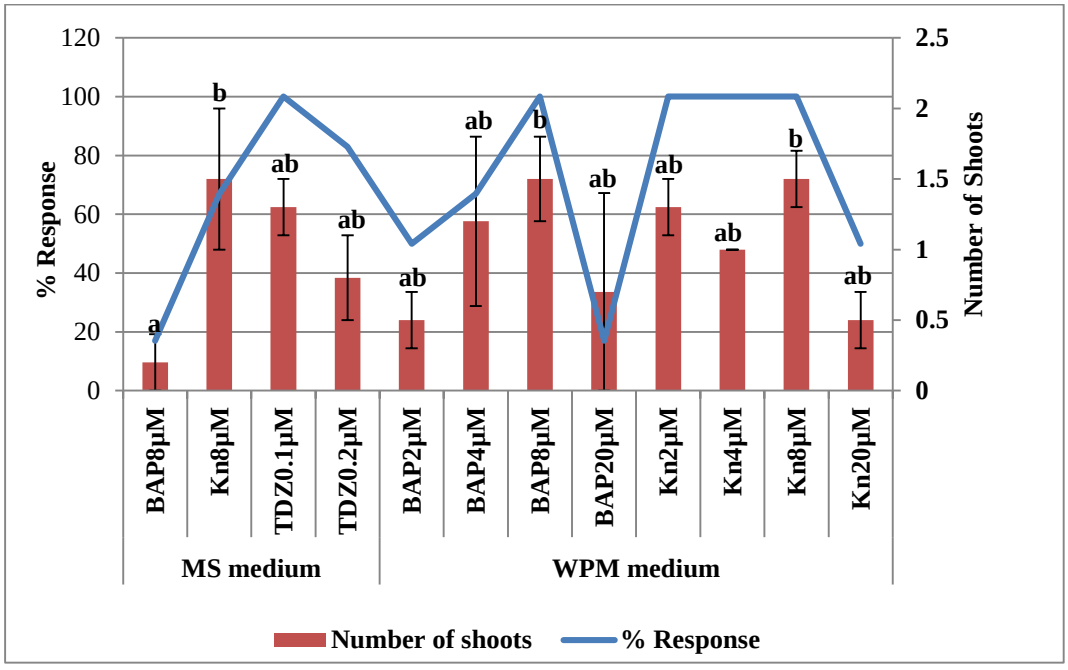

Fig. 59: Effect of individual cytokinins in inducing multiples from *in vitro* nodes of *S.suaveolens* after 8 weeks

Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

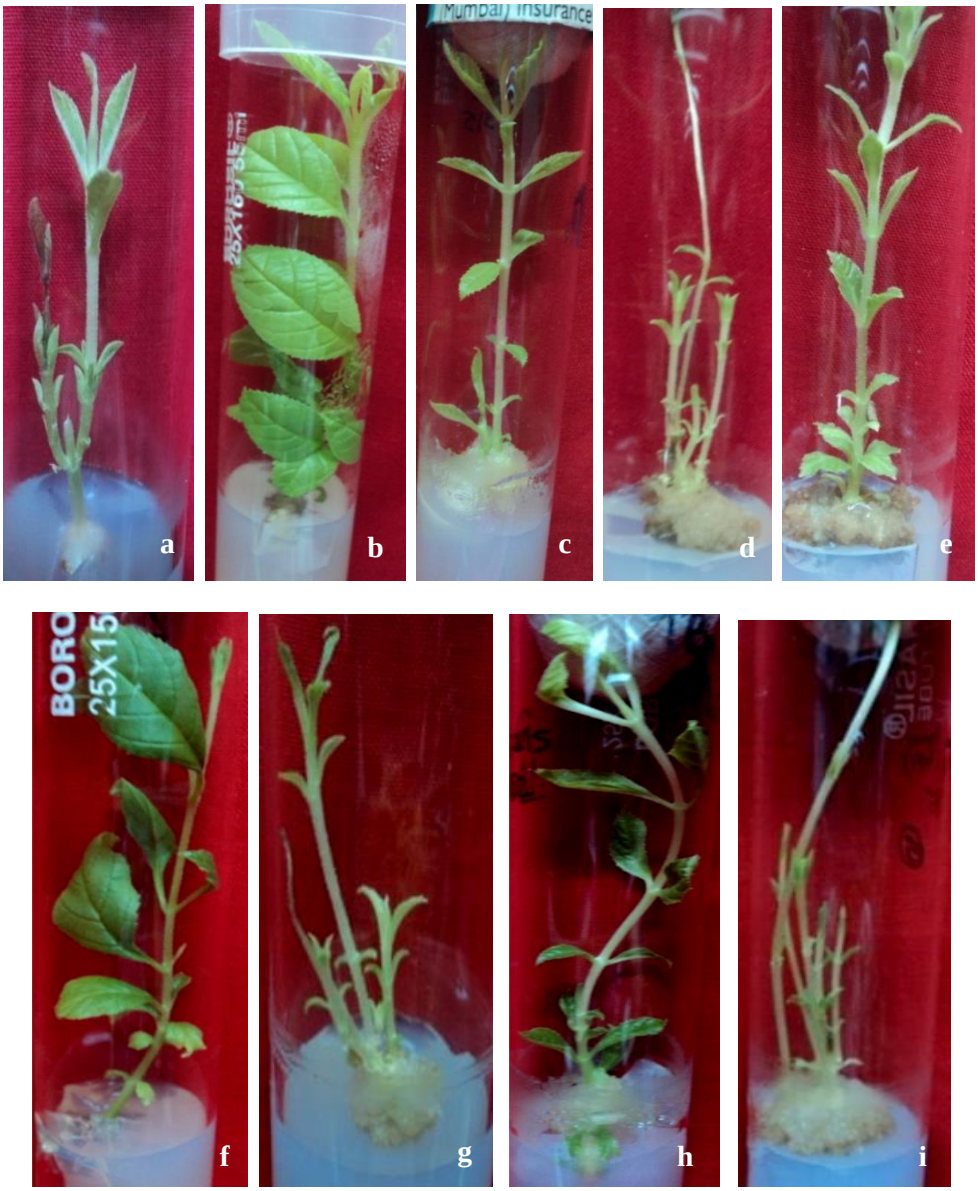

Fig. 60: Development of shoots from *in vitro* nodes of *S.suaveolens* in MS and WPM medium

a. One or two shoots developed in MS+Kn(8µM) after 8 weeks
b. Healthy shoot with enlarged leaves in MS+TDZ(0.1µM) after 8 weeks
c. Shoot developed in MS+TDZ(0.2µM) after 8 weeks
d. Multiples developed with minute leaves in WPM+BAP(4µM) after 8 weeks
e. Single elongated shoot in WPM+BAP(8µM) after 8 weeks
f. Healthy shoot formed in WPM+Kn(2µM) after 8 weeks
g. Mutiples formed in WPM+Kn(8µM) after 8 weeks
h. Formation of long shoot in MS+TDZ (0.2µM) after 12 weeks
i. Multiples developed in WPM+Kn (8µM) after 12 weeks

In WPM medium the *in vitro* nodes from cotyledonary node were subcultured in BAP(2 µM,4µM,8µM,20µM), Kn(2 µM,4µM,8µM,16µM,20µM) and TDZ(0.2µM,TDZ0.25 µM).
At the end of 8 weeks it was observed that the Kn (16µM), TDZ (0.2µM), TDZ(0.25 µM) failed to develop shoots. While in rest combinations single or two shoots were formed (Fig.59). In presence of BAP (2µM, 4µM and 8µM) the *in vitro* buds elongated into shoots which varied in percent response (Fig.59) This response in culture reached to 67% and 100% at 4 and 8 µM forming healthy shoots (Fig.60d and e) and decreased to 17% at higher concentration of BAP (20µM). At Kn 2µM, 4µM and 8µM concentrations a 100% response was observed but there was variation in the number of shoots. At 2µM healthy single shoots were formed which elongated (Fig.60f)and formed large leaves and at 8µM the number of shoots formed were more 1.5 ± 0.2 (Fig.60g).Increase in Kn to 20 µM failed to enhance the number of shoots as single shoot developed(Fig.59).

The single *in vitro* nodes were again excised from *in vitro* shoots and subcultured in fresh medium which had induced a response. After 12 weeks only MS medium fortified with TDZ (0.1µM) and (0.2µM) responded out of all the concentrations while BAP (8µM) and Kn (8µM) failed to develop shoots. The observation revealed that TDZ at 0.1µM was initially able to induce a 100% response but after 12 weeks this response decreased to 50% with 0.5± 0.2 number of shoots (Fig.61).Whereas TDZ at 0.2 µM was able to induce same percent response (83%) even after 12 weeks and it also slightly increased in the number (Fig.61) and length of shoots (Fig.60h).
The WPM medium fortified with BAP(2µM,4µM,8µM 20µM) and Kn (2µM, 4µM and 8 µM) were subcultured , but all the concentrations failed to form shoots, except at Kn8 µM, in which 100% shoots were formed with an average of 3.1± 0.5 in number by the end of 12 weeks (Fig.60i). The shoots formed were long and thin with very minute leaves (Fig.61).

The MS medium fortified with TDZ (0.1µM, 0.2µM) and WPM medium fortified with Kn8µM failed to enhance the number in further passages.

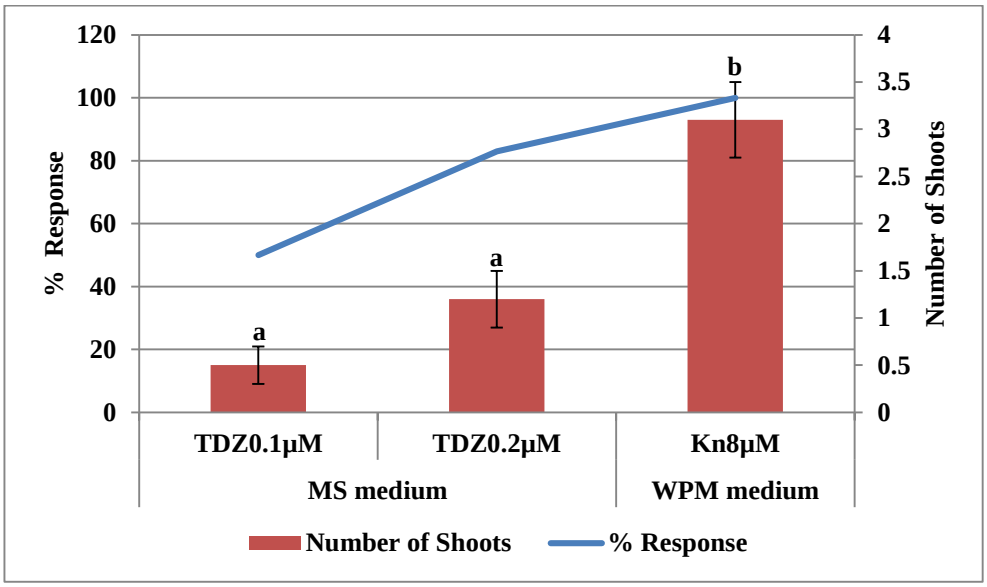

Fig. 61: Effect of individual cytokinins in inducing multiples from *in vitro* nodes of *S.suaveolens* after 12 weeks

Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

The MS medium fortified with TDZ (0.1, 0.2µM) and WPM medium fortified with Kn(8 µM) were able to enhance the number of shoots only upto 12 weeks. The WPM medium supplemented was considered as optimum concentration as it induced maximum number of multiple shoots.

As the cotyledonary node explants of *S.suaveolens* also possessed the regenerating ability to form shoots in individual cytokinin hence the same was assessed with combinations of two cytokinins and cytokinin with auxins.

4.4.2.1.5 Effect of two cytokinins on shoot induction from cotyledonary node explants

To evaluate the synergistic effect of cytokinins on shoot induction cotyledonary nodes were placed on medium fortified with different concentrations of two cytokinins in combinations ie. BAP and Kn, Kn and TDZ and BAP with TDZ.

❖ **Synergistic effect in MS medium**

- **BAP+Kn**

The first combination of two cytokinins tried was in MS medium fortified with BAP and Kn for cotyledonary node explants. Medium supplemented with BAP (8µM) and lower concentrations of Kn (2,4 and 8µM) it was observed that Kn at 4µM, 83 % cultures responded and 2.0 ± 1.2 number of shoots developed which was maximum among all the combinations tried (Fig.62a)(Table 23). Whereas when BAP (16µM) was combined with Kn (2, 4 and 8µM) there was formation of single shoot in all the concentrations. Increasing BAP to 20µM and combining with Kn (2, 4 and 8µM) resulted in a very less percent response and number of shoots (Table 23).

- **BAP+TDZ**

Another combination of cytokinins was fortified with BAP and TDZ. When the BAP (8, 16 and 20µM) was coupled with TDZ (0.1, 0.2, 0.25µM) concentrations it obtained a poor response in terms of shoot induction. Out of all the concentrations in BAP at 20µM with TDZ (0.25µM) induced a maximum of 83% response and formed 0.8 ± 0.2 shoots (Table 23).

- **Kn +TDZ**

The last synergistic combination tried was Kn with TDZ where an improved response was observed when Kn (2µM) was added with TDZ (0.1µM, 0.2µM and 0.25µM).It resulted in single shoot formation in 83% cultures with 2.0 ± 0.6 nodes at 0.1µM TDZ (Plate 4b). Kn at 4µM with different concentrations of TDZ (0.1, 0.2, 0.25µM) had synergistic effect as there was 100 % response in cultures with 1.0 ± 0.0 number of shoots. In this Kn at 4µM with TDZ at 0.2µM resulted in forming healthy shoots with many nodes (Fig.62c).Increase in Kn (8µM) with all TDZ concentrations resulted in less number of shoots and percent response (Table 23).

Therefore observations revealed that the explants could induce shoot proliferation but there was only slight variation in terms of increase in percent response and number of shoots as combinations of cytokinins failed to enhance the number after four weeks. The maximum number of shoots were induced in MS medium fortified with BAP(8µM) and Kn(4 µM) with 2.0 ± 1.2 number.

Table 23. Effect of two cyokinins on shoot induction from cotyledonary node explants of *S.suaveolens* in MS medium after 4 weeks

Cytokinins (µM)			MS medium		
BAP	**Kn**	**TDZ**	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
8	2	-	33	0.3 ± 0.2^{ab}	0.7 ± 0.5^{abcd}
8	**4**	-	**83**	$\mathbf{2.0\pm1.2^{c}}$	1.0 ± 1.3^{abcd}
8	8	-	33	0.3 ± 0.2^{ab}	0.8 ± 0.5^{abcd}
16	2	-	50	0.8 ± 0.5^{ab}	1.0 ± 0.6^{abcd}
16	4	-	66	0.7 ± 0.2^{ab}	0.7 ± 0.2^{abcd}
16	8	-	66	0.7 ± 0.2^{ab}	0.7 ± 0.2^{abcd}
20	2	-	33	0.3 ± 0.2^{ab}	0.5 ± 0.3^{abc}
20	4	-	17	0.2 ± 0.2^{ab}	0.7 ± 0.7^{abcd}
20	8	-	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}
8	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
8	-	0.2	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}
8	-	0.25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16	-	0.1	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
16	-	0.2	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
16	-	0.25	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}
20	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20	-	0.2	50	0.5 ± 0.2^{ab}	0.7 ± 0.3^{abcd}
20	-	0.25	83	0.8 ± 0.2^{ab}	1.3 ± 0.3^{abcd}
-	2	0.1	83	0.8 ± 0.2^{ab}	2.0 ± 0.6^{d}
-	2	0.2	66	0.7 ± 0.2^{ab}	0.7 ± 0.2^{abcd}
-	2	0.25	66	0.7 ± 0.2^{ab}	0.7 ± 0.2^{abcd}
-	4	0.1	100	1.0 ± 0.0^{b}	1.8 ± 0.4^{cd}
-	**4**	**0.2**	**100**	1.0 ± 0.0^{b}	$\mathbf{2.0\pm0.5^{d}}$
-	4	0.25	100	1.0 ± 0.0^{b}	1.7 ± 0.3^{cd}
-	8	0.1	17	0.2 ± 0.2^{ab}	0.3 ± 0.3^{ab}
-	8	0.2	83	0.8 ± 0.2^{ab}	1.5 ± 0.4^{bcd}
-	8	0.25	33	0.3 ± 0.2^{ab}	1.5 ± 1.1^{bcd}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test.

Fig. 62: Shoot formation from cotyledonary node explants of *S. suaveolens* in presence of two cytokinins after 4 weeks

a. Development of multiple shoots in MS+BAP (8µM)+ Kn(4µM)
b. Single shoot formation in MS+ Kn(2µM) +TDZ(0.1 µM)
c. Healthy shoot in MS +Kn(4µM) +TDZ(0.2µM)
d. Single shoot with many nodes in WPM+BAP (4µM) +TDZ (0.2µM)
e. Formation of healthy shoot in WPM +Kn(2µM)+TDZ(0.2 µM)
f. Single shoot in WPM+ Kn(4µM) +TDZ(0.25µM)

❖ **Synergistic effect in WPM medium**

The cotyledonary node explants were placed on WPM medium fortified with combinations of cytokinins by taking lower concentrations of BAP as they were effective individually and therefore combined with Kn and TDZ.

- **BAP+Kn**

When BAP 2 µM was added with Kn (2µM, 4µM, 8µM) concentrations it resulted in 100 % response with Kn(2µM) and (4µM) with an average of 1.0 ± 0.0 shoot number. While with Kn (8µM) the axillary bud proliferated but failed to form shoots. A combined effect resulted when BAP at 4µM was incorporated with different concentrations of Kn, which at Kn 2µM the response reached to 83% response forming single shoot with many nodes (2.7 ± 0.6).Increasing BAP to 8µM when combined with (2 and 4µM) of Kn failed to enhance the morphogenic response but at 8µM 67% cultures formed single shoot with many nodes (2.2 ± 1.1)(Table 24).

- **BAP+TDZ**

Adding BAP and TDZ together improved the response in cultures in almost all the combinations. A maximum of 100% response was observed when BAP was at 2µM with all TDZ concentrations(Table 24) The combinations of BAP(4µM) and (8µM) with all TDZ concentrations obtained less percent response with single shoot formation. But out of all the BAP and TDZ combinations maximum average number of nodes (3.8 ± 2.6) were obtained in BAP(4µM) with TDZ(0.2µM) (Fig.62d) .

- **Kn+TDZ**

Kn and TDZ combinations also proved better in terms of percent response in WPM medium and resulted in forming healthy single shoots with many nodes. A 100% response was obtained in Kn (2µM) with TDZ(0.2µM) with 1.0±0.0 number of shoots and 2.8 ± 0.5 nodes(Fig.62e) and Kn(4µM) with TDZ(0.25µM) also resulted in similar response(100%) with 1.0 ± 0.0 shoot number(Fig.62f) and 2.8 ± 0.4 number of nodes. Comparatively the combinations of Kn (8µM) with TDZ (0.1, 0.2, 0.25µM) resulted in a poor response(Table 24).

Table 24. Effect of two cyokinins on shoot induction from cotyledonary node explants of *S.suaveolens* in WPM Medium after 4 weeks

Cytokinins (µM)			WPM medium		
BAP	**Kn**	**TDZ**	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
2	2	-	100	1.0 ± 0.0^{d}	1.0 ± 0.0^{abc}
2	4	-	100	1.0 ± 0.0^{d}	1.0 ± 0.0^{abc}
2	8	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
4	2	-	83	0.8 ± 0.2^{cd}	2.7 ± 0.6^{bcd}
4	4	-	67	0.7 ± 0.2^{bcd}	2.0 ± 0.6^{abcd}
4	8	-	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
8	2	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
8	4	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
8	8	-	67	0.7 ± 0.2^{bcd}	2.2 ± 1.1^{abcd}
2	-	0.1	100	1.0 ± 0.0^{d}	1.8 ± 0.8^{abcd}
2	-	0.2	100	1.0 ± 0.0^{d}	1.0 ± 0.0^{abc}
2	-	0.25	100	1.0 ± 0.0^{d}	1.8 ± 0.5^{abcd}
4	-	0.1	67	0.7 ± 0.2^{bcd}	2.0 ± 1.0^{abcd}
4	-	0.2	33	0.3 ± 0.2^{ab}	3.8 ± 2.6^{d}
4	-	0.25	50	0.5 ± 0.2^{bc}	2.7 ± 1.4^{bcd}
8	-	0.1	50	0.5 ± 0.2^{bc}	1.5 ± 1.1^{abcd}
8	-	0.2	50	0.5 ± 0.2^{bc}	0.5 ± 0.2^{abc}
8	-	0.25	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
-	2	0.1	83	0.8 ± 0.2^{cd}	2.3 ± 0.8^{abcd}
-	2	0.2	100	1.0 ± 0.0^{d}	2.8 ± 0.5^{cd}
-	2	0.25	67	0.7 ± 0.2^{bcd}	2.0 ± 0.8^{abcd}
-	4	0.1	83	0.8 ± 0.2^{cd}	2.2 ± 0.7^{abcd}
-	4	0.2	83	0.8 ± 0.2^{cd}	2.8 ± 0.6^{cd}
-	**4**	**0.25**	**100**	**1.0 ± 0.0^{d}**	**2.8 ± 0.4^{cd}**
-	8	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	0.2	83	0.8 ± 0.2^{cd}	2.7 ± 0.8^{bcd}
-	8	0.25	50	0.5 ± 0.2^{bc}	1.5 ± 0.7^{abcd}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test.

From the above experiment with cotyledonary node explants, it was able to form maximum of 2.0 ± 1.2 average number of shoots in MS medium fortified with BAP (8µM) and Kn (4µM) amongst all the combinations tried. While in WPM medium fortified with BAP (4µM) and

TDZ (0.2µM) resulted in highest number of nodes (3.8 ± 2.6). Thus the synergistic combination of cytokinin induced single shoot in various combinations. The microshoots were excised into single nodes for multiplying the shoots.

4.4.2.1.5.1 Multiplication of shoots from *in vitro* nodes in presence of two cytokinins

The *in vitro* nodes were subcultured in MS and WPM medium combinations in which response was obtained after 4 weeks. But out of all the combinations only in MS medium fortified BAP(8µM) with Kn(2µM), BAP(8µM) with Kn(4µM) ,BAP(20µM) with Kn(4, 8µM) response was observed after 8 weeks. Whereas in WPM medium all the respective combinations which were subcultured failed to enhance the number as they dried up after 10 weeks except in combinations BAP(20µM) with Kn(2µM,4µM), BAP(2µM) with TDZ(0.2µM) response was observed.

In MS medium fortified with BAP (8µM) with Kn (2µM, 4µM) after 8 weeks failed to enhance the shoot number. Whereas in BAP (20µM) with Kn(4µM) resulted in forming1.2 ± 0.4 shoots with 67% response after 8 weeks (Fig.63) and in BAP(20µM) with Kn(8µM) formed single shoots which were accompanied with basal callus(Fig.63).

In WPM medium fortified with BAP(20µM) with Kn (2µM,4µM) combinations single shoots were developed with an average of 0.7 ± 0.5 and 1.0 ± 0.5 shoots in only 33% and 50% cultures respectively and slight callus was also formed. Whereas in BAP (2µM) with TDZ (0.2µM) more than two shoots were formed with an average of 2.0 ± 0.6 in 100% cultures (Fig.63).

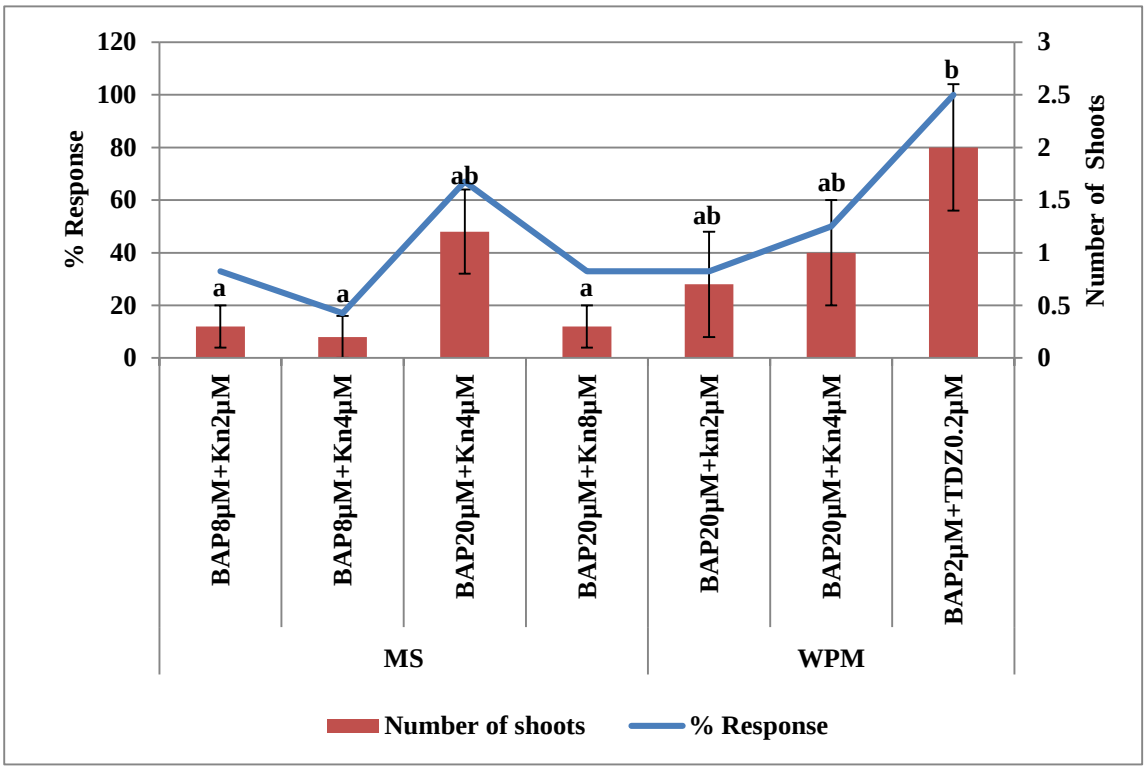

Fig. 63: Effect of two cytokinins from *in vitro* nodes of *S.suaveolens* in inducing multiples after 8 weeks

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

On further transfer all the above combinations failed to respond except in MS medium fortified with BAP (8µM) and Kn(4µM) response improved slightly after 12 weeks with an average of 1.0 ± 0.6 shoots in 50% cultures.

As there was no appreciable number of shoots obtained in presence of cytokinins, the cotyledonary node explants were placed in presence of cytokinins and auxins.

4.4.2.1.6 Effect of cytokinins and auxins on shoot induction from cotyledonary node explants

When cotyledonary node explants were placed on **MS medium** fortified with cytokinin and auxins, in many combinations the axillary buds remain dormant and failed to produce shoots. BAP (20µM), Kn (8µM) when combined with IAA/NAA (0.1, 0.5, 1 µM) it resulted in poor response in terms of shoot formation as they were weak and unhealthy. Whereas the response slightly improved when TDZ was combined with auxins. Combination of TDZ (0.2µM) with different concentrations of IAA, it induced healthy single shoots with maximum number of

nodes among all the cytokinin and auxin combinations. A maximum of 67% response was obtained in presence of TDZ at 0.2µM with IAA at 0.1 µM and single shoots had an average of 2.0 ± 1.0 nodes (Fig.64a)whereas TDZ (0.2µM) with IAA (0.5µM) induced highest number of nodes (2.7 ± 1.4) (Fig.64b). When IAA was replaced with NAA a highest response (83%) was obtained in TDZ (0.2µM) and NAA (0.1µM) (Fig.64c) out of all the combinations tried with an average of 0.8 ± 0.2 number of shoots (Table 25).

Thus in MS medium fortified with BAP or Kn with auxins resulted in poor response and maximum shoot induction (83%) was observed in TDZ (0.2µM) with NAA (0.1µM).In **WPM medium** when BAP (8µM) was combined with different concentrations of IAA and NAA, in all the combinations single shoot developed but varied in percent response and number of nodes.

Table 25. Effect of cyokinins and auxins on shoot induction from cotyledonary node explants of *S.suaveolens* in MS medium after 4 weeks

Cytokinins (µM)			Auxins (µM)		% Response	Number of shoots*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**	**IAA**	**NAA**			
20	-	-	0.1	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
20	-	-	0.5	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
20	-	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20	-	-	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20	-	-	-	0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20	-	-	-	1	17	0.2 ± 0.2^{ab}	0.5 ± 0.5^{a}
-	8	-	0.1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	0.5	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
-	8	-	1	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	8	-	-	0.1	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
-	8	-	-	0.5	33	0.3 ± 0.2^{abc}	0.3 ± 0.2^{a}
-	8	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.2	0.1	-	67	0.7 ± 0.2^{cd}	2.0 ± 1.0^{a}
-	-	**0.2**	**0.5**	-	50	0.5 ± 0.2^{bcd}	$\mathbf{2.7\pm1.4^{b}}$
-	-	0.2	1	-	17	0.2 ± 0.2^{ab}	1.0 ± 0.9^{a}
-	-	**0.2**	**-**	**0.1**	**83**	$\mathbf{0.8\pm0.2^{d}}$	0.8 ± 0.2^{a}
-	-	0.2	-	0.5	50	0.5 ± 0.2^{bcd}	0.7 ± 0.3^{a}
-	-	0.2	-	1	33	0.3 ± 0.2^{abc}	0.3 ± 0.2^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test.

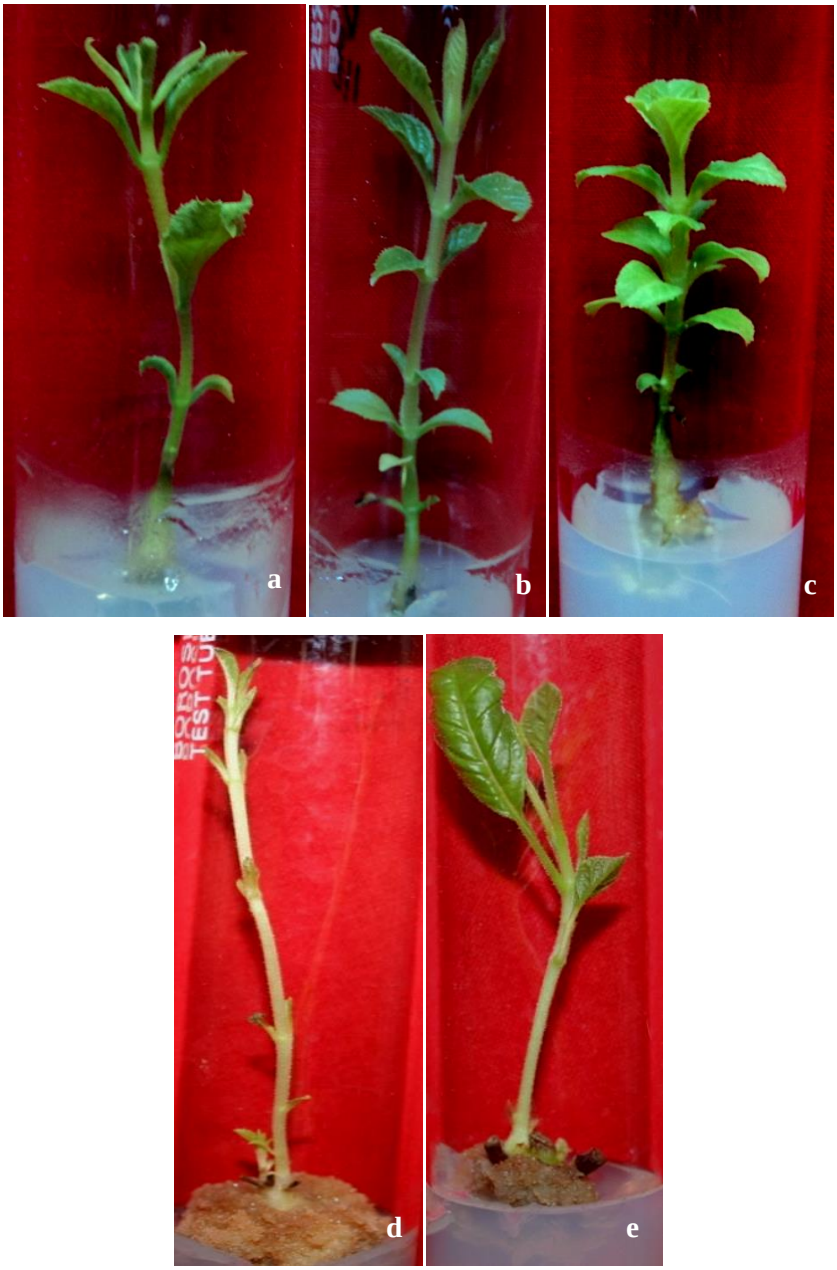

Fig.64: Shoot induction from cotyledonary node explants of *S.suaveolens* in presence of cytokinins and auxins after 4 weeks
a. Single shoot developed in MS+TDZ(0.2µM)+IAA (0.1µM)
b. Healthy shoot formed in MS+TDZ(0.2µM) +IAA(0.5µM)
c. Development of healthy shoot with many nodes in MS+ TDZ (0.2µM) +NAA (0.1µM)
d. Elongated shoot with minute leaves in WPM +Kn (2µM) +1AA (0.1µM)
e. Single shoot formed in WPM +Kn(2µM) +1AA(1µM)

There were 67% cultures resulted in BAP (8µM) with IAA (0.5 µM) forming single shoot and an average of 1.2 ± 0.7 nodes whereas in BAP (8 µM) with NAA (0.1µM) resulted in single shoot formation only in 33% cultures.

When Kn (2µM) was combined with IAA and NAA it proved to be effective in terms of percent response compared to BAP. Combination of Kn (2µM) and IAA (0.1µM) resulted in 100 % response with 1.0±0.0 number of shoots having 2.5 ± 0.7 number of nodes (Fig.64d). When concentration of IAA was increased to 1 µM single healthy shoot with 2.2 ± 1.2 nodes were formed (Fig.64e). Combinations of Kn (2µM) with NAA resulted in less percent response with single formation. The TDZ combinations with auxins in WPM medium were not much effective in terms of percent response and shoot number. Out of various combinations of TDZ with IAA and NAA, TDZ (0.1µM) and IAA (0.1µM) resulted in maximum of 67% response (Table 26).

Table 26. Effect of cyokinins and auxins on shoot induction from cotyledonary node explants of *S.suaveolens* in WPM medium after 4 weeks

Cytokinins (µM)			**Auxins (µM)**		**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
BAP	**Kn**	**TDZ**	**IAA**	**NAA**			
8	-	-	0.1	-	50	0.5 ± 0.2^{ab}	$0.5\pm0.c^{abc}$
8	-	-	0.5	-	50	0.5 ± 0.2^{ab}	1.2 ± 0.7^{abc}
8	-	-	1	-	67	0.7 ± 0.2^{ab}	0.7 ± 0.2^{abc}
8	-	-	-	0.1	33	0.3 ± 0.2^{ab}	1.3 ± 1.1^{abc}
8	-	-	-	0.5	67	0.7 ± 0.2^{ab}	0.7 ± 0.2^{abc}
8	-	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	**2**	-	**0.1**	-	**100**	**1.0 ± 0.0^{b}**	**2.5 ± 0.7^{c}**
-	2	-	0.5	-	33	0.3 ± 0.2^{ab}	1.7 ± 1.1^{abc}
-	2	-	1	-	50	0.5 ± 0.2^{ab}	2.2 ± 1.2^{bc}
-	2	-	-	0.1	33	0.3 ± 0.2^{ab}	1.0 ± 0.6^{abc}
-	2	-	-	0.5	33	0.3 ± 0.2^{ab}	1.0 ± 0.8^{abc}
-	2	-	-	1	67	0.7 ± 0.2^{ab}	0.8 ± 0.3^{abc}
-	-	0.1	0.1	-	67	0.7 ± 0.2^{ab}	2.5 ± 1.1^{c}
-	-	0.1	0.5	-	50	0.5 ± 0.2^{ab}	0.8 ± 0.4^{abc}
-	-	0.1	1	-	17	0.2 ± 0.2^{ab}	0.3 ± 0.3^{ab}
-	-	0.1	-	0.1	50	0.5 ± 0.2^{ab}	0.5 ± 0.2^{abc}
-	-	0.1	-	0.5	50	0.5 ± 0.2^{ab}	0.7 ± 0.3^{abc}
-	-	0.1	-	1	33	0.8 ± 0.7^{ab}	0.5 ± 0.3^{abc}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test.

Therefore compared to MS in WPM medium the combinations of BAP, Kn and TDZ with different concentrations of IAA and NAA improved the response with maximum of 100 % response in Kn (2µM) and IAA (0.1µM).

4.4.2.1.6.1 Multiplication of shoots from *in vitro* nodes in presence of cytokinins and auxins

By the end of 4 weeks as only single shoots were formed in respective cytokinin and auxin combinations these shoots were excised into single nodes on the respective media for multiplication of shoots.

It was observed that the cytokinin and auxin combinations failed to enhance number of shoots. As after 6 weeks the axillary buds proliferated into single shoot and later on by the end of 8 weeks failed to survive.

Hence in *S.suaveolens* the fourth explant used which was taken up for establishment and multiplication of shoots was nodal explant.

The regenerative ability of nodal explants was assessed in presence of individual cytokinin as follows:

4.4.2.1.7 Effect of individual cytokinins on shoot induction in MS and WPM medium from nodal explants

The nodal explants (1.5cm) were also placed in **MS medium** without or with PGRs. Observation after 4 weeks revealed that the explants failed to respond in MS basal medium. When the explants were inoculated in MS medium fortified with individual cytokinins, the media supplemented with BAP resulted in poor response in terms of % shoot formation. At 2µM only 50% of cultures were formed with single shoot which were weak. As the concentration increases the percent response decreased and became nil at 30 µM. When medium was fortified with different concentrations of Kn, there was an improvement in terms of percent response. Lower concentrations of Kn (2µM, 4µM and 8 µM) were effective compared to higher concentrations. At 2µM a maximum of 0.8 ± 0.2 number of shoots and 1.7±0.6 number of nodes were induced in 83% cultures (Table 27) (Fig.65a). Incorporating TDZ in the medium the nodal explant showed a varied morphogenic response. At lower concentrations (0.1-0.2µM) the percent response was similar (50%) but 0.2µM obtained 1.5 ± 0.8 number of nodes(Table 27) (Fig.65b).But a maximum of 83% cultures with 0.8 ± 0.2

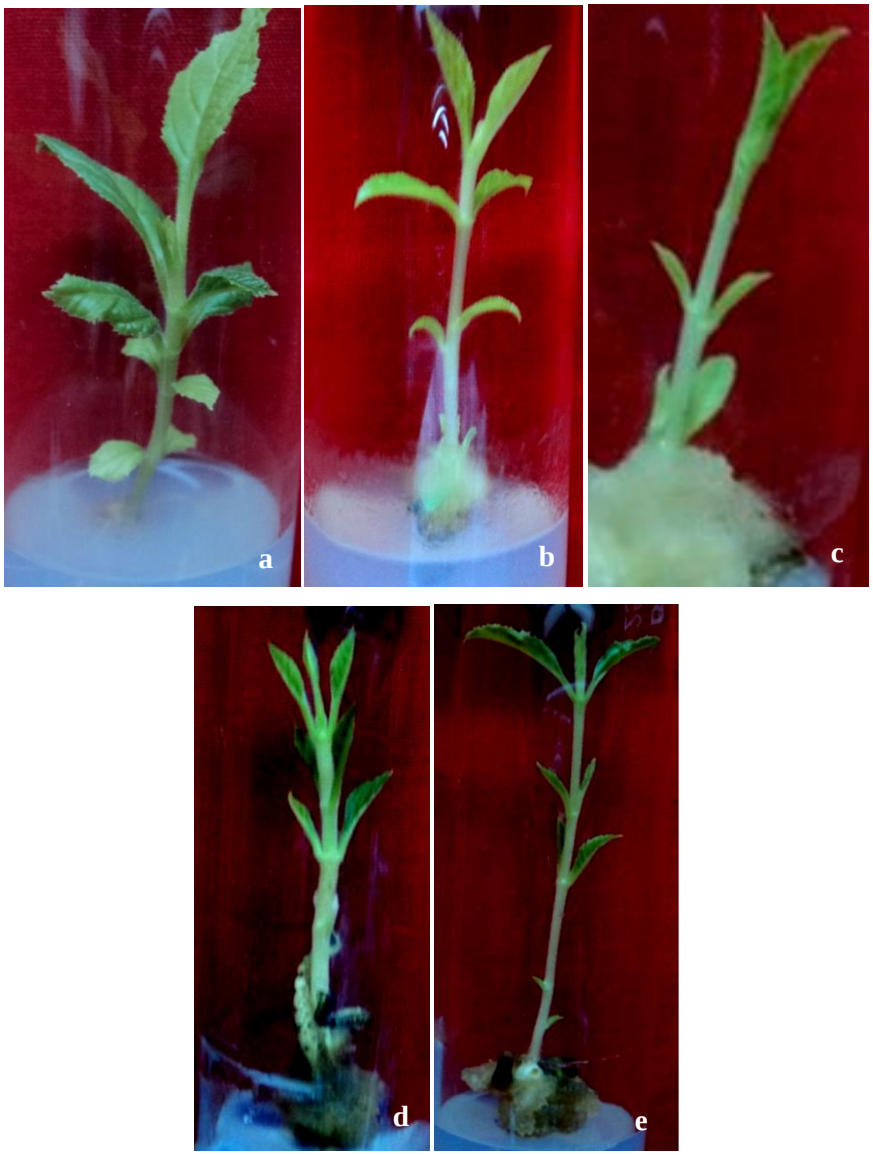

Fig.65: Shoot induction from nodal explants of *S.suaveolens* in presence of individual cytokinin after 4 weeks
a. Single healthy shoot formed in MS+ Kn(2 µM)
b. Shoot developed in MS+TDZ (0.2µM)
c. Shoot formation with friable basal callus in MS+TDZ (1µM)
d. Formation of single shoot in WPM+TDZ (0.1 µM)
e. Long shoot developed with many nodes in WPM+ TDZ (0.2µM)

number of shoots and 2.3 ± 0.8 number of nodes were formed at 1µM but the shoots were associated with basal callusing (Fig. 65c).

Nodal explants when placed in **WPM** basal **medium** only 20% cultures responded. But when medium was fortified with cytokinins, there was an improved response in all the BAP concentrations compared to MS medium. A maximum of 80% response wasobtained at 8µM with 1.4 ± 0.5 number of shoot. While at 16 µM the single shoot was formed with an average of 1.8 ± 0.6 number of nodes (Table 27). At higher concentrations (20 µM - 30 µM) percent response decreases and became nil. When Kn was added in the WPM medium there was an increase and decrease in percent response observed. The percent response decreased upto 8µM then, again it increased at 20µM and become nil at 25µM and 30µM.

Table 27. Effect of individual cyokinins on shoot induction from nodal explants of *S.suaveolens* after 4 weeks

Cytokinins (µM)			MS medium			WPM medium		
BAP	**Kn**	**TDZ**	**% Response**	**Number of shoots***	**Number of *in vitro* nodes**	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
0	0	0	0	0.0± 0.0^{a}	0.0±0.0 ^{a}	20	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}
2	-	-	50	0.5± 0.2^{b}	0.7±0.3^{abc}	80	1.2 ± 0.4^{a}	1.7 ± 0.5^{a}
4	-	-	33	0.3 ± 0.2^{ab}	0.5±0.3^{abc}	40	0.6 ± 0.4^{a}	1.1 ±0.7^{a}
8	-	-	16	0.2 ± 0.2^{ab}	0.2±0.2^{ab}	80	1.4 ± 0.5^{a}	1.5 ± 0.6^{a}
16	-	-	0	0.0± 0.0^{a}	0.0±0.0 ^{a}	75	1.0 ± 0.4^{a}	1.8± 0.6^{a}
20	-	-	16	0.2 ± 0.2^{ab}	0.5±0.5^{abc}	50	0.5 ± 0.3^{a}	1.5 ± 0.9^{a}
25	-	-	0	0.0 ± 0.0^{a}	0.0±0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
30			0	0.0 ± 0.0^{a}	0.0±0.0^{a}	0	0.0 ± 0.0^{a}	0.0±0.0 ^{a}
-	2	-	83	0.8 ± 0.2^{c}	1.7±0.6^{cd}	80	0.8 ± 0.2^{a}	2.8 ± 0.8^{b}
-	4	-	50	0.5 ± 0.2^{bc}	1.0±0.5^{abc}	60	0.6 ± 0.2^{a}	1.8 ± 0.7^{b}
-	8	-	67	0.7 ± 0.2^{c}	1.5±0.6^{bcd}	50	0.5± 0.1^{a}	0.5 ± 0.2^{a}
-	16	-	0	0.0 ± 0.0^{a}	0.0±0.0^{a}	66	0.7 ± 0.3^{a}	1.7± 0.9^{ab}
-	20	-	16	0.2 ± 0.2^{ab}	0.2±0.2^{ab}	75	0.8 ± 0.3^{a}	1.8 ± 0.8^{ab}
-	25	-	0	0.0 ± 0.0^{ab}	0.0±0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30		0	0.0 ± 0.0^{a}	0.0±0.0^{a}	0	0.0 ± 0.0^{a}	0.0±0.0 ^{a}
-	-	0.1	50	0.5 ± 0.2^{ab}	1.3±0.7^{abcd}	80	1.0 ± 0.3^{b}	2.3 ± 0.7^{b}
-	-	0.2	50	0.5 ± 0.2^{abc}	1.5±0.8^{bcd}	**100**	**1.8 ± 0.8^{c}**	**3.6 ± 0.7^{c}**
-	-	0.25	16	0.2 ± 0.2^{a}	0.2±0.2^{ab}	40	0.4 ± 0.2^{a}	1.8 ± 1.2^{b}
-	-	0.5	50	0.5±0.2^{abc}	1.2±0.6^{abcd}	10	0.1±0.1^{a}	0.1± 0.1^{a}
-	-	**1**	**83**	**0.8 ± 0.2 ^{c}**	**2.3±0.8^{d}**	10	0.1± 0.1^{a}	0.1± 0.1^{a}
-	-	2	66	0.7 ± 0.2^{bc}	1.2±0.5^{abcd}	20	0.2 ± 0.1^{a}	0.3± 0.2^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

In all concentrations the number of shoots formed were single and long with many nodes, but the maximum node number (2.8 ± 0.8) was obtained at Kn 2µM. When medium was fortified with TDZ it proved to be effective at lower concentrations whereas the higher concentrations resulted in formation of weak shoots. At 0.1 µM, 83 % cultures were forming single shoots with 2.3±0.7 nodes (Fig.65d) (Table 27). Whereas at 0.2µM, highest (100 %) response with maximum of 1.8 ± 0.8 number of shoots and 3.6 ± 0.7 nodes were obtained(Fig.65e). But all the TDZ concentrations were associated with callus at the base.

Hence the from the above experiment it was concluded that the nodal explants had the regeneration capacity for forming healthy shoots in presence of the three individual cytokinins and among all the cytokinins TDZ was effective in giving highest percent response. As in all the respective combinations single shoot were formed they were subcultured in the same medium by excising single nodes in order to develop multiple shoots.

4.4.2.1.7.1 Multiplication from *in vitro* nodes in presence of individual cytokinins

The *in vitro* shoots developed from nodal explants (those which obtained response after 4weeks) were cut into single nodes and subcultured in respective medium fortified with PGRs. The MS medium fortified with BAP(20µM) , Kn (2µM, 4µM, 8µM),TDZ(0.1µM, 0.2µM, 0.5µM and 1µM) and in WPM medium supplemented with BAP(8µM), Kn(2µM ,4µM,8µM and 20µM) were the concentrations in which the explants respond after 8 weeks.
In the MS medium fortified with BAP (20µM), Kn (2µM, 4µM, 8µM), TDZ (0.5, 1µM) induced single shoot after 8 weeks. Whereas at TDZ (0.1µM) and TDZ (0.2µM) more than one shoots developed with an average of 1.0 ± 0.4 in 67 % cultures and 1.0 ± 0.3 shoots in 83% cultures (Fig.66).In both concentrations the shoots formed were long and healthy (Fig.67 a and b).

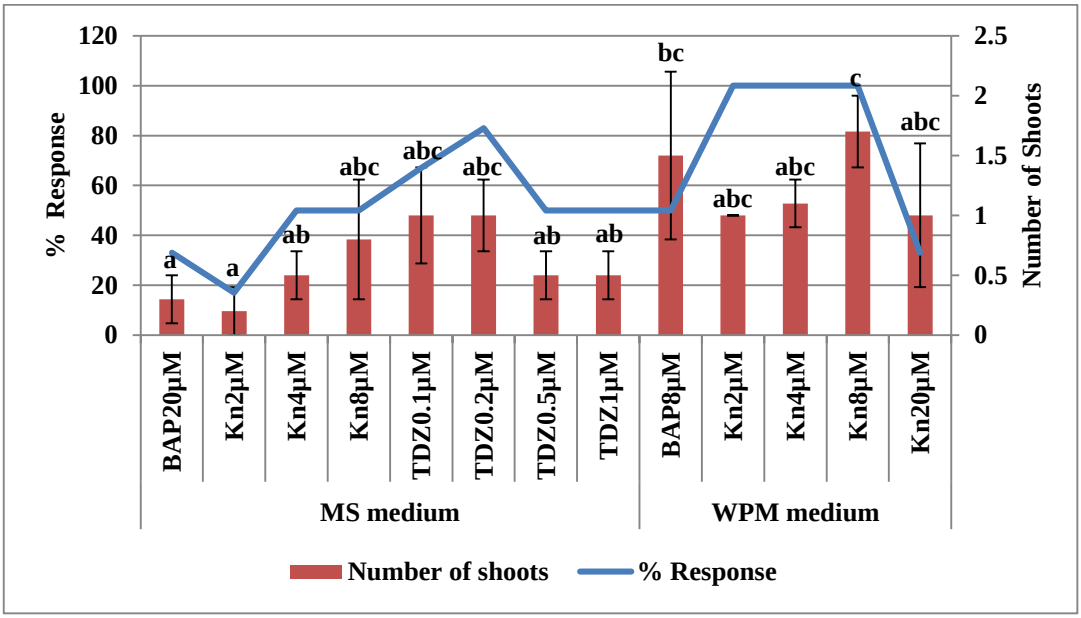

Fig.66: Effect of individual cytokinins in inducing multiples from *in vitro* nodes of *S.suaveolens* after 8 weeks

Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

In WPM medium fortified with BAP(8µM) resulted in 50% response only whereas a 100% response was observed at Kn (2µM, 4µM and 8µM) where the single shoot developed were long and healthy with large leaves at 2µM, 4µM (Fig.67c and d) while at 8µM the number reached to 1.7±0.5 (Fig.66;Fig.67e).

A least response (33%) was observed in WPM medium fortified with Kn (20µM) by the end of 8 weeks (Fig.66).

All the above combinations when subcultured into same medium, only in MS medium fortified with TDZ at 0.1µM, and 0.2µM and in WPM medium fortified with Kn 4µM and Kn 8µM shoots were formed after 12 weeks. In MS medium fortified with TDZ (0.1µM) 80% response was obtained and the shoots formed were healthy and long which failed to enhance in number and obtained increase in terms of length only (Fig.67f).When medium was fortified with TDZ (0.2µM) 100% shoots were formed with an average of 1.9 ± 0.3 in number (Fig.67g;Fig.68).

Fig.67: Effect of individual cytokinins on inducing multiples from *in vitro* nodes of *S.suaveolens*

a. Development of one or two healthy elongated shoots in MS+TDZ(0.1µM) after 8 weeks

b. Long shoots developed in MS+TDZ(0.2µM) after 8 weeks

c. Single shoot with larged healthy leaves in WPM+Kn(2µM) after 8 weeks

d. Formation of single shoot in WPM +Kn(4µM) after 8 weeks

e. Small multiples devloped in WPM+Kn(8µM) after 8 weeks

f. Single healthy shoot formed in MS+TDZ (0.1µM) after 12 weeks

g. Shoot formation in MS+TDZ (0.2µM) after 12 weeks

h. Healthy multiples developed in WPM+Kn(8µM) after 12 weeks

In WPM medium fortified with individual cytokinins, Kn at 4µM resulted in forming one or two shoots per node with an average of 1.7 ± 0.2 number in 80 % cultures. Whereas Kn at

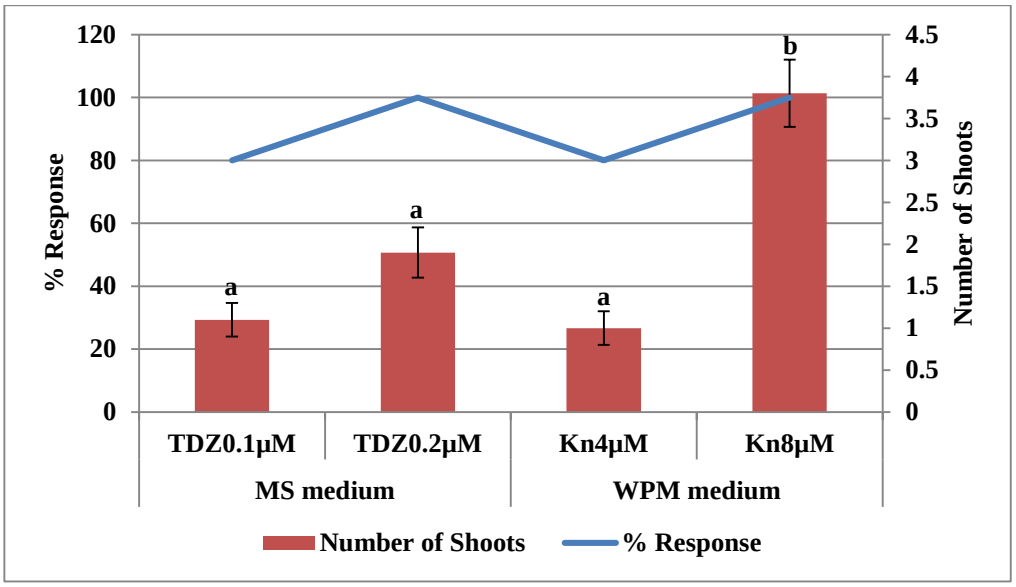

Fig.68: Effect of individual cytokinins in inducing multiples from *in vitro* nodes of *S.suaveolens* after 12 weeks

Values represent mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

8µM induced maximum number of shoots (3.8 ± 0.6) in 100% cultures after 12 weeks (Fig.67h; Fig.68). The shoots obtained from this explants were healthy with well developed leaves as compared to cotyledonary node.

The *in vitro* nodes of shoots which developed in Kn(4µM) failed to proliferate after 18 weeks whereas in MS medium fortified with TDZ(0.1µM), (0.2µM) and WPM medium fortified with Kn (8µM) concentrations shoots were developed at subsequent passages with 100% response, hence they were compared in terms of shoot number and length.

At TDZ (0.1µM) there was enhancement at every passage in terms of shoot number but forming only single or two shoots per node but there was marked increase in terms of length at every passage reaching 6.9 ± 0.6cms by the end of 32 weeks (Fig.69) (Fig.70a-c).

At TDZ (0.2µM) concentration the number of shoots forming per node at each passage were more compared to TDZ (0.1µM) with an average of 2.6±0.4 by the end of 24 weeks which decreased by the end of 32 weeks (Fig.70d-f).The shoots formed were healthy and long with an average of 5.7 ± 0.8 cms by the end of 32 weeks which was less compared to 0.1µM (Fig.69).

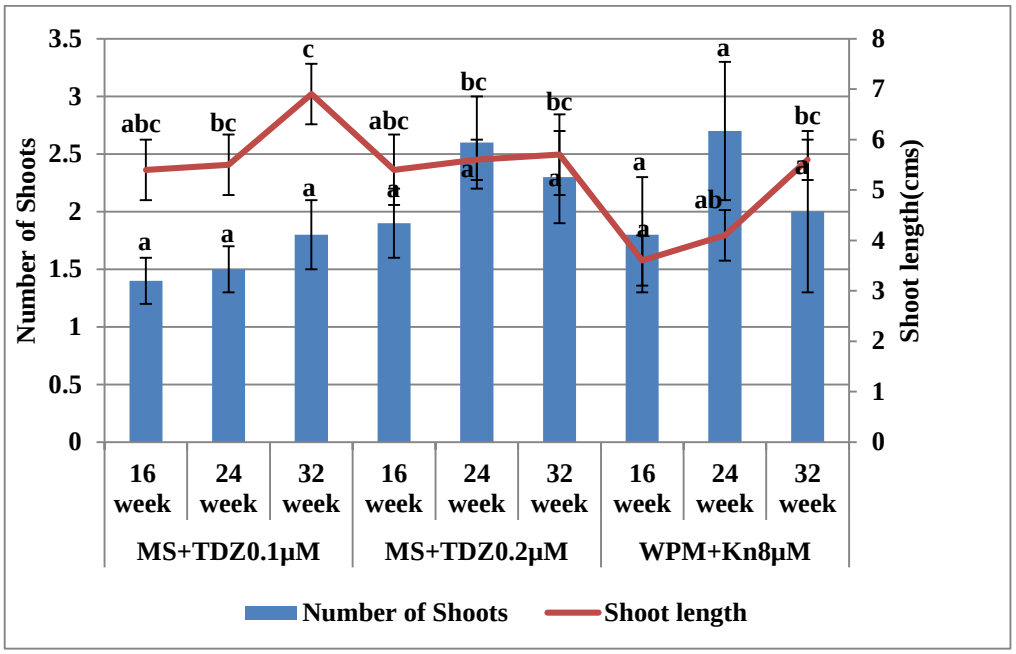

Fig. 69: Effect of individual cytokinins from *in vitro* nodes of *S. suaveolens* on shoot number and length after 16,24 and 32 weeks

Values represent mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

In WPM medium fortified with Kn (8µM) there was increase in number of shoots till 24 weeks with an average of 2.7 ± 0.6 shoots(Fig.69) which was slightly near to MS+TDZ(0.2µM) concentration. The shoot formed in this concentration were healthy but short in length (Fig.70 g-i) compared to MS+TDZ (0.1µM, 0.2µM).

Therefore all the concentrations resulted in forming an average of only two to three shoots per node and failed to form multiples and there was marked increase only in terms of length forming healthy and long shoots.

Fig.70: Effect of MS + TDZ(0.1µM),MS+TDZ(0.2µM) and WPM+Kn(8µM) on shoot number and shoot length of *S.suaveolens* in different passages

a-c MS+TDZ(0.1µM) a.16 weeks b.24 weeks c.32 weeks

d-f MS+ TDZ(0.2µM) d.16 weeks e.24 week f.32 weeks

g-i WPM+ Kn(8µM) f. 16 weeks g.24 weeks h.32 weeks

As an appreciable number were not formed in the individual cytokinin ie. MS medium with TDZ (0.1µM), TDZ (0.2µM) and WPM medium with Kn(8µM) different additives like $AgNO_3$(20mg/l), PVP(100mg/l), Calcium pantothenate (0.5mg/l) and Casein hydrolysate (1g/l) were added. After 32 weeks it was observed that there was a slight increase in number of shoot in MS medium supplemented with TDZ(0.2µM) and $AgNO_3$(20mg/l)as well as TDZ(0.2µM) and calcium pantothenate (0.5mg/l) compared to individual TDZ(0.2µM) concentration. Out of all the combinations MS+TDZ(0.2µM) +$AgNO_3$(20mg/l) resulted in maximum number(3.0 ± 1.0)(Fig.71;Fig.72a) whereas MS+TDZ(0.2µM)+calcium

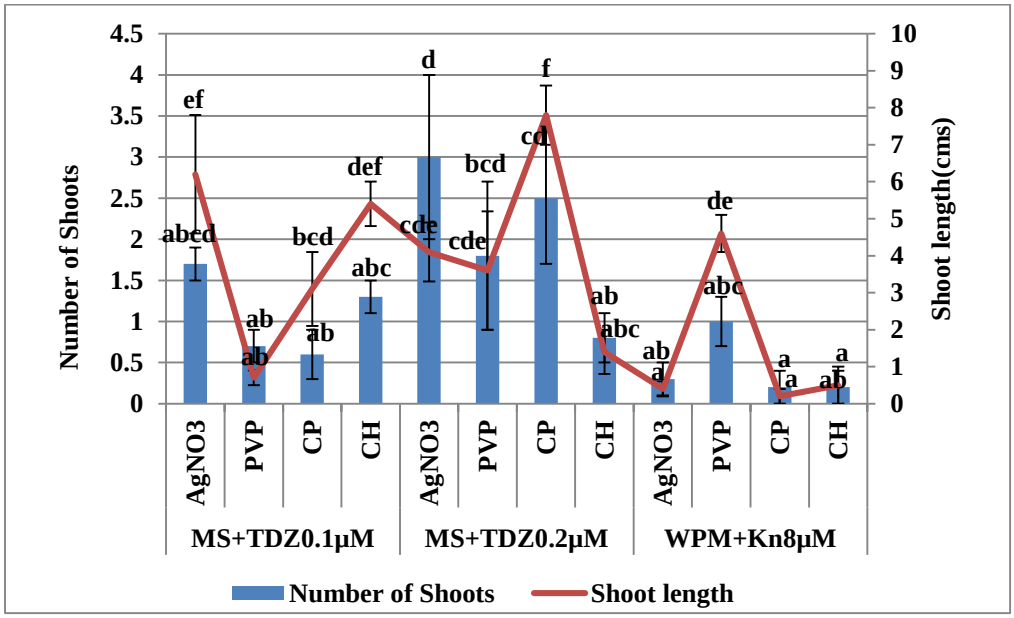

Fig.71: Effect of additives on shoot number and length of *S.suaveolens* after 32 weeks

$AgNO_3$-Silver nitrate ; PVP- Polyvinylpyrolidone; CP-Calcium pantothenate ;CH-Casien hydrolysate

Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

pantothenate (0.5mg/l) resulted in maximum shoot length (7.8 ± 0.8cms)(Fig.71;Fig.72b).The MS+TDZ(0.2µM)+PVP(100mg/l) (Fig.71;Fig.72c) resulted in healthy single shoot formation while adding casein hydrolysate obtained poor response .In MS medium fortified with TDZ(0.1µM) and all the additives the TDZ(0.1µM) and $AgNO_3$(20mg/l) resulted in highest shoot length but failed to enhance the number (Fig.72d)

Fig.72: Development of healthy shoots of *S.suaveolens* in MS and WPM medium supplemented with additives

a. **Healthy multiple shoots formed in MS+TDZ(0.2µM)+ $AgNO_3$(20mg/l)**
b. **Elongated shoots developed in MS+TDZ(0.2µM)+Calcium pantothenate (0.5mg/l)**
c. **Development of single shoot in MS+TDZ(0.2µM)+PVP (100mg/l)**
d. **Shoots formed in MS+TDZ(0.1 µM) +$AgNO_3$ (20mg/l)**
e. **Healthy single shoot formed in WPM+ Kn(8µM)+PVP (100mg/l)**

and in WPM medium supplemented with Kn(8µM) and PVP(100mg/l) resulted in highest shoot length(Fig.72e) while the other additive failed to increase the number of shoots.

The nodal explants produced healthy shoots (2-3 per node) in presence of individual cytokinins compared to cotyledonary node explants. Hence in order to enhance the number the nodal explants were placed in combinations of cytokinins.

4.4.2.1.8 Effect of two cytokinins on shoot induction from nodal explants

To evaluate the synergistic effect of cytokinins on shoot induction from nodal explants they were placed on medium fortified with different concentrations of two cytokinins ie. BAP and Kn, Kn and TDZ and BAP with TDZ. Observations revealed that the explants could induce shoot proliferation but there was only slight variation in terms of percent response and number of shoots as combinations of cytokinins failed to enhance the number after four weeks.

❖ **Synergistic effect in MS medium**

- **BAP+Kn**

Compared to cotyledonary node explants the BAP/Kn combination was effective in terms of shoot formation and number of shoots in nodal explants. In MS medium when the BAP and Kn combinations were at same concentrations ie.2µM, 4µM and BAP(4µM) with Kn(8µM) ,BAP(8µM) with Kn(2µM) 100% shoot formation was observed. Maximum number of 1.5 ± 0.3 shoots were formed in BAP (4µM) with Kn (4µM) and with an average number of 2.8 ± 0.5 nodes (Table 28) (Fig.73a) followed by BAP(4µM) with Kn(8µM) (Fig.73b) and BAP(8µM) with Kn(2µM) (Fig.73c) with nearly same shoot and node number.

- **BAP+TDZ**

The other combinations of cytokinin (BAP and TDZ) in MS medium also proved to be effective in terms of shoot formation compared to cotyledonary node. Among all the concentrations of BAP (2µM) with different concentrations of TDZ(0.1,0.2,0.25 µM), TDZ(0.2µM) resulted in highest 100% response with 1.5 ± 0.2 number of shoots. BAP(4 µM) and BAP(8µM) with TDZ(0.1,0.2,0.25 µM) resulted in nearly similar response in terms of percent response and number of shoots but differed in number of nodes formation. Out of all the combinations, BAP at 8µM with TDZ (0.2µM) induced a 83% cultures and formed 0.8 ± 0.2 shoots with 3.3 ± 1.3 number of nodes (Table 28) (Fig.73d).

- **Kn+TDZ**

The Kn/TDZ combination was less effective in terms of percent response and shoot number as compared to above two combinations of cytokinins. Out of all the combinations the Kn(4 µM) with TDZ(0.25µM) resulted in 100% response with an average of 1.0 ± 0.0 shoots whereas Kn(8µM) with TDZ(0.2µM) formed a single shoot with 2.0 ± 1.1 nodes which was highest among all the other combinations (Table 28)**.**

❖ **Synergistic effect in WPM medium**

Similar combinations of cytokinins were tried in WPM medium the results are as follows:

- **BAP+Kn**

In this medium also BAP and Kn combination proved to be effective as 100% shoots were formed in BAP(2µM) with Kn (2µM) (Fig.73e), BAP(2µM) with (8µM)(Fig.73f) ,BAP(4µM) with Kn(4µM) and BAP(4µM) with (8µM) (Fig.73g) combinations but the number of shoots were maximum (1.3 ± 0.2) in the later three BAP(2µM) with (8µM), BAP(4µM) with Kn (4µM) and BAP(4µM) with (8µM) while in BAP(2µM) with Kn (2 µM) number of nodes was higher ie.4.3 ± 0.3. Compared to BAP (2µM), BAP (4µM) the combinations of BAP (8µM) with Kn (2µM, 4µM and 8µM) resulted in less percent response in terms of shoot formation (Table 28).

- **BAP+TDZ**

In comparison to MS medium the morphogenic response in terms of percent response, number of shoots and nodes was less in WPM medium fortified with BAP/TDZ combinations. All the combinations resulted in formation of single shoot with less number of nodes. BAP (2µM) with TDZ (0.1, 0.2 and 0.25µM) resulted in single but stunted shoots. BAP (4µM) with TDZ (0.1, 0.2 and 0.25µM) also resulted in less response. Whereas about 83% cultures responded with an average of 1.0 ± 0.3 shoots and 1.6±0.5 number of nodes in BAP (8µM) with TDZ (0.1µM) which was maximum among all BAP/TDZ combinations (Table 28).

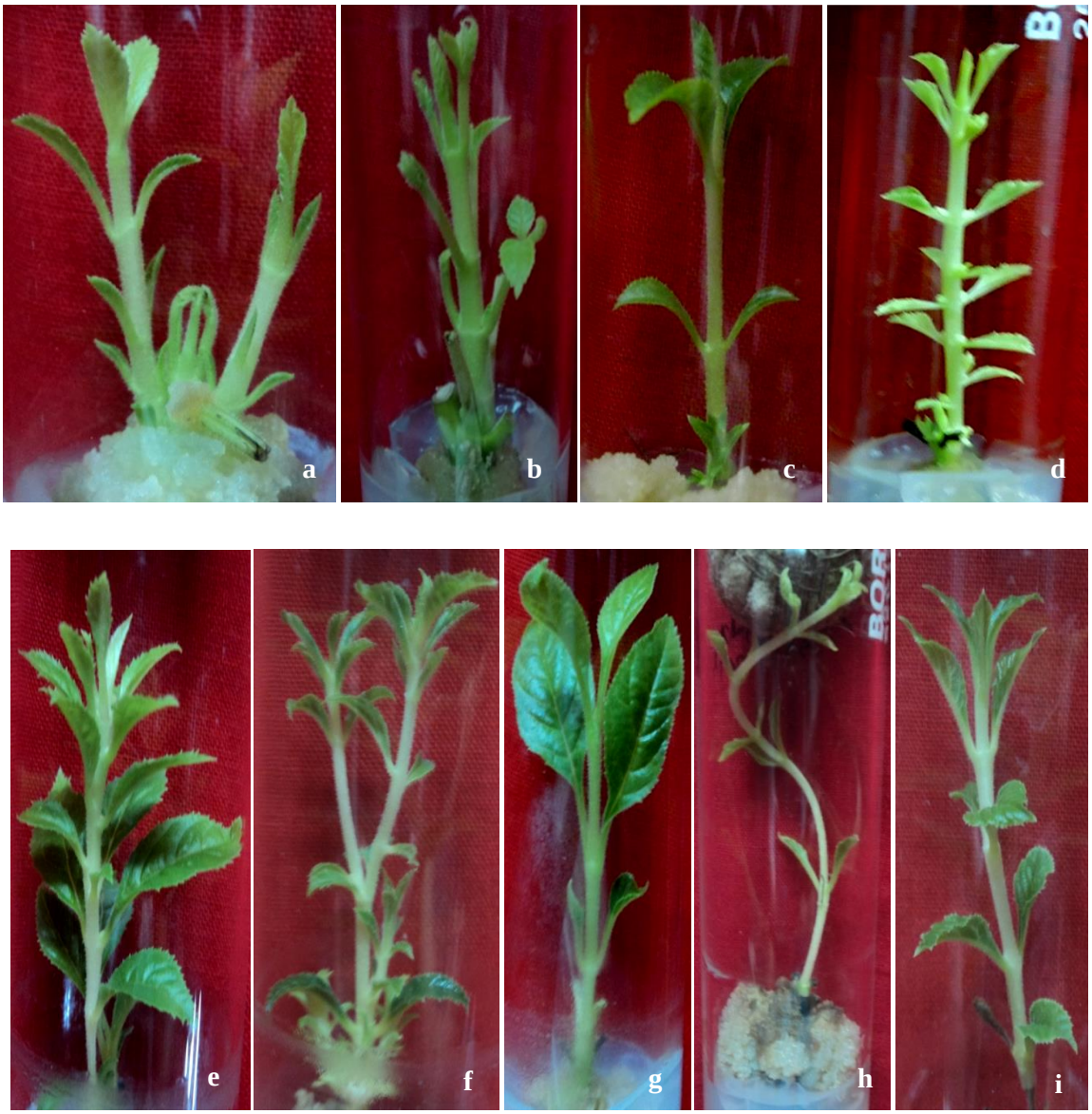

Fig.73: Shoot induction from nodal explants of *S.suaveolens* in MS and WPM medium fortified with two cyokinins after 4 weeks

a. Shoots induced in MS+BAP (4µM) + Kn (4µM)
b. Development of single shoot in MS+BAP (4µM) + Kn (8µM)
c. Single shoot formed in MS+BAP (8 µM) +Kn (2µM)
d. Shoot formed with many nodes in MS+BAP (8 µM) +TDZ (0.2µM)
e. Healthy shoot formed in WPM+BAP (2µM) +Kn(2µM)
f. Shoots proliferated in WPM+BAP (2µM) +Kn(8µM)
g. Single shoot in WPM+BAP (4µM)+Kn(8µM)
h. Elongated shoot formed with basal callus in WPM +Kn (2µM) +TDZ (0.1µM)
i. Shoot developed in WPM+Kn (2µM)+TDZ(0.25µM)

Table 28. Effect of two cyokinins on shoot induction from nodal explants of *S.suaveolens* after 4 weeks

Cytokinins (µM)			MS medium			WPM medium		
BAP	**Kn**	**TDZ**	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***	**% Response**	**Number of shoots***	**Number of *in vitro* nodes***
2	**2**	-	100	1.0±0.0^{cde}	1.5±0.2^{abcdef}	**100**	1.0±0.0^{cde}	**4.3±0.3^{h}**
2	4	-	67	0.7±0.2^{bcd}	2.2±1.1^{bcdef}	67	0.7±0.2^{abcde}	1.8±0.6^{abcdefg}
2	8	-	67	0.7±0.2^{bcd}	1.7±0.7^{abcdef}	100	1.3±0.2^{e}	2.8±0.4^{efgh}
4	**2**	-	83	0.8±0.2^{bcd}	**3.0±0.7^{ef}**	67	0.7±0.2^{abcde}	1.7±0.7^{abcdefg}
4	**4**	-	**100**	**1.5±0.3^{e}**	2.8±0.5^{def}	100	1.3±0.2 ^{e}	1.3±0.2^{abcdef}
4	**8**	-	100	1.3±0.2^{de}	2.5±0.7^{cdef}	**100**	**1.3±0.2 ^{e}**	3.3±0.3^{gh}
8	2	-	100	1.3±0.2^{de}	2.5±0.4^{cdef}	33	0.7±0.2^{abcde}	0.5±0.3^{abc}
8	4	-	0	0.0±0.0^{a}	0.0±0.0^{a}	50	1.0±0.5^{cde}	1.2±0.5^{abcdef}
8	8	-	67	0.7±0.2^{bcd}	0.7±0.2^{abc}	33	0.3±0.2^{abc}	2.2±1.4^{bcdefg}
2	-	0.1	33	0.7±0.4^{bcd}	0.5±0.3^{ab}	67	0.7±0.2^{abcde}	1.3±0.5^{abcdef}
2	-	0.2	100	1.5±0.2^{e}	1.2±0.2^{abcde}	83	0.8±0.2^{bcde}	0.8±0.2^{abcde}
2	-	0.25	66	0.7±0.2^{bcd}	0.7±0.2^{abc}	50	0.5±0.2^{abcd}	1.3±0.7^{abcdef}
4	-	0.1	83	0.8±0.2^{bcd}	0.8±0.2^{abc}	50	0.5±0.2^{abcd}	0.8±0.4^{abcde}
4	-	0.2	83	0.8±0.2^{bcd}	1.0±0.3^{abcd}	66	0.7±0.2^{abcde}	0.7±0.2^{abcd}
4	-	0.25	67	0.7±0.2^{bcd}	0.7±0.2^{abc}	33	0.3±0.2^{abc}	0.3±0.2^{ab}
8	-	0.1	83	1.0 ± 0.3^{cde}	2.3±0.6^{abc}	83	1.0±0.3^{cde}	1.6±0.5^{abcdefg}
8	-	0.2	83	0.8±0.2^{bcd}	3.3±1.3^{f}	33	0.3±0.2^{bc}	0.3±0.2^{ab}
8	-	0.25	67	0.7±0.2^{bcd}	1.0±0.4^{abcd}	0	0.0±0.0 ^{a}	0.0±0.0^{a}
-	2	0.1	83	0.8±0.2^{bcd}	0.8±0.2^{abc}	83	1.2±0.3^{de}	3.1±1.1^{fgh}
-	2	0.2	67	0.7±0.2^{bcd}	0.7±0.2^{abc}	66	0.7±0.2^{abcde}	2.5±0.8^{cdefgh}
-	2	0.25	67	0.7±0.2^{bcd}	0.7±0.2^{abc}	100	1.2±0.2^{de}	2.7±0.7^{defgh}
-	4	0.1	33	0.3±0.2^{ab}	0.5±0.3^{ab}	50	0.5±0.2^{abcd}	1±0.5^{abcde}
-	4	0.2	67	0.7±0.2^{bcd}	1.0±0.4^{abcd}	66	0.7±0.2^{abcde}	1.7±0.8^{abcdefg}
-	4	0.25	100	1.0±0.0^{cde}	1.2±0.2^{abcde}	33	0.3±0.2^{abc}	1.5±1.0^{abcdefg}
-	8	0.1	33	0.3±0.2^{ab}	0.8±0.5^{abc}	33	0.3±0.2^{abc}	1.3±1.0^{abcdef}
	8	0.2	50	0.5±0.2^{abc}	2.0±1.1^{bcdef}	33	0.3±0.2^{abc}	1.7±1.1^{abcdefg}
	8	0.25	50	0.5±0.2^{abc}	1.0±0.6^{abcd}	17	0.2±0.2^{ab}	0.2±0.2^{ab}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

- **Kn+TDZ**

In WPM medium fortified with Kn/TDZ combinations the response slightly improved. Medium fortified Kn (2 µM) and TDZ(0.1µM) resulted in forming maximum of 1.2 ± 0.3 number of shoots and 3.1 ± 1.1 nodes in 83% cultures (Fig.73h). While Kn (2µM) with TDZ

(0.25µM) combination resulted in 100% response with similar 1.2 ± 0.2 number of shoots (Fig.73i).But number of nodes were slightly less (2.7 ± 0.7) compared to Kn (2µM) with TDZ (0.1µM). The combinations of Kn(4µM), Kn(8µM) with TDZ(0.1µM,0.2 µM,0.25µM) obtained less response compared to Kn(2µM)(Table 28) .

In both the media fortified with combinations of cytokinins (BAP with Kn, BAP with TDZ and Kn with TDZ) the nodal explants formed of healthy long shoots with many nodes. Highest number of shoots were obtained in MS medium fortified with BAP (4µM) with Kn(4µM) and BAP(2µM) with TDZ(0.2µM) combination while highest nodes (4.3±0.3) were obtained in WPM medium fortified with BAP(2µM) and Kn(2µM) combination. But in order to enhance the number of shoots the *in vitro* nodes from the shoots developed in respective combinations of cytokinins were subcultured in the same medium.

4.4.2.1.8.1 Multiplication from *in vitro* nodes in presence of two cytokinins

To further enhance the multiple shoot formation the *in vitro* nodes were excised from the shoots and placed in the combinations which had induced a response.

Out of various combinations response was obtained in MS medium fortified with BAP(4µM) with(Kn2µM, 4µM, 8µM), BAP(8µM) with Kn(2µM), BAP(8µM) with TDZ(0.1µM,0.2µM), Kn(8µM) with TDZ(0.2µM,0.25µM) and WPM medium fortified with Kn (2µM) with TDZ(0.2µM, 0.25µM) obtained response while rest other failed to respond in terms of shoot formation.

After 8 weeks it was observed that BAP (4µM) with Kn (2µM, 4µM) was unable to enhance the number and response was observed in 33% cultures. But in BAP(4µM) with Kn(8µM) combination there was a slight increase in number of shoots (1.5 ± 0.6) with 67% response (Fig.74) (Fig.75a).While BAP(8µM) with Kn (2µM) failed to the improve number of shoots resulting in only 50 % response (Fig.75b). BAP (8µM) with TDZ (0.1 µM) also resulted in forming healthy single shoot after 8 weeks (Fig.75c).

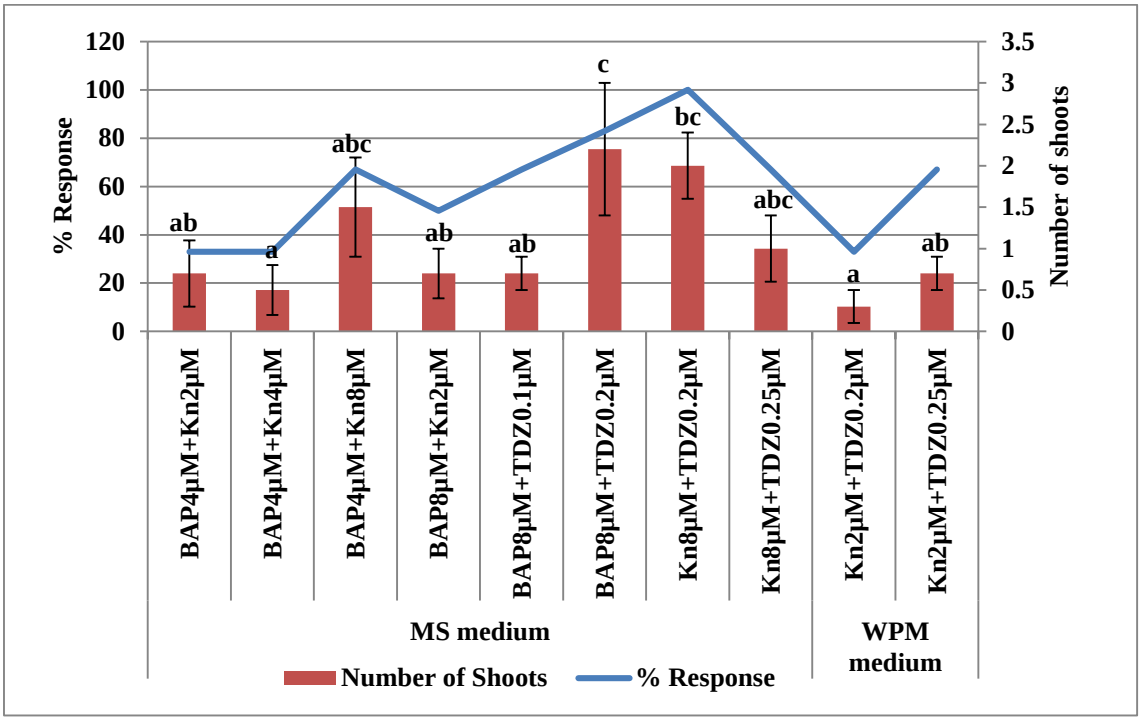

Fig.74: Synergistic effect of cytokinins on inducing multiples from *in vitro* nodes of *S.suaveolens* after 8 weeks

Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

Increase in shoot multiplication rate was observed in MS medium supplemented with BAP (8µM) with TDZ (0.2µM) with an average of 2.2 ± 0.8 shoots (Fig.75d) which was more compared to 4 weeks and highest among all the other combinations. The shoot number also get enhanced in combination Kn(8µM) with TDZ(0.2µM) after 8 weeks (Fig.74;Fig.75e) as the number increased upto 2.0 ± 0.4 compared to 4 weeks. Whereas in Kn(8µM) with TDZ(0.25µM) combination the *in vitro* nodes developed into one or two healthy long shoots(Fig.75f).The WPM medium fortified with Kn(2µM) with TDZ(0.2µM,0.25µM)(Fig.74;Fig.75g) resulted in single shoot formation.

All the above combinations were subcultured by excising the *in vitro* nodes but the combinations which reported enhancement in number at every passage were: MS medium fortified with BAP (4µM) with Kn (8µM), Kn(8µM) with TDZ(0.2µM), and BAP(8µM) with TDZ(0.1µM),(0.2µM). The other combinations like MS medium supplemented with BAP(4µM) with Kn(2µM,4µM), ,BAP(8µM) with Kn(2µM), Kn(8 µM) with TDZ(0.25µM) and WPM medium fortified with Kn(2µM) with TDZ(0.2 µM,TDZ0.25µM) failed to respond.

Fig.75: Development of healthy shoot from *in vitro* nodes of *S.suaveolens* after 8 weeks

a. Small multiples developed in MS+BAP (4 µM) +Kn(8 µM)
b. Multiple shoots formed in MS+BAP (8 µM) +Kn(2 µM)
c. Single shoot development in MS+BAP (8 µM) +TDZ (0.1 µM)
d. Multiple shoots proliferated in MS+BAP (8 µM) +TDZ (0.2µM)
e. Formation of more than two shoots in MS+Kn(8µM)+TDZ(0.2 µM)
f. Development of one or two shoots in MS+ Kn(8µM) +TDZ(0.25µM)
g. Single shoot formed in WPM + Kn(2µM) +TDZ(0.25µM)

In MS medium with BAP(4µM) and Kn(8µM) combination there was formation of healthy multiple shoots at each passage which increase in number till 16 weeks with an average of 5.9 ± 1.0 shoots but after 24 weeks the number reduced to 3.4 ± 0.3 and got slightly increased to 3.9 ± 0.5 after 32 weeks(Fig.76;Fig.77a-d).

Whereas in MS medium with BAP (8µM) with TDZ(0.1µM) combination there was enhancement in terms of number observed at every passage with an average of 5.6 ± 0.3 by the end of 32 weeks. The shoots formed were healthy and long (Fig.76;Fig.77e-h).

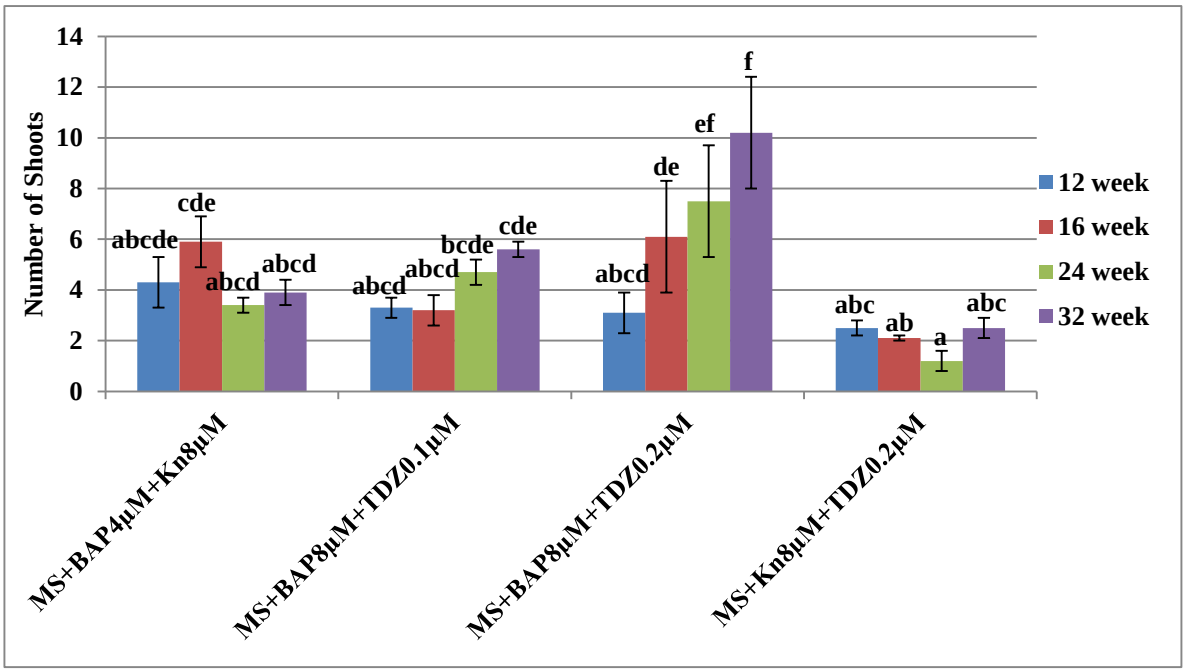

Fig.76:Effect of two cytokinins on inducing multiples from *in vitro* nodes of *S.suaveolens* at different passages

Values represent mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

In medium fortified BAP (8µM) with TDZ (0.2µM) there was an increase in number at each passages reaching (10.2 ± 2.2) in number by the end of 32 weeks. This was the highest shoot number among the other combinations (Fig.76).The shoots formed were very healthy with well developed leaves (Fig.78 a-d).

The Kn(8µM) with TDZ(0.2µM) combination the number of shoots formed were least among all the other combinations in each passages (Fig.76) (Fig.78e-h) but this combination resulted

Fig.77: Enhancement of *S.suaveolens* shoots from *in vitro* nodes in combinations of two cytokinins ie. MS+BAP(4µM)+Kn(8µM), MS+BAP(8µM)+TDZ(0.1µM)

a-d Healthy multiples developed in MS+BAP(4µM)+Kn(8µM) at each passage

a. 12 weeks
b. 16 weeks
c. 24 weeks
d. 32 weeks

e-h Healthy multiples formed in MS+ BAP(8 µM)+TDZ(0.1µM) at each passage

e. 12 weeks
f. 16 weeks
g. 24 weeks
h. 32 weeks

Fig.78: Multiple shoot formation from *in vitro* nodes of *S.suaveolens* in combinations of two cytokinins ie. MS+BAP (8µM)+TDZ(0.2µM), MS + Kn(8µM)+TDZ(0.2µM)

a-d Multiple shoot formation in MS+BAP(8µM)+TDZ(0.2µM) at every passages

- **a. 12 weeks**
- **b. 16 weeks**
- **c. 24 weeks**
- **d. 32 weeks**

e-h Healthy shoots in MS + Kn(8µM) +TDZ(0.2µM) at every passages

- **e. 12 weeks**
- **f. 16 weeks**
- **g. 24 weeks**
- **h. 32 weeks**

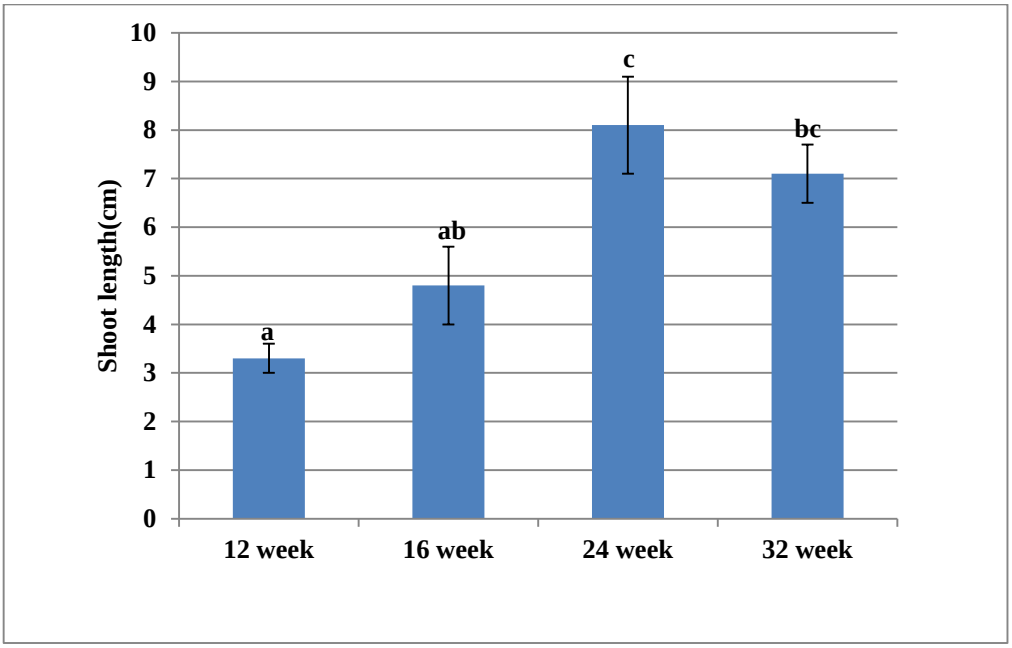

Fig.79: Effect of MS medium fortified with Kn (8µM) and TDZ (0.2µM) on shoot length of *S.suaveolens* at each passage

Values represent mean ± S.E of ten replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

in forming elongated healthy shoots. There was increase in shoot length at each passage which increased to 8.1 ± 1.0 cms by the end of 24 weeks (Fig.79).

Hence from the above results in *S.suaveolens* MS medium in comparison to WPM medium proved to be optimum for producing healthy shoots in terms of number as well as length.The MS medium supplemented with BAP (8µM) with TDZ(0.2µM) combination was the optimum combination for regenerating into healthy multiples shoots.

4.4.2.1.9 Effect of cytokinins and auxins on nodal explants

As a combination of cytokinins induced axillary buds and *in vitro* buds to proliferate and develop into single shoot or multiple the effect of a cytokinin and auxin was also evaluated .Similar to cotyledonary node explants the synergistic effect of cytokinin with auxin was also evaluated by selecting the optimised concentration (one with highest percent response) from BAP/Kn/TDZ and combined with 0.1, 0.5 and 1µM concentrations of auxins (IAA and NAA). When explants were placed on medium containing combinations of cytokinin and auxins it was observed that only single or two shoot were obtained from both cotyledonary and nodal explants after four weeks.

- **Shoot induction in MS medium**

When BAP (2µM) was added with different concentrations of IAA in MS medium it obtained poor response in terms of percent and number of shoots. Incorporating NAA in medium the response improved slightly with maximum of 83% cultures resulted in BAP (2µM) with NAA (0.5µM) combination with 0.8 ± 0.2 shoots and 2.0 ± 0.7 nodes. When Kn was added with auxins the response improved in terms of number of nodes. At IAA (1µM) single shoot were obtained with maximum of 1.4 ± 1.0 nodes. Whereas combination of Kn (2µM) with NAA (0.5µM) resulted in 100% response with an average of (1.0± 0.0) number of shoots and highest number (2.5±0.6) of nodes. Incorporating TDZ with auxins obtained poor response except in TDZ (1µM) with IAA (0.1µM) combination 83% cultures responded with single shoot and 1.2±0.4 nodes (Table 29).

Thus MS medium fortified with Kn (2µM) with NAA (0.5µM) resulted in 100% response with an average of (1.0 ± 0.0) number of shoots and highest number (2.5 ± 0.6) of nodes which was maximum among all the other combinations.

Table 29. Effect of cyokinins and auxins on shoot induction from nodal explants of *S.suaveolens* in MS medium after 4 weeks

Cytokinins (µM)			Auxins (µM)		% Response	Number of shoots*	Number of *in vitro* node*
BAP	**Kn**	**TDZ**	**IAA**	**NAA**			
2	-	-	0.1	-	33	0.5 ± 0.3^{abcd}	1.0 ± 0.6^{abc}
2	-	-	0.5	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
2	-	-	1	-	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
2	-	-	-	0.1	67	0.7 ± 0.2^{bcd}	0.8 ± 0.3^{ab}
2	-	-	-	0.5	83	0.8 ± 0.2^{cd}	2.0 ± 0.7^{bc}
2	-	-	-	1	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
-	2	-	0.1	-	17	0.2 ± 0.2^{ab}	0.5 ± 0.5^{ab}
-	2	-	0.5	-	33	0.3 ± 0.2^{abc}	1.2 ± 0.7^{abc}
-	2	-	1	-	50	0.7 ± 0.3^{bcd}	1.4 ± 1.0^{abc}
-	2	-	-	0.1	50	0.5 ± 0.2^{abcd}	1.3 ± 0.7^{abc}
-	**2**	-	-	**0.5**	**100**	**1.0 ± 0.0^{d}**	**2.5 ± 0.6^{c}**
-	2	-	-	1	17	0.2 ± 0.2^{ab}	0.5 ± 0.5^{ab}
-	-	1	0.1	-	83	0.8 ± 0.2^{cd}	1.2 ± 0.4^{abc}
-	-	1	0.5	-	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	1	1	-	33	0.3 ± 0.2^{abc}	0.3 ± 0.2^{a}
-	-	1	-	0.1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	1	-	0.5	17	0.2 ± 0.2^{ab}	0.2 ± 0.2^{a}
-	-	1	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test

- **Shoot induction in WPM medium**

In WPM medium the morphogenic response was better compared to MS in terms of percent response. When BAP (8µM) was combined with different concentrations of IAA and NAA, all combinations formed single shoot but varied in percent response and number of nodes. The BAP (8µM) with IAA (0.1µM) combination resulted in 100% response with an average of 1.3 ± 0.2 number of shoots and 1.2 ± 0.2 nodes. Whereas when NAA was added it obtained maximum 1.9 ± 0.9 number of nodes in BAP (8µM) with NAA (0.1µM) combination**.** The Kn with auxins combinations proved to be better compared to BAP, as the Kn (2µM) with IAA(0.1µM) combination resulted in 100% response with maximum of 1.7±0.2 shoots in number whereas Kn(2µM)+IAA(1µM) combinations obtained maximum (2.7±0.8) number of nodes. Incorporation of TDZ (0.2µM) along with IAA and NAA in medium there was not much variation observed in terms of shoot number. Among all the combinations tried, TDZ (0.2µM) with IAA (0.5µM) resulted in 83% response with only 0.8±0.2 shoots formed and maximum of 1.5 ± 0.5 nodes. Whereas in TDZ (0.2µM) with NAA (0.5µM) combinations 67% cultures formed single shoot with 2.0±1.0 nodes (Table 30).

Thus out of all the combinations in WPM medium the Kn(2µM) with IAA(0.1µM) combination resulted in 100% response with maximum of 1.7 ± 0.2 shoots.

Overall from the experiments it can be concluded that both the auxins when combined with cytokinin failed to improve the number of shoots from nodal explants. From all the combinations IAA proved to be effective in nodal explants with 100% response in Kn(2 µM) + IAA(0.1 µM) and Kn (2µM) +IAA(1µM) but the earlier obtained highest 1.7 ± 0.2 number of shoots while later with highest number of nodes (2.7 ± 0.8).

4.4.2.1.9.1 Multiplication from *in vitro* nodes in presence of cytokinins and auxins

The *in vitro* nodes from the shoots developed in combinations of cytokinin and auxin were subcultured in respective media but all the combinations failed to respond except in WPM medium fortified with Kn(8µM) with NAA(0.1µM) there was formation of two elongated shoots with many nodes after 8 weeks but after subculture it failed to survive.

Therefore in *S.suaveolens* the combinations of cytokinins and auxins induced a response but resulted in forming one or two shoots only and failed to form multiples from nodal explants or *in vitro* nodes.

Table 30.Effect of cyokinins and auxins on shoot induction from nodal explants of *S.suaveolens* in WPM medium after 4 weeks

Cytokinins (µM)			Auxins (µM)		% Response	Number of shoot*	Number of *in vitro* nodes*
BAP	**Kn**	**TDZ**	**IAA**	**NAA**			
8	-	-	0.1	-	100	1.3 ± 0.2^{d}	1.2 ± 0.2^{abcd}
8	-	-	0.5	-	50	0.5 ± 0.2^{abc}	0.7 ± 0.3^{abc}
8	-	-	1	-	33	0.3 ± 0.2^{ab}	0.5 ± 0.3^{abc}
8	-	-	-	0.1	67	1.0 ± 0.4^{cd}	1.9 ± 0.9^{de}
8	-	-	-	0.5	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
8	-	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	**2**	-	**0.1**	-	**100**	**1.7 ± 0.2^{e}**	1.8 ± 0.4^{cde}
-	2	-	0.5	-	67	0.7 ± 0.2^{bcd}	0.8 ± 0.3^{abcd}
-	**2**	-	**1**	-	**100**	1.0 ± 0.0^{cd}	**2.7 ± 0.8^{ab}**
-	2	-	-	0.1	67	0.7 ± 0.2^{bcd}	1.3 ± 0.5^{abcde}
-	2	-	-	0.5	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	2	-	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
-	-	0.2	0.1	-	67	0.8 ± 0.3^{bcd}	0.7 ± 0.2^{abc}
-	-	0.2	0.5	-	83	0.8 ± 0.2^{bcd}	1.5 ± 0.5^{bcde}
-	-	0.2	1	-	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
-	-	0.2	-	0.1	67	0.7 ± 0.2^{bcd}	0.7 ± 0.2^{abc}
-	-	0.2	-	0.5	67	0.7 ± 0.2^{bcd}	2.0 ± 1.0^{de}
-	-	0.2	-	1	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq0.05$ according to Duncan's Multiple range test.

4.4.3 Indirect organogenesis

In few combinations of WPM medium fortified with PGRs a morphogenic callus was induced at the base of cotyledonary node explants which was transferred for shoot bud differentiation.

4.4.3.1 Individual cytokinin

The basal callus with shoot buds formed in presence of BAP (16 µM) was subcultured and few shoot buds differentiated from it after 8 weeks. Therefore in order to enhance the number $AgNO_3$ (20mg/l) was added in the medium, but it failed to enhance the shoot number instead large amount of stunted shoots were formed (Fig.80a) after 12 weeks. In order to elongate these shoots they were transferred to medium fortified with coconut water (10%) medium which resulted in significant increase in shoot number as well as shoot length. When the callus was transferred to a fresh medium after 16,24 and 32 weeks the shoot number and

Fig.80: Indirect shoot formation in WPM medium fortified with individual cytokinins in *S.suaveolens*

a. Shoot buds developed in WPM+BAP (16µM) +$AgNO_3$ (20mg/l) after 12weeks

b. and c. Shoot elongated after transferring to WPM + coconut water (10%) medium after 24 and 32 weeks respectively

d. Multiples emerged from morphogenic callus in WPM+BAP (20µM) after 8 weeks

e. Increase in shoot number in WPM+ BAP (20µM) after 12 weeks

length enhanced to 22.5 ± 3.9 and 8.5 ± 3.5cms in length respectively by the end of 32 weeks(Fig.80b,c;Fig.81).

Presence of BAP at 20µM also resulted in formation of basal callus with shoot buds which when transferred there was formation of shoots (Fig.80d) with increased in number after 8 weeks and the shoot number after 12 weeks (Fig.80e) reached to 15.0± 0.0.

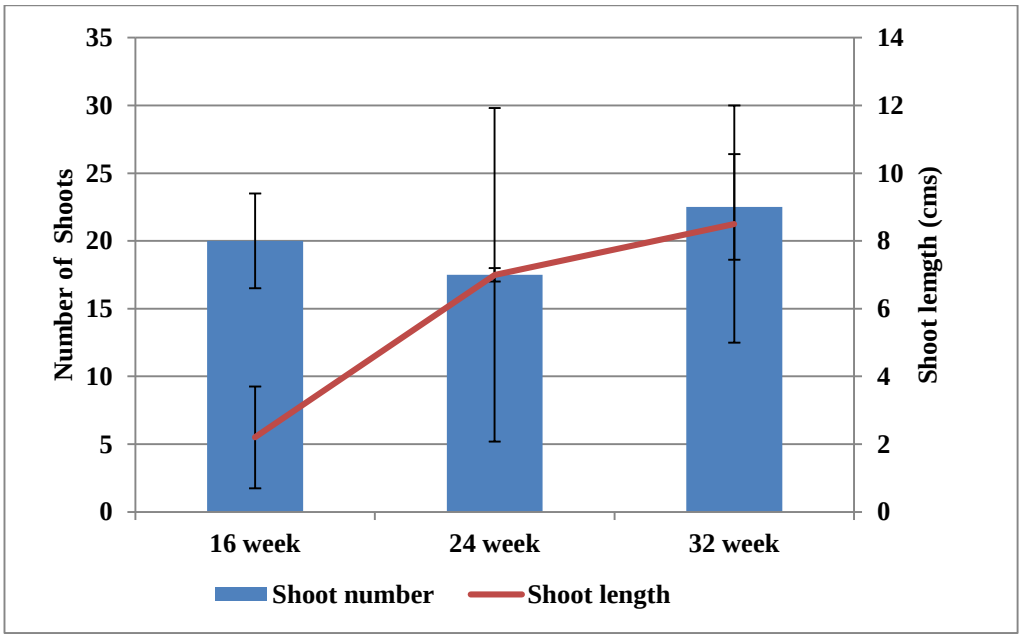

Fig.81: Effect of WPM medium fortified with coconut water (10%) on shoot number and length of *S.suaveolens* at three passages.

4.4.3.2 Synergistic effect of cytokinins

A combination of BAP (20µM) with Kn (2µM, 4µM) formed single shoot accompanied with callus at the basal portion of shoot. New shoots started proliferating after 6 weeks. But the shoots failed to survive after 8 weeks.

4.4.3.3 Effect of cytokinins and auxins

In the combination of WPM medium with BAP (20µM) with NAA(1µM) there was formation of single long shoot growing from callus after 8 weeks. This when subcultured further, there was an increased in only callus mass in large amount after 12 weeks but the shoots get dried up.

In WPM medium with Kn (8µM) and IAA (0.1µM) from the callus with shoot buds one

or two shoots developed along with nodular callus at base after 8 weeks (Fig.83a,b) this nodular callus was transferred further in fresh medium .After 12 and 16 weeks there was formation of single shoot only which get elongated within few days into healthy and long shoot with an average of 6.1±0.3 cms in length(Fig.82;Fig.83c).

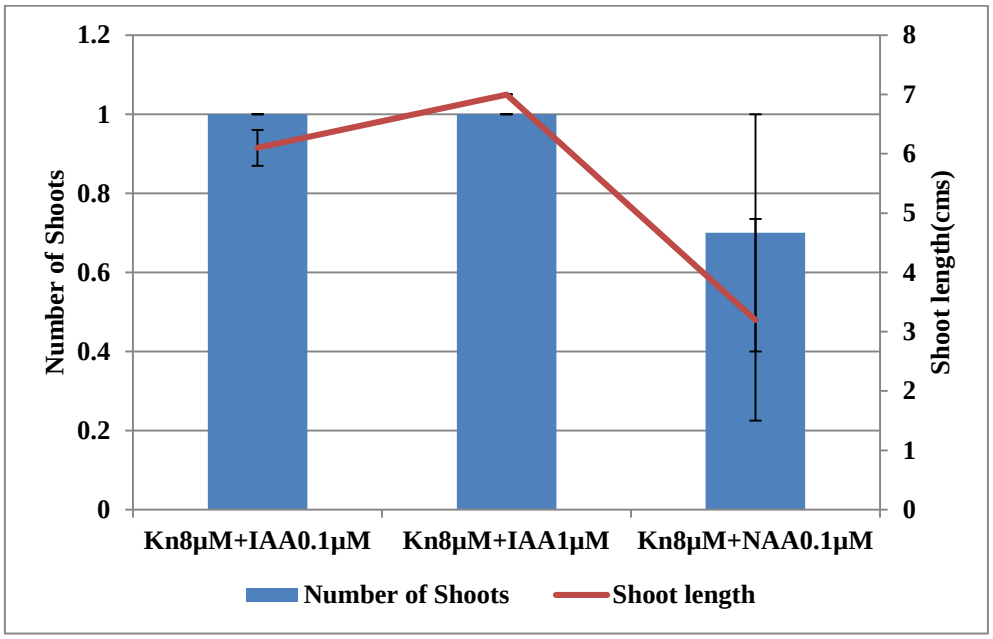

Fig.82 Indirect organogenesis in WPM medium fortified with Kn (8µM) with IAA (0.1µM, 1µM), Kn(8µM) with NAA(0.1µM) after 12 weeks in *S.suaveolens*

In Kn(8µM) with IAA(1µM) from nodular callus single shoots proliferated after 8 weeks which was transfer further in fresh medium(Fig.83d,e). After 12 weeks there was no multiples formed but the single shoot growth was observed in terms of length upto 7cm (Fig.82; Fig.83f).

In Kn(8µM) +NAA(0.1µM) there was formation of nodular callus which was (Fig.83g) transferred to medium by lowering the Kn concentration ie Kn(4µM) with NAA(0.1µM) but the shoot obtained poor growth and there was formation of nodular callus which was utilised and transferred to same medium. It was observed that there was a development of the shoot with healthy growth from nodular callus which got elongated upto 3.4±1.7cms in length (Fig.82) (Fig.83h,i).

Fig.83: Indirect organogenesis in presence of cytokinin and auxin in *S.suaveolens*

a. Shoot induced from basal callus in WPM+ Kn(8µM)+ IAA(0.1µM)
b. Increased in shoot length after 8 weeks
c. Healthy shoots elongated after 12 weeks
d. Callus along with shoot bud in WPM+ Kn (8 µM) +IAA (1µM)
e. Shoot bud proliferated into shoot after 8 weeks
f. Increase in shoot length after 12 weeks
g. Nodular callus developed at base of shoot in WPM+Kn(8µM) +NAA(0.1 µM)
h. Shoot growth occurred after 8 weeks
i. Increase in shoot length after 12 weeks

4.4.4 *In vitro* rooting of *S.suaveolens* microshoots

In *S. suaveolens* roots were induced in MS and WPM (liquid and static) medium fortified with different concentrations of IBA and NAA and obtained varied response.

- **Rooting in MS medium**

The liquid and static medium of half and full strength were tried for root induction. It was observed that in medium without PGRs obtained resulted in less response in half strength MS (liquid and static) medium while in full strength also similar response in terms of roots formation was observed.

When half strength MS liquid and static medium was fortified with different concentrations of IBA it obtained very good response in terms of percentage and number of roots but roots were small in length. In MS liquid medium 2µM and 2.5µM concentrations induce 100% roots but 2.5µM was efficient in inducing optimum number (9.3 ± 0.8) of roots (Fig.84a) whereas in static medium fortified with IBA(1µM) resulted in the maximum number of roots (8.8 ± 3.8) in 50% cultures (Fig.84b). When, half strength medium was incorporated with NAA it proved to be better compared to IBA in terms of percent response and root length but number of roots induced were less (Table 31). Liquid medium fortified with NAA at 1µM, 2µM, 5µM evoked 100% root formation in which a maximum number of 4.5 ± 0.8 roots were induced at 5µM (Fig.84c).The maximum root length was in NAA (1µM) (Table 31).Static medium fortified with NAA at 1µM and 2µM also induced 100% response in cultures and number of roots formed were almost similar to those formed in liquid medium.

In full strength MS medium fortified with IBA also induced roots at all concentrations but lower concentrations (1µM and 2µM) were less responsive whereas higher concentration induced maximum of 100% roots at 2.5µM and 5µM with 6.8 ± 1.3 roots at 5µM (Fig.84d). Whereas in static medium with all IBA concentrations (1µM-5µM) roots were less in number and short in length. When NAA was added the percent response was less in liquid medium of all the concentrations used while in static medium NAA at 2µM induced 100% roots. The maximum of 3.7±1.8 number were formed in liquid NAA(2µM) (Fig.84e) while root length was maximum at liquid NAA (1µM) (2.3 ± 0.9cms) (Table 31).

Table 31. Effect of half and full strength MS medium fortified with IBA and NAA on root induction of *S.suaveolens* after 4 weeks

Medium	Auxin (µM)		% Response	Number of roots*	Root Length* (cms)	% Response	Number of roots*	Root Length* (cms)
			Liquid			Static		
Half strength	0		33	0.5 ± 0.3^{a}	0.9± 0.6^{ab}	50	2.0 ± 0.9^{ab}	0.8 ± 0.4^{ab}
	IBA	**NAA**						
	1		50	1.5±0.8^{ab}	1.1 ±0.5^{ab}	66	8.8±3.8^{cd}	1.0±0.4^{ab}
	2		100	5.5±1.7^{bc}	4.1±0.9^{ef}	50	1.0±0.4^{a}	0.6±0.2^{a}
	2.5		**100**	**9.3±0.8^{d}**	0.8±0.1^{ab}	33	1.2±0.7^{ab}	0.2±0.1^{a}
	5		50	4.0±1.8^{ab}	0.7±0.1^{ab}	50	1.0±0.4^{a}	0.2±0.1^{a}
		1	100	2.8± 0.5^{ab}	4.3±0.6^{f}	100	2.3±0.4^{ab}	2.3±0.5^{bcd}
		2	100	3.0±0.5^{ab}	1.4±0.4^{abc}	100	3.2±0.4^{ab}	0.8±0.2^{ab}
		2.5	66	2.2±1.1^{ab}	3.9±1.3^{def}	66	2.2±0.7^{ab}	1.2±0.5^{ab}
		5	100	4.5±0.8^{ab}	2.7±0.3^{cde}	66	2.5±1.0^{ab}	0.9±0.4^{ab}
Full strength	0		0	0.0±0.0^{a}	0.0±0.0^{a}	33	0.3 ± 0.2^{a}	0.3±0.2^{abc}
	IBA	**NAA**						
	1		33	1.2±0.7^{ab}	1.0±0.7^{abcde}	0	0.0±0.0^{a}	0.0±0.0^{a}
	2		33	0.3±0.2^{a}	0.2±0.2^{ab}	50	0.7±0.3^{a}	0.5±0.4^{abc}
	2.5		100	6.3±1.1^{cd}	4.2±0.8^{f}	66	2.0±0.9^{ab}	1.1±0.4^{abcd}
	5		100	6.8±1.3^{d}	2.5±0.7^{ef}	66	1.3±0.5^{ab}	1.2±0.7^{abcd}
		1	66	2.3±0.9^{ab}	2.3±0.9^{de}	66	2.2±0.7^{ab}	0.6±0.2^{abcd}
		2	66	3.7±1.8^{bc}	1.8±0.8^{bcde}	100	2.0±0.5^{ab}	1.6±0.4^{abcd}
		2.5	66	1.0±0.4^{ab}	2.0±0.9^{cde}	50	2.0±1.1^{ab}	0.4±0.2^{abc}
		5	50	2.0±1.1^{ab}	1.1 0.6^{abcde}	0	0.0±0.0^{a}	0.0±0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤0.05 according to Duncan's Multiple range test.

- **Rooting in WPM medium**

The rooting of shoots was also achieved in half and full strength WPM (liquid and static) medium fortified with different concentrations IBA and NAA.

Both the half and full strength liquid and static medium were able to induce roots. In WPM medium also the liquid medium proved to be effective in induction of roots as compared to static. But the later medium proved to be better in terms of percent response.

The half strength basal liquid medium resulted in formation of single long roots with highest root length of 4.9 ± 3.2 cms in 50 % cultures. In static the root number and length was less. When the medium was fortified with different concentrations of IBA all the concentrations were able to induce roots but highest 100% with an average of 8.5 ± 3.1 roots were formed at half strength WPM liquid media fortified with IBA (2µM) (Fig.84f).

Fig. 84: Root induction of *S.suaveolens* shoots in MS and WPM medium after 4 weeks

a. Many small roots developed in ½ MS liquid +IBA (2.5µM)
b. Thick and stout roots formed in ½ MS static +IBA (1µM)
c. Roots induced in ½ MS liquid + NAA (5µM)
d. Root formation in MS liquid + IBA (5µM)
e. Very thick roots developed in MS liquid + NAA (2µM)
f. Small roots developed in ½ WPM liquid + IBA (2µM)
g. Thin roots formed in ½ WPM liquid+ NAA (2µM)
h. Roots arise with small laterals in ½ WPM liquid +NAA (2.5µM)
i. Thick small roots developed in WPM static +IBA (5µM)
j. Long many healthy roots formed at the base in WPM liquid +NAA (1µM)
k. Many roots formed in WPM liquid+ NAA(5µM)

Table 32. Effect of half and full strength WPM medium fortified with IBA and NAA on root induction of *S.suaveolens* after 4 weeks

Medium	Auxin (µM)		% Response	Number of roots*	Root Length* (cms)	% Response	Number of roots*	Root Length* (cms)
			Liquid			Static		
Half strength	**0**		50	0.75± 0.5^{a}	**4.9 ± 3.2^{b}**	66	0.7 ± 0.3^{a}	1.8 ± 1.4^{b}
	IBA	**NAA**						
	1		50	1.75 ± 0.5 ^{a}	0.9± 0.8^{ab}	20	0.2±0.2 ^{a}	0.02± 0.02^{a}
	2		**100**	**8.5 ± 3.1 ^{b}**	1.3 ± 0.8^{ab}	66	0.7 ±0.7^{a}	0.1 ± 0.1^{a}
	2.5		50	1.7 ± 0.8 ^{a}	1.6 ± 0.8^{ab}	60	1.2 ±1.0^{a}	0.5 ± 0.4^{ab}
	5		75	2.0 ± 1.4 ^{a}	0.2 ± 0.1^{a}	75	1.3 ± 1.3^{a}	0.2 ± 0.2^{a}
		1	0	0.0±0.0^{a}	0.0±0.0^{a}	67	2.5±1.1^{bcd}	0.9±0.3^{ab}
		2	100	2.0±0.4^{bc}	1.8±0.4^{abc}	0	0.0±0.0^{a}	0.0±0.0^{a}
		2.5	67	4.3±1.5^{d}	0.6±0.2^{ab}	0	0.0±0.0^{a}	0.0±0.0^{a}
		5	67	4.0±1.3^{d}	3.3±1.0^{cd}	67	0.7±0.2^{ab}	0.6±0.2^{ab}
Full strength	0		50	0.75±0.5^{a}	1.4 ±0.8^{ab}	33	1.0 ± 1.0^{a}	0.5±0.5^{b}
	IBA	**NAA**						
	1		80	2.0±0.5^{ab}	0.7±0.3^{a}	83	1.7 ± 0.3^{ab}	0.3±0.1^{ab}
	2		75	1.5 ±0.9^{a}	1.3±0 .4^{ab}	100	3.7 ± 0.9^{b}	1.1± 0.3^{b}
	2.5		40	2.2 ± 1.4^{b}	0.5 ±0.4^{a}	0	0.0 ± 0.0^{a}	0.0± 0.0^{a}
	5		40	1.6 ± 1.0^{ab}	1.2± 0.8^{b}	40	3.8 ± 3.1^{b}	0.1± 0.1^{a}
		1	83	4.6±1.2^{cd}	2.8±0.9^{abc}	100	3.5±0.6^{bcd}	1.3±0.2^{ab}
		2	50	1.7±0.8^{a}	1.0±0.5^{a}	100	1.8±0.7^{a}	1.9±0.4^{ab}
		2.5	50	2.5±1.1^{abc}	0.7±0.3^{a}	100	4.5±0.7^{cd}	1.1±0.1^{ab}
		5	83	5.5±1.6^{d}	1.9±0.5^{ab}	83	1.3±0.3^{ab}	1.6±0.6^{ab}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at p≤ 0.05 according to Duncan's Multiple range test.

While in the other concentrations less number of roots were induced. The length of roots in all the concentrations of IBA were less compared to control. When medium was fortified with NAA at 2µM (Fig.84g) induced 100% response but number of roots were less as compared to 2.5µM where 4.3 ± 1.5 number of roots were induced (Fig.84h). Increase in concentration to 5µM roots formed reached to 3.3 ± 1.0 cm in length after 4 weeks (Table 32).

Full strength liquid and static basal medium only single roots were induced. When medium was supplemented with IBA and NAA, the liquid medium with IBA at 1µM evoked a maximum of 80% response but number of roots formed were less. 100% roots were formed in static IBA (2µM) medium but average number of roots were maximum at 5µM ie. 3.8 ± 3.1 (Fig.84i).

When NAA was added there was not much improvement in terms of percent response in liquid medium but the number of roots formed were highest(5.5 ± 1.6) at NAA(5µM). Whereas in static medium 100% root induction were observed at NAA (1µM, 2µM and 2.5µM). In which root length was maximum (2.8 ± 0.9) at NAA (1µM) (Fig.84j) while NAA (5µM) (Fig.84k) induced maximum (5.5 ± 1.6) number of roots.

Overall results depicts that half strength was effective compared to full strength, liquid media was better than static media and IBA was suitable for root induction compared to NAA in *S.suaveolens*. Both MS and WPM media induced roots but MS medium was suitable for roots formation. Out of all the combinations tried the half strength MS liquid medium fortified with IBA (2.5µM) induced optimum number of roots.

4.4.5 Hardening of *S. suaveolens* plantlets

Hardening of *in vitro* raised plantlets is essential for their successful establishment under greenhouse and field conditions. Hence the *in vitro* plants of *S.suaveolens* were transferred to different natural planting substrates, and then hardened off in two ways:

1. Plantlets were placed in culture room conditions and then transfer to greenhouse
2. The plantlets were directly transfer to greenhouse

4.4.5.1 Under Lab conditions

The following planting substrates were sterilised before hardening and plantlets were grown and kept under culture room conditions (Fig.85a).

- Cocopeat
- Cocopeat: Sand(1:1)
- Cocopeat: Soil(1:1)
- Cocopeat: Sand: Soil (1:1:1)

Observations after one month revealed that the plants obtained growth in Cocopeat:Sand:Soil and Cocopeat substrate with 50% response in each whereas in the other substrate the plantlets cease to grow. In cocopeat, growth of the shoots started by the end of second week and after 3 weeks, induction of new leaves and increase in root length was observed(Fig.85b,c).

Fig.85: Hardening of *S.suaveolens* in Cocopeat under culture room conditions

a. Plantlets placed in cocopeat filled cups
b. New leaves developed at the apex
c. Increased in length of leaves and roots
d. Plantlet transferred to polybags filled with soil and placed in greenhouse

These plantlets were transferred to polybag filled with soil and placed in greenhouse(Fig.85d), but it failed to survive within a week.

Since the plants were not successfully hardened under culture room conditions another method by directly transfer to greenhouse was tried.

4.4.5.2 Under greenhouse conditions

The hardening under greenhouse different substrates were selected without sterilising them and the *in vitro* plantlets with well developed roots were placed in them by removing the traces of medium and washed with water. Prior to transfer the initial data for number of roots, root length, shoot length and plant height was recorded.

The plants were hardened in following different substrates and obtained varied results.

- Cocopeat
- Sand
- Soil
- Sand: Soil (1:1)
- Cocopeat: Sand (1:1)
- Cocopeat: Soil (1:1)
- Cocopeat: Sand: Soil (1:1:1)

Observations after four weeks revealed that in each substrate the percent survival varied and there was an increase in shoot and root length as well as plant height was observed with emergence of new leaves. The results of hardening in different substrates under greenhouse conditions is as follows:

Cocopeat

The rooted plants were transferred to cocopeat substrate (Fig.86a) out of which only 50% by the end of four weeks(Fig.87).In these plants there was development of new leaves(4.0 ± 0.6) observed within two weeks which increased in length to 2.4 ± 0.3 cms(Fig.86b)(Fig.88). In this substrate there was not much variation observed in terms of increase in shoot length, root length and plant height observed from the initial. The shoot length from 3.2 ± 0.4 to 3.6 ± 0.6cms, the root length reached from 3.3±0.5cms to 4.3 ± 1.1 cms(Fig.89). The growth of plants in terms of new secondary roots formation was observed with increased in number of roots (Fig.86c).These plants were transferred in plastic pots filled with soil for their further growth (Fig.86d) and the pots were covered with polythene bags for 4-5 days and later on

Fig.86: Hardening of *S.suaveolens* plants in Cocopeat substrate

a. Rooted plantlets transferred to substrate

b. Emergence of new leaves at the apex which increased in length after 4 weeks

c. Plant showing growth with formation of new secondary roots after 4 weeks

d. Plantlet transferred to pots filled with soil

removed. But within a week of transfer the plants failed to survived and hence no further growth was seen.

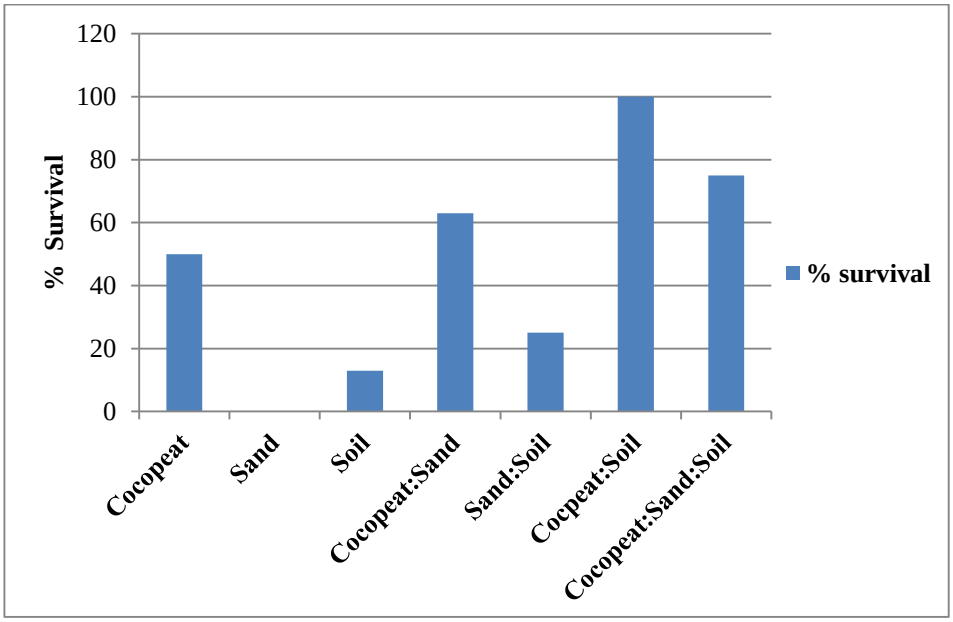

Fig.87: Percent survival of *S.suaveolens* after four weeks in different substrates under greenhouse conditions

Sand

The rooted plants were transferred to thermocol cup filled with individual sand substrate for hardening but in sand the percent response was nil(Fig.87) as none of the plants could survive within 2- 3 weeks of transfer.

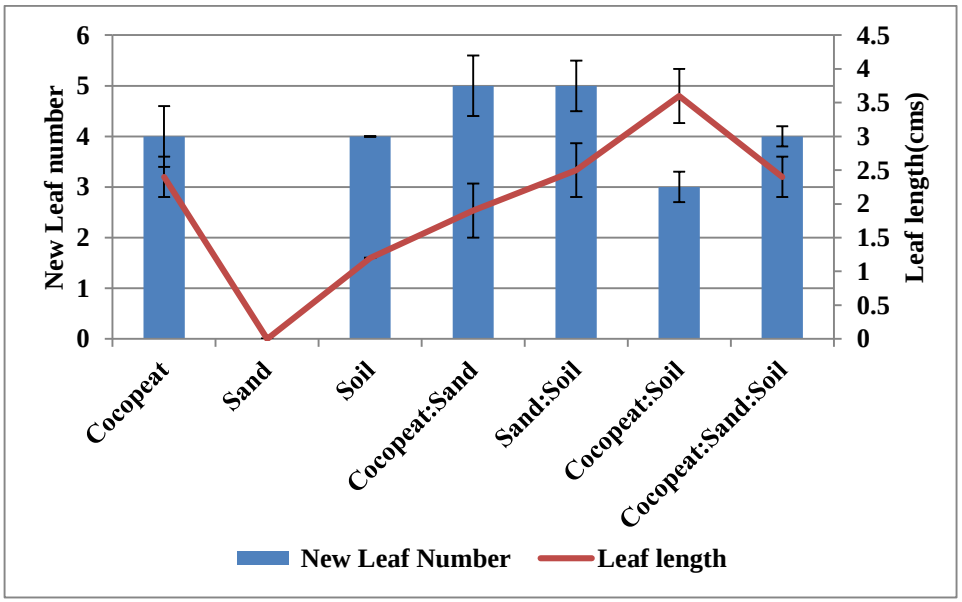

Fig.88: Leaf number and leaf length of *S.suaveolens* in different substrates after 4 weeks

Soil

The different plantlets were transferred in thermocol cups filled with soil.Similar to sand the soil substrate obtained poor response as only 13% of the plants survived (Fig.87). The plant also failed to obtained growth in terms of increase in shoot and root length and plant height.

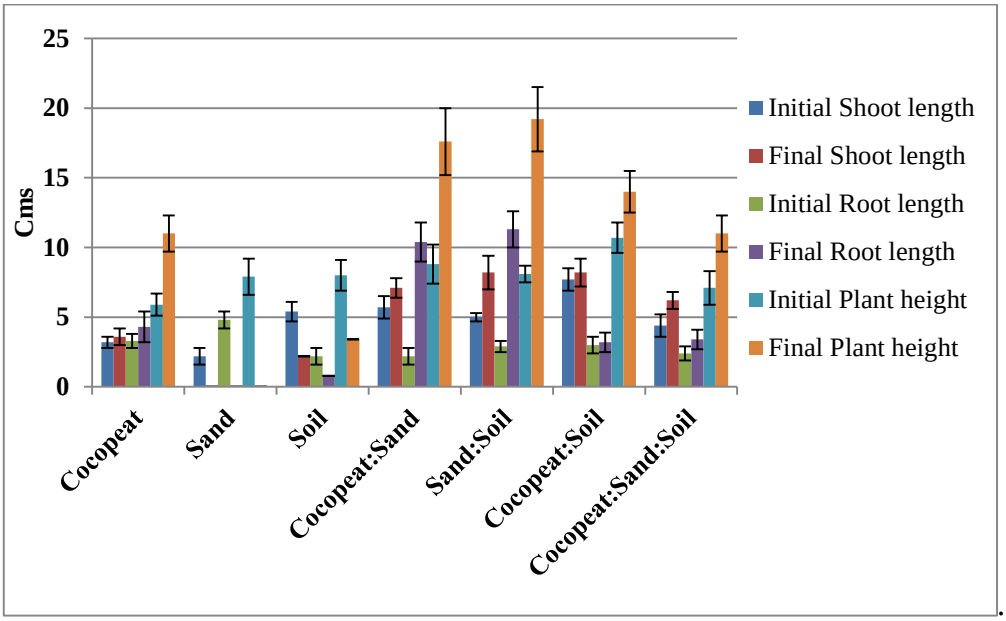

Fig. 89: Initial and final shoot, root length and plant height of *S.suaveolens* in different substrates

Cocopeat: Sand

The *in vitro* rooted plants were transfered to thermocol cups filled with cocopeat:sand (Fig.90a,b)and out of all 63% of them survived by end of four weeks(Fig.87).The development of more than four new leaves took place(5.0 ± 0.6) but the leaf growth was not much healthy as they were smaller in size (1.9 ±0.4cms)compared to other substrates(Fig.88). There was an increase in shoot length from 5.7 ± 0.8 to 7.1 ± 0.7cms and root length from 2.2 ± 0.6 to 10.4 ± 1.4cms. The plant height reached to 17.6 ± 2.4cms by the end of four weeks (Fig.89) which was more compared to cocopeat:soil (Fig.90c,d). These plants were also transferred to plastic pots filled with soil (Fig.90e) for their further growth. But they ceased to grow and resulted in mortality within 2 weeks of transfer.

Fig.90: Hardening of *S.suaveolens* plants in Cocopeat: Sand substrate

a. *In vitro* plantlet

b. Plantlet transferred in Cocopeat:Sand substrate

c. and d. Plant growth with increase in shoot and root length after 4 weeks

e. Plant transferred to pot filled with soil

Fig.91: Hardening of *S.suaveolens* plants in Sand:Soil substrate

a. *In vitro* plantlet
b. Plantlet placed in Sand:Soil substrate
c. Plantlet covered with polybag showing growth with emergence of new leaves
d. and e. Increased in shoot and root length after 4 weeks
f. Plant transferred to plastic pot filled with soil

Sand: Soil

Since sand and soil failed to help individually for hardening of plants the in vitro plants were transferred to sand and soil in combination (Fig.91a,b).It was observed that the percent survival rate of plantlets increased to 25% by the end of four weeks (Fig.87).There was formation of more than two new leaves at the apices within two weeks(Fig.88;Fig.91c). In the plants there was growth observed in terms of shoot length, root length and plant height in the plants (Fig.91d,e).The shoot length from 5.0 ± 0.3cms to 8.2 ± 1.2cms ,while root length drastically increased from 2.9 ± 0.4 to 11.3 ± 1.3cms with plant height reaching 19.2 ± 2.3cms which was maximum among all the substrates(Fig.89). These plants after transferring to plastic pots filled with soil (Fig.91f) failed to show any further growth.

Cocopeat: Soil

The *in vitro* plantlets were transferred to cocopeat:soil (1:1) substrate(Fig.92a,b).It was observed that all the plants (100%) survived by the end of four weeks(Fig.87) .The plants developed new healthy leaves (Fig.92c) which by the end of four weeks increased in number as well as in length ie.3.6 ± 0.4cms (Fig.88)which was maximum among all the other substrates. New secondary roots were formed which increased in number as well as length by the end of four weeks. Healthy growth of plants was observed with slight increase in shoot (7.7 ± 0.8 to 8.2 ± 1.0cms) and root length (3.0 ± 0.6 to 3.2 ± 0.7cms) (Fig.89d) .The plant height from 10.7 ± 1.1cms reached to 14.0 ± 1.5cms which compared to sand:soil substrate was less(Fig.89). These plants were transferred to plastic pots filled with soil for further growth and pots were covered with transparent polybags for 4-5 days. By the end of eight weeks there was a healthy growth observed in the plants (Fig.92e). The leaves texture become rough and thick and there was a marked increase in shoot and root length of plant ie.7.4 ± 0.4 and 11.9 ± 2.7cms and the plant height reached to 19.2±1.8cms. Therefore the plant was finally transferred to garden pots (Fig.92f) for their normal growth which then developed into a healthy plant.

Cocopeat: Sand: Soil

When cocopeat: sand: soil was used in combinations the rate of survival of the plantlets increased to 75% compared to individual substrates by the end of four weeks (Fig.87). The *in vitro* plantlets transferred obtained growth with emergence of new leaves at the apices (Fig.93a,b) within 10-15 days (2 weeks) and increased in length by end of four weeks with

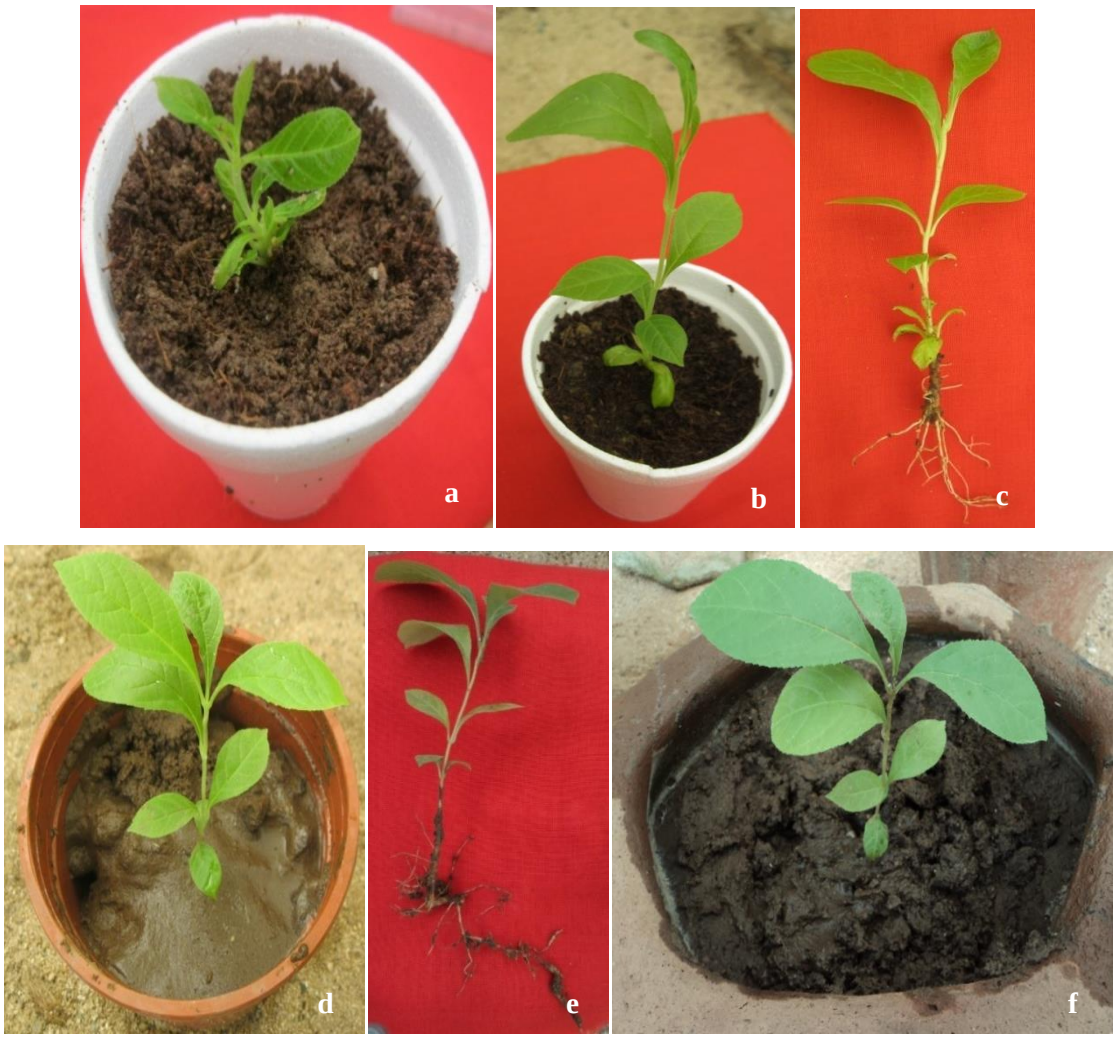

Fig.92: Hardening of *S.suaveolens* plants in Cocopeat:Soil substrate

a. The *in vitro* plantlet placed in Cocopeat:Soil substrate
b. Plant growth with emergence of new leaves
c. Increase in shoot and root growth after 4 weeks
d. Plant showing growth with expanded healthy leaves in pot filled with soil by the end of 8 weeks
e. Plant with increased shoot and root length
f. Plant growth in garden pot filled with soil after 12 weeks

Fig.93: Hardening of *S.suaveolens* plants in Cocopeat: Sand: Soil substrate

a. *In vitro* plantlet
b. Plantlet transferred to Cocopeat: Sand: Soil filled with thermocol cup
c. Plant showing healthy growth with emergence of new leaves after 4 weeks
d. Increased in plant height, shoot and root length after 4 weeks
e. Plant transferred to pot for further growth

2.4 ± 0.3cm (Fig.88;Fig.93c).There was a healthy development of plants occurred in term of increase in plant height which reached to 11.0 ± 1.3cms by the end of four weeks(Fig.93d) as similar to individual cocopeat substrate. The shoot length increased from 4.4 ± 0.8 to 6.2 ± 0.6cms and root length slightly increased from 2.4 ± 0.5 to 3.4 ± 0.7cms (Fig.89).Similar to other substrates these plants were also transferred to plastic pots filled with soil (Fig.93e) but these plants ceased to grow.

Therefore from the above studies it can be concluded that the individual substrates cocopeat, sand and soil were not effective for the growth and development of *S.suaveolens* plants. The cocopeat worked well in combination with soil and sand with good survival rate as well as growth of plants (cocopeat:sand, cocopeat:soil, cocopeat:sand:soil). But out of all the substrates cocopeat:soil was optimum with 100 % survival rate and proved to be beneficial in terms of growth of *S.suaveolens* plant.

Hence the regeneration studies in *S.suaveolens* concludes that

- The observations revealed that cotyledonary leaf and hypocotyl explants failed to establish shoot cultures in MS and WPM medium fortified with individual cytokinin. Cotyledonary node and nodal explants formed shoots exhibiting their regeneration capacity in presence of cytokinin.
- The nodal explants were suitable in terms of regeneration of shoots as compared to cotyledonary node.
- Although both the media helped in shoot and root induction but the overall results depicts that MS medium was effective in *S.suaveolens*. TDZ was the suitable cytokinin among all the cytokinins used individually as well as in combination.
- The half strength MS medium fortified with IBA was the suitable auxin in terms of root induction.
- Cocopeat:Soil substrate was optimum for the survival and growth of *S.suaveolens* plants.

4.4.6 Section III: Synthetic seed studies

The synthetic seed studies were carried out in *S.sauveolens* as follows:

4.4.6.1 Effect of various encapsulation matrices on synseed germination in regenerative media with different storage period

In *S. suaveolens* also the effect of different combination of matrices and regenerative media at various storage period on germination of synseeds was observed. The *in vitro* nodes were used as a explants source for the preparation of synseeds. These nodes were encapsulated in 3% sodium alginate mixed with medium fortified without or with PGRs and incubated in 75mM $CaCl_2$ aqueous solution. Since from the regeneration studies MS medium proved to be effective for multiple shoot formation in *S.suaveolens* therefore all the matrix and regenerative medium were prepared in the same.

These nodes were encapsulated in different matrices of half or full strength basal medium (control) and fortified with PGRs and placed on different regenerative medium(liquid and static) immediately at 0 day while the rest were stored at 4ºC in petridish containing filter paper with liquid half strength or full strength MS medium. The synseeds were placed on respective regenerative media(liquid and static) after different (7day,15day and 30day) storage period and observations for germination in terms of shoot or shoot and root were recorded after 4 weeks.

The different half strength and full strength matrices and their regenerative medium are discussed below:

4.4.6.1.1 Effect of half strength and full strength MS medium matrix on synseed germination

The *in vitro* nodes encapsulated in half strength basal medium matrix were placed in MS basal regenerative medium which served as control and the medium was fortified with PGRs ie.IBA (2µM), NAA (2µM), GA_3 (10µM) and an organic supplement coconut water (10%).All the synseeds formed resulted with varied response in terms of % shoot emergence and % shoot and root formation in different regenerative medium at various storage period.

The encapsulated *in vitro* nodes transferred in liquid basal regenerative medium failed to germinate while in static poor response was observed at each time interval.

When liquid and static medium was fortified with an auxin IBA (2µM), the liquid medium obtained poor response and in static there was 17% germination of synseeds into 0.2 ± 0.2 shoot from synseeds transferred at 0 day but with increase in storage period to 7 days reached to 50% in terms of 0.5 ± 0.2 shoots. After 15 and 30 days storage none of the synseeds remain viable. When medium was replaced by NAA (2µM) the liquid medium was better in comparison to static as the synseeds remain viable till 15 days of storage. The synseeds when transferred at 0 day 33 % shoot emergence was observed which increased to 67% germination into an average of 0.7 ± 0.2 shoots after 7 days of storage, while it again decreased to 33% germination into small shoots after 15 days of storage. The auxins were then replaced by GA_3 (10µM) and the synseeds were transferred at 0 day in liquid medium which resulted in 67 % germination into 0.7 ± 0.2 shoots after 4 weeks,but the germination frequency decreased with increase in storage to 7 days, in both liquid and static as only 33% synseeds germinated into 0.3 ± 0.2 shoots. When coconut water in medium was taken it improved the response as it resulted in 100 % germination into 1.0±0.0 shoots in liquid medium after 4 weeks when the synseeds were transfered at 0 day while in static 67% synseeds emerged into 0.7 ± 0.2 shoots. But after storage of 7, 15 and 30 days synseeds transferred in liquid and static medium lost the germination frequency (Table 33).

Compared to half strength when *in vitro* nodes were encapsulated in full strength basal medium matrix the response was improved.

It was observed that the static regenerative media proved to be effective in terms of germination of synseeds after 4 weeks. The synseeds transferred in liquid and static basal regenerative medium at 0 day or after storage of 7 days resulted in nearly same response in terms of shoot emergence while in static the germination was observed into 0.3 ± 0.2 shoots even after storage of 15 days (Table 33).

Incorporation of auxins in medium ie. IBA (2µM) and NAA (2µM) the response improved. The synseeds transferred at 0day resulted in 83% response in terms of 0.8±0.2 shoots emergence in liquid IBA(2µM) medium whereas in static maximum of 100% germination into an average of 1.0 ± 0.0 shoots and 2.5 ± 0.8 roots was observed after 4 weeks(Fig.94a). The synseeds after 15 days of storage obtained germination in static medium only, which reached to 67% with emergence of 0.7 ± 0.2 shoots. Similarly synseeds transferred at 0 day in the liquid medium fortified with NAA (2µM) obtained less response while in static medium**,**

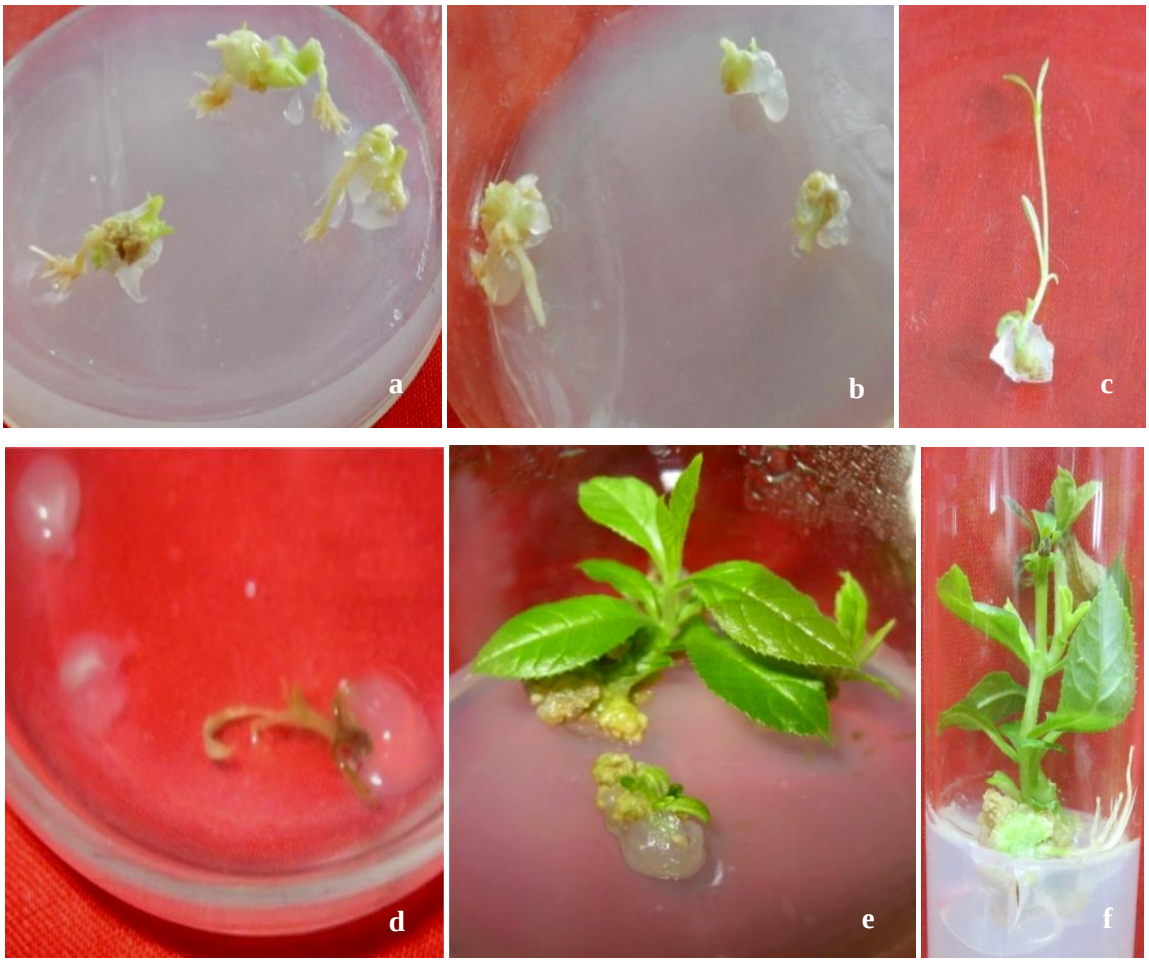

Fig.94: Effect of MS basal medium matrix on *S.suaveolens* synseed germination after 4 weeks

a. Emergence of shoot and root from synseeds placed in MS regenerative medium fortified with IBA(2µM) transferred at 0 day

b. Synseeds germinated in MS+NAA (2µM) regenerative medium placed on 0 day

c. Development of single shoot from synseeds placed at 0 day in MS+GA_3 (10 µM) regenerative medium

d. Formation of single shoot from synseeds placed 15 days of storage in MS+GA_3 (10µM) regenerative medium

e. Emergence of healthy shoot from synseeds placed at 0 day in MS + coconut water (10%)

f. Elongation of shoot in MS+CW (10%) after 4 weeks

100 % germination was observed of which 33% synseeds emerged into shoots and 67 % synseeds emerged into shoots and roots but they remain stunted (Fig.94b) with a total of 1.0±0.4 shoots and 0.7±0.2 roots. Further the germination frequency in terms of shoot and root decreased to 50 % after storage of 7 and 15 days. When medium was supplemented with liquid GA_3 (10µM) the synseeds transferred in the same at 0 day and after storage of 7 and 15 days germinated into shoot with 33% response (Fig.94c,d) while in static 100% synseeds germination occurred at 0day transfer (Fig.94e)but failed to induce response after storage. The synseeds were also transferred to liquid and static medium fortified with coconut water at 0 day which resulted in varied % shoots emergence after 4 weeks but the number remained same and after storage of 7 days the percent and number of shoots emerged differed (Table 33).

The synseeds were then transferred to medium fortified with coconut water for elongation. The shoots and roots developed from synseeds placed after 7 days of storage in full strength static medium fortified with NAA (2µM) when transferred to coconut water medium there was an increase root length but shoot length remain as it is. While the shoots developed from synseeds placed in at 0 day in static coconut water regenerative medium when transferred to same medium get elongated to single healthy shoot with big leaves (Fig.94f).
Therefore the basal medium matrix was helpful in germination of synseeds in terms of shoot and shoot and root emergence.

Table 33. Effect of half and full strength MS medium matrix on *S.suaveolens* synseed germination in after 4 weeks

		Half MS basal medium matrix							
RM	**Storage days**	**%Shoot emergence**		**% Shoot and Root emergence**		**Number of shoots***		**Number of roots***	
Half strength		**liquid**	**static**	**liquid**	**static**	**liquid**	**static**	**liquid**	**static**
BM	0	0	17	0	0	0.0 ± 0.0^{a}	0.2 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	$0.0\pm.0.0^{a}$	$0.0\pm.0.0^{a}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	17	17	0	0	0.2 ± 0.2^{ab}	0.2 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	50	0	0	0.0 ± 0.0^{a}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

	15	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
NAA(2µM)	0	33	33	0	0	0.3±0.2^{ab}	0.3±0.2^{ab}	0.0±0.0^{a}	0.0±0.0^{a}
	7	67	0	0	0	0.7±0.2^{c}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	15	33	0	0	0	0.3±0.2^{ab}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
GA_3 (10µM)	0	67	0	0	0	0.7±0.2^{c}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	7	33	33	0	0	0.3±0.2^{ab}	0.3±0.2^{ab}	0.0±0.0^{a}	0.0±0.0^{a}
	15	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
CW (10%)	0	100	67	0	0	1.0±0.0^{c}	0.7±0.2^{c}	0.0±0.0^{a}	0.0±0.0^{a}
	7	67	0	0	0	0.7±0.2^{c}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	15	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
		Full strength MS basal medium							
RM	**Storage days**	**%Shoot emergence**		**% Shoot and Root emergence**		**Number of shoots***		**Number of roots***	
Full strength		**liquid**	**static**	**liquid**	**static**	**liquid**	**static**	**liquid**	**static**
BM	0	17	50	0	0	0.2±0.2^{ab}	0.5±0.2^{abcd}	0.0±0.0^{a}	0.0±0.0^{a}
	7	17	17	0	0	0.2±0.2^{ab}	0.2±0.2^{ab}	0.0±0.0^{a}	0.0±0.0^{a}
	15	0	33	0	0	0.0±0.0^{a}	0.3±0.2^{abc}	0.0±0.0^{a}	0.0±.00^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
IBA(2µM)	0	83	0	0	100	0.8±0.2^{cd}	1.0±0.0^{c}	0.0±0.0^{a}	2.5±0.8^{c}
	7	0	50	0	0	0.0±0.0^{a}	0.5±0.2^{abcd}	0.0±0.0^{a}	0.0±0.0^{a}
	15	0	67	0	0	0.0±0.0^{a}	0.7±0.2^{bcd}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
NAA(2µM)	0	50	33	0	67	0.5±0.2^{abcd}	1.0±0.4^{c}	0.0±0.0^{a}	0.7±0.2^{b}
	7	33	0	0	50	0.3±0.2^{abc}	0.5±0.2^{abcd}	0.0±0.0^{a}	0.5±0.2^{b}
	15	0	0	0	50	0.0±.0.0^{a}	0.5±0.2^{abcd}	0.0±0.0^{a}	0.5±0.2^{b}
	30	0	0	0	0	0.0±0.0^{a}	0.0.±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
GA_3 (10µM)	0	33	100	0	0	0.3±0.2^{abc}	1.0±0.0^{c}	0.0±0.0^{a}	0.0±0.0^{a}
	7	33	0	0	0	0.3±0.2^{abc}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	15	33	0	0	0	0.3±0.2^{abc}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
CW(10%)	0	33	100	0	0	1.0±0.6^{c}	1.0±0.0^{c}	0.0±0.0^{a}	0.0±0.0^{a}
	7	100	33	0	0	1.0±0.0^{c}	0.3±0.2^{abc}	0.0±0.0^{a}	0.0±0.0^{a}
	15	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	30	0	0	0	0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test.

4.4.6.1.2 Effect of half strength MS medium matrix fortified with BAP(20µM) on synseed germination

The *in vitro* nodes encapsulated in this matrix when transferred to liquid basal medium at 0day resulted in 67% germination into 0.7±0.2 shoots after 4 weeks whereas in static medium 33% germination was observed into 0.3±0.2 shoots but with increase in storage period the viability decreased (Table 34).

Similar response was observed in liquid medium fortified with IBA (2µM) as 67% synseeds germinated into 0.7±0.2 shoots whereas in static 33% synseeds germinated into 0.3 ± 0.2 shoots. The synseeds after storing of 7, 15 and 30 days in both liquid and static medium resulted in similar response. Whereas the synseeds transferred at 0 day in liquid medium fortified with NAA (2µM) resulted in 100% germination into 1.0±0.0 shoots and in static medium 50 % germination was observed but later on with increase in storage period the germination frequency decreased (Table 34). When medium was supplemented with liquid Kn (8µM) medium the synseeds placed at 0day resulted in 33% germination after 4 weeks, which when transferred after storage of 7, 15 days failed to germinate but after 30 days storage it regains the germination frequency with 33% response.In static medium the germination was observed after 7 days of storage only with 67% response in terms of 0.7±0.2 shoots which remain stunted after emergence (Table 34).

The synseeds developed in full strength MS medium fortified with BAP (20µM) matrix resulted in poor response in all the regenerative media at each storage period. When transferred at 0day it resulted in 17 % germination in liquid basal regenerative medium only, IBA (2µM) and NAA (2µM) whereas static medium failed to germinate the synseeds at each interval (Table 34).

The shoots developed in half strength static medium fortified with Kn (8µM) also get elongated after transferring to coconut water medium.

Overall results proves that this matrix failed to germinate the synseeds in terms of shoot and root emergence.

Table 34. Effect of half and full strength MS medium matrix fortified with BAP(20µM) on *S.suaveolens* synseed germination after 4 weeks

		½ MS+BAP(20µM)			
RM	**Storage days**	**% Shoot emergence**		**Number of shoots**	
Half strength		**liquid**	**static**	**liquid**	**static**
BM	0	67	33	0.7 ± 0.2^{bc}	0.3 ± 0.2^{ab}
	7	0	33	0.0 ± 0.0^{a}	0.3 ± 0.2^{ab}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	67	33	0.7 ± 0.2^{bc}	0.3 ± 0.2^{ab}
	7	33	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
NAA(2µM)	0	100	50	1.0 ± 0.0^{c}	0.5 ± 0.2^{b}
	7	0	33	0.0 ± 0.0^{a}	0.3 ± 0.2^{ab}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn(8µM)	0	33	0	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}
	7	0	67	0.0 ± 0.0^{a}	0.7 ± 0.2^{bc}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	33	0	0.3 ± 0.2^{a}	0.0 ± 0.0^{a}
Full strength		**MS+BAP(20µM)**			
BM	0	17	0	0.2 ± 0.2^{b}	0.0 ± 0.0^{a}
	7	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	17	0	0.2 ± 0.2^{b}	0.0 ± 0.0^{a}
	7	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
NAA(2µM)	0	17	0	0.2 ± 0.2^{b}	0.0 ± 0.0^{a}
	7	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn(8µM)	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test.

4.4.6.1.3 Effect of half strength and full strength MS medium matrix fortified with Kn (8µM) on synseed germination

The *in vitro* nodes encapsulated in half strength medium fortified with Kn(8µM) matrix were also placed in various media at 0 day and after storage of 7,15 and 30 days.

It was observed that the synseeds transferred at 0day in both liquid and static basal regenerative medium failed to germinate after 4 weeks except in static medium synseeds stored for 7 days germinated into 50% shoots after 4 weeks.

Incorporation of PGRs slightly improved the response. The synseeds transferred at 0day in liquid MS medium fortified with IBA (2µM) failed to germinate but when transferred after 7 days of storage it resulted in 100 % synseeds germination of which 67% germinated into small shoots and 33% emerged into plantlets forming a total of 1.0 ± 0.4 shoots and 0.7 ± 0.4 roots (Fig.95a) while in static medium 33% synseeds germinated into only 0.3 ± 0.2 shoots. With increase in storage period to 15 days in liquid medium the germination into 0.3 ± 0.2 shoots and 1.0 ± 0.6 roots with 33% response was observed (Fig.95b). While the liquid and static medium fortified with NAA (2µM) failed to germinate the synseeds at any time interval except after 7 days of storage resulted in 67% germination into 0.7 ± 0.2 shoots. When medium was supplemented with two cytokinins ie.BAP(4µM) with Kn(8µM) the response varied. The synseeds placed in liquid medium obtained poor response in terms of germination. Whereas in static medium the germination frequency increased with increase in storage ie. it retains it viability even after 7 days and 15 days of storage with 67% shoot emergence and maximum number of 1.3 ± 0.4 and 1.0 ± 0.4 shoots respectively (Fig.95c,e) (Table 35).

The *in vitro* nodes encapsulated in full strength matrix when transferred to liquid basal regenerative medium resulted in better response compared to half strength as there was 100 % germination in terms of 1.0 ± 0.0 shoots emergence (Fig.95f) whereas in static medium 67% synseed transferred at 0 day germinated into 0.7 ± 0.2 shoots, but with increase in storage period there was nil germination (Table 35).

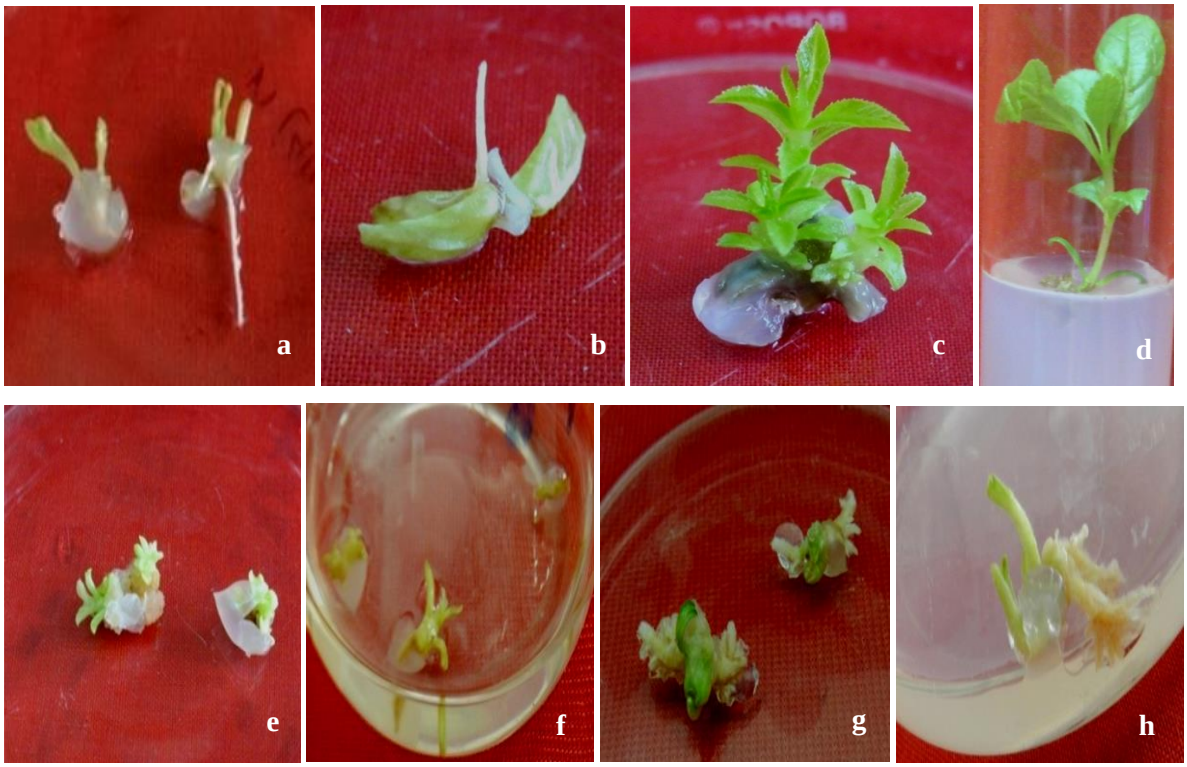

Fig.95: a-d Effect of ½ MS+Kn(8µM)matrix on *S.suaveolens* synseed germination after 4 weeks

a. **Emergence of shoot and root from synseed placed after 7 days of storage in liquid regenerative medium fortified with IBA(2µM)**
b. **Synseeds stored for 15 day emerged into shoot and root when placed in liquid IBA (2µM)**
c. **Development of multiple shoot from synseeds transfer after 7 days of storage to BAP(4 µM) +Kn(8µM) regenerative medium**
d. **Shoot which developed in BAP(4µM)+Kn(8µM) elongated after transferring to MS+CW(10%) medium**
e. **Stunted shoots emergence from 15 day stored synseeds placed in BAP(4µM) +Kn(8µM) regenerative medium**

f-h Effect of full strength MS+Kn(8µM) matrix on synseed germination after 4 weeks

f. **Shoot emergence from synseeds placed at 0day in liquid MS Basal medium**
g. **Shoot and root emergence from synseed placed at 0day in MS+NAA(2µM)**
h. **Emergence of shoot and root from after 15 day stored synseeds when placed in MS+NAA(2µM) regenerative medium**

When synseeds were placed in both liquid and static MS medium supplemented with IBA (2µM) at 0day it resulted in 67% germination into 0.7 ± 0.2 shoots. But after storage the synseeds transferred in the liquid medium failed to germinate while in static the germination initially decreased after 7 days of storage and reached to 67% after 15 days of storage in terms of 0.7 ± 0.2 shoots emergence. Whereas the synseeds placed in liquid NAA(2µM) medium at 0 day and after 7 day storage resulted in shoot emergence but varied in percent response and number and in static NAA(2µM) medium all the synseeds transfered at 0day germinated after 4 weeks of which 67% germinated into small shoot with roots (Fig.95g) and 33% into only shoots with a total 1.0 ± 0.4 number of shoots and 1.3 ± 0.4 roots. It retain the viability even after storage of 15 days as synseeds germinated into 0.5 ± 0.2 shoots and 1.5 ± 0.7 roots in static medium (Fig.95h). Transfer of synseeds at 0day and after storage of 7 days to liquid BAP(4µM) with Kn(8µM) medium 67% germination into 0.7±0.2 shoots was observed but in static 33% germinated into shoot and 33% into shoot and root, with a total of 0.7 ± 0.4 shoots and 0.3 ± 0.2 roots after 4 weeks. In both liquid and static medium the germination frequency decreased with increased storage period (Table 35).

Since shoots and roots emerged after 7 days of storage from synseeds placed in full strength static medium fortified with NAA (2µM) were small they were transfered to medium with only coconut water (10%) for elongation. After 4 weeks it was observed that there was increase in root length only while shoots failed to elongate .Also the shoots emerged after 7 days of storage in full strength medium fortified with IBA(2µM) were stunted and hence transferred in the same which resulted in elongation of shoots after 4 weeks of transfer.

When the small shoots developed in half strength liquid medium fortified with BAP(4 µM) with Kn(8µM) after 7 days of storage were transferred to medium supplemented with coconut water(10%) resulted in increased in length (Fig.95d). Also the shoots developed after 7 days of storage in liquid medium with IBA (2µM) showed increase in shoot and root growth in terms of length after transferring to coconut water medium.

This matrix was helpful in germination of synseeds in terms of shoot and root emergence.

Table 35: Effect of half and full strength MS medium matrix fortified with Kn(8µM) on *S.suaveolens* synseed germination after 4 weeks

		½ MS+Kn(8µM) matrix							
RM	**Storage days**	**%Shoot emergence**		**%Shoot and Root emergence**		**Number of shoots**		**Number of roots**	
Half strength		**liquid**	**static**	**liquid**	**static**	**liquid**	**static**	**liquid**	**static**
BM	0	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	50	0	0	0.0 ± 0.0^{a}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	0	33	0	0	0.0 ± 0.0^{a}	0.3 ± 0.2^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	67	33	33	0	1.0 ± 0.4^{cd}	0.3 ± 0.2^{a}	0.7 ± 0.4^{a}	0.0 ± 0.0^{a}
	15	0	0	33	0	0.3 ± 0.2^{a}	0.0 ± 0.0^{a}	1.0 ± 0.6^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
NAA(2µM)	0	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	67	0	0	0	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
BAP(4µM)+ Kn(8µM)	0	0	50	0	0	0.0 ± 0.0^{a}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	33	67	0	0	0.3 ± 0.2^{a}	1.3 ± 0.4^{d}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	67	0	0	0.0 ± 0.0^{a}	1.0 ± 0.4^{cd}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Full strength		**MS+Kn(8µM) matrix**							
BM	0	100	67	0	0	1.0 ± 0.0^{c}	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	67	67	0	0	0.7 ± 0.2^{bc}	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	17	0	0	0.0 ± 0.0^{a}	0.2 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	67	0	0	0.0 ± 0.0^{a}	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
NAA(2µM)	0	33	33	0	67	0.3 ± 0.2^{ab}	1.0 ± 0.4^{c}	0.0 ± 0.0^{a}	1.3 ± 0.4^{b}
	7	67	0	0	0	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	50	0.0 ± 0.0^{a}	0.5 ± 0.2^{abc}	0.0 ± 0.0^{a}	1.5 ± 0.7^{b}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
BAP(4µM)+ Kn(8µM)	0	67	33	0	33	0.7 ± 0.2^{bc}	0.7 ± 0.4^{bc}	0.0 ± 0.0^{a}	0.3 ± 0.2^{a}
	7	67	67	0	0	1.0 ± 0.4^{c}	1.0 ± 0.4^{c}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	33	0	0	0	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test.

4.4.6.1.4 Effect of half and full strength MS medium matrix fortified with TDZ (0.2 µM) on synseed germination

The synseeds developed by encapsulating *in vitro* nodes in half strength MS medium fortified with TDZ(0.2µM) matrix failed to germinate when transferred to half strength basal medium, TDZ(0.2µM), IBA(2µM) and Kn(8µM) with TDZ(0.2µM) regenerative medium.

In full strength the synseeds transferred to liquid basal regenerative medium at 0 day resulted in 33 % germination in terms of 0.3 ± 0.2 shoots after 4 weeks whereas in static medium 50% synseeds germinated into 0.5 ± 0.2 shoots .But with increase in storage period the germination frequency decreased in both the media(Table 36). Therefore the synseeds were transferred to liquid medium supplemented with individual cytokinin TDZ(0.2µM) at 0 day which resulted in 50% synseeds germination into 0.5 ± 0.2 shoots while in static medium 67% shoot emergence was observed with 0.7 ± 0.2 number.

Table 36. Effect of full strength MS medium matrix fortified with TDZ(0.2µM) on *S.suaveolens* synseed germination after 4 weeks

RM	Storage days	%Shoot emergence		% Shoot and Root emergence		Number of shoots*		Number of roots*	
Full strength		liquid	static	liquid	static	liquid	static	liquid	static
		MS+TDZ(0.2µM) matrix							
BM	0	33	50	0	0	0.3 ± 0.2^{ab}	0.5 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	33	0	0	0.0 ± 0.0^{a}	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
TDZ(0.2µM)	0	50	67	0	0	0.5 ± 0.2^{bc}	0.7 ± 0.2^{c}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	33	33	0	0	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	0	67	0	17	0.0 ± 0.0^{a}	0.8 ± 0.2^{c}	0.0 ± 0.0^{a}	0.2 ± 0.2^{ab}
	7	50	0	0	17	0.5 ± 0.2^{bc}	0.2 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.3 ± 0.3^{b}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn(8µM) +TDZ(0.2µM)	0	67	0	0	0	0.7 ± 0.2^{c}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	33	0	0	0.0 ± 0.0^{a}	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p \leq 0.05$ according to Duncan's Multiple range test.

After storage of 7 days in both liquid and static medium the percent germination reduced to 33% in terms of shoot emergence and became nil after 15 and 30 days storage. Replacing the cytokinin with individual auxin IBA (2µM) none of the synseeds germinated in liquid medium transferred at 0day while in static 67% germinated into shoot (Fig.96a) and 17% sprouted into shoot and root with an average of 0.8 ± 0.2 shoots and 0.2 ± 0.2 roots.

Whereas synseeds transferred after storage of 7 days in liquid medium 50% emerged into 0.5 ± 0.2 shoots while in static 17% germinated into 0.2 ± 0.2 shoot and 0.3 ± 0.3 roots. The synseeds transferred at 0day in another liquid Kn (8µM) with TDZ (0.2µM) regenerative medium resulted in 67% germination into shoot after 4 weeks but failed to germinate at each storage period while in static poor response was observed except the synseeds transferred after 7 days of storage germinated into 0.3 ± 0.2 shoots with 33% response after 4 weeks (Fig.96b) (Table 36).

4.4.6.1.5 Effect of half and full strength MS medium matrix fortified with BAP(8µM) +TDZ(0.2µM) on synseed germination

The another matrix tried for encapsulation of *in vitro* nodes was half strength medium supplemented with BAP (8µM) with TDZ (0.2µM).

The synseeds transferred to liquid basal medium at 0day or after storage failed to induce morphogenic response while in static basal medium there was only 50% shoot emergence with 0.5 ± 0.2 number after 4 weeks. With increase in storage period the regenerating ability of synseeds ceased. When medium was supplemented with liquid and static IBA(2µM) medium there was not much improvement observed. The synseeds transferred to liquid and static medium at 0day resulted in similar 33% germination into shoots with varied number (Table 37), but after storage of 7 days synseeds germinated only in static medium reaching to 50% in terms of shoot emergence. At 0 day transferred of synseeds to liquid NAA(2µM) medium failed to respond whereas when transferred to static medium resulted in 33% emergence of 0.3 ± 0.2 shoots which remained same even after storage of 15 days (Fig.96c).

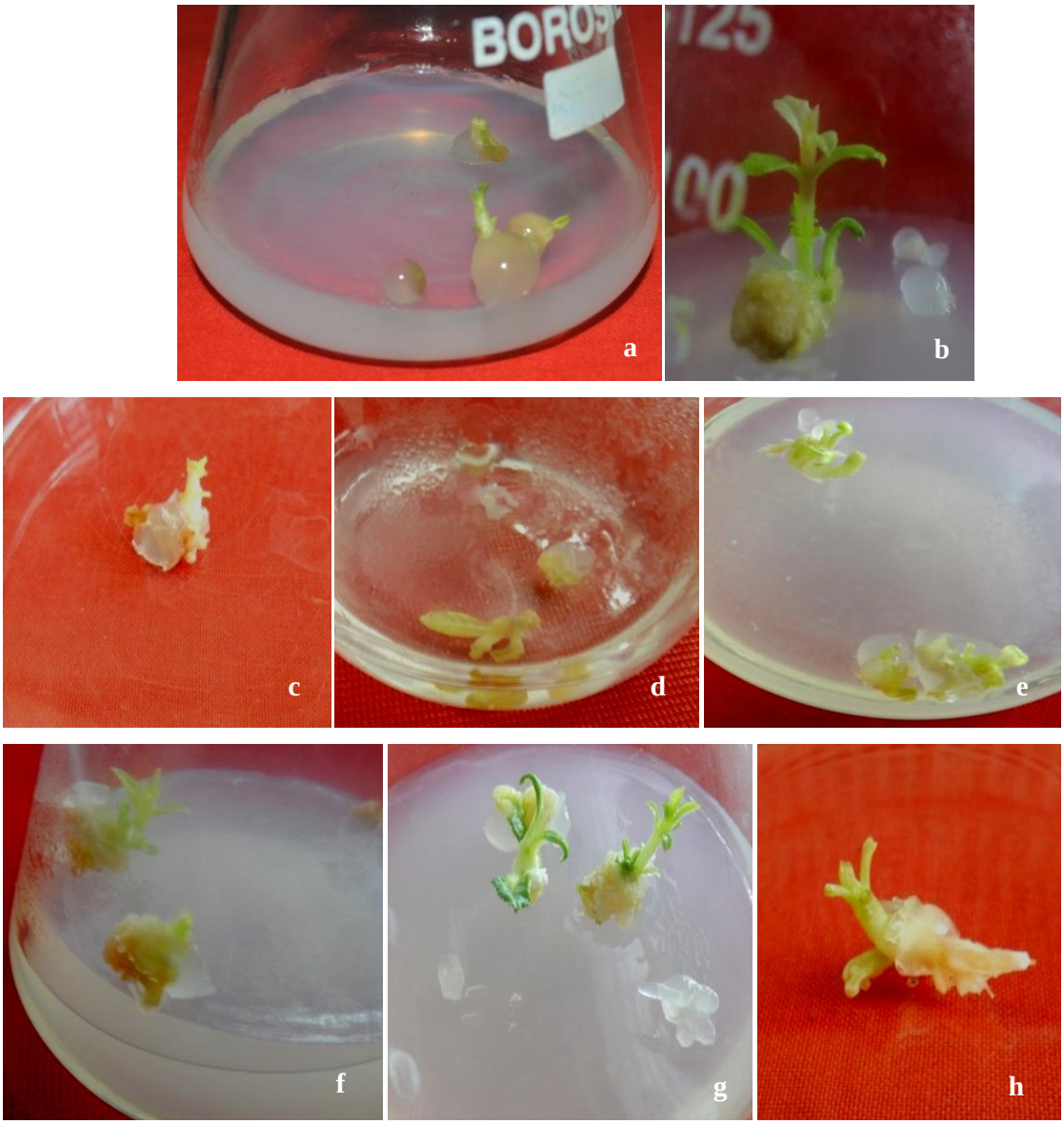

Fig.96:Effect of full strength MS+TDZ(0.2µM) matrix on *S.suaveolens* synseed germination after 4 weeks
a. Germination of synseeds placed at 0 day in IBA (2µM) regenerative medium
b. Single shoot emerged from 7 day stored synseeds placed in Kn(8 µM) +TDZ(0.2 µM) in regenerative medium
Effect of half strength MS+BAP (8µM)+TDZ(0.2µM) matrix on synseed germination
c. Small shoot developed from 15 day stored synseeds in NAA (2µM) medium
Effect of full strength MS+BAP (8µM) +TDZ (0.2µM) matrix on synseed germination after 4 weeks
d. Shoot developed from synseeds placed at 0day in liquid IBA (2µM) medium
e.Shoot and root emerged from synseeds placed at 0day in static IBA(2µM) regenerative medium
f. Shoot and root developed in IBA (2µM) after 15 day storage
g. Synseeds germinated into when placed in BAP (8µM)+TDZ(0.2µM) medium at 0day
h.Shoot and root emergence from 15 day stored synseeds placed in BAP (8µM)+TDZ(0.2µM) regenerative medium

Increasing the strength of medium fortified with 3% sucrose there was an improvement in germination frequency. When the synseeds were placed in full strength liquid and static basal regenerative medium at 0 day resulted in 67% synseeds germination into small shoots (0.7 ± 0.2) and the response was similar in static medium when transferred after storage of 7 days. The synseeds placed in liquid and static medium fortified with IBA (2µM) retain their viability even after 15 days of storage. At 0day transfer of synseeds to liquid medium resulted in 33% germination into 0.3 ± 0.2 shoots (Fig.96d) but the maximum germination frequency was observed in static medium ie total of 100% of which 83% emerged into shoot and 17% emerged into shoot and root (Fig.96e) with an average of 1.0 ± 0.3 shoots and 0.3 ± 0.3 roots. When the synseeds were transferred to liquid and static medium after storage of 7 days resulted in similar response ie 67% germination in terms of (0.7 ± 0.2) shoots emergence. But after 15 days of storage synseeds placed in liquid medium 33% emerged into 0.3 ± 0.2 shoot and in static 33% synseeds germinated into 0.3 ± 0.2 shoots and 0.3 ± 0.2 roots (Fig.96f). In another regenerative medium fortified with liquid and static medium NAA(2µM) synseeds transferred at 0day failed to germinate while after storage of 7 days when transferred to liquid medium 100% synseeds germinated into 1.0 ± 0.0 shoots and in static only 50% response was observed. In static medium the response remained the same even (50%) after 15 days of storage.

The shoot and root developed in static medium fortified with IBA (2µM) (0 day) when transfered to medium supplemented with coconut water, it failed to elongate while increased in root length was observed.

This matrix was also helpful in germination of synseeds in terms of shoot and root emergence.

Table 37. Effect of half and full strength MS medium matrix fortified with BAP(8µM) +TDZ(0.2µM) on *S.suaveolens* synseed germination after 4 weeks

		½ MS+BAP(8µM)+TDZ(0.2 µM)matrix							
RM	**Storage days**	**% Shoot emergence**		**%Shoot and Root emergence**		**Number of shoots**		**Number of roots**	
Half strength		**liquid**	**static**	**liquid**	**static**	**liquid**	**static**	**liquid**	**static**
BM	0	0	50	0	0	0.0 ± 0.0^{a}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	33	33	0	0	0.3 ± 0.2^{ab}	0.6 ± 0.4^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	50	0	0	0.0 ± 0.0^{a}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
NAA(2µM)	0	0	33	0	0	0.0 ± 0.0^{a}	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	33	0	0	0	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	33	0	0	0.0 ± 0.0^{a}	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}.
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Full strength		**MS+BAP(8µM)+TDZ(0.2µM) matrix**							
BM	0	67	67	0	0	0.7 ± 0.2^{bc}	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	0	67	0	0	0.0 ± 0.0^{a}	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
IBA(2µM)	0	33	83	0	17	0.3 ± 0.2^{ab}	1.0 ± 0.3^{c}	0.0 ± 0.0^{a}	0.3 ± 0.3^{b}
	7	67	67	0	0	0.7 ± 0.2^{bc}	0.7 ± 0.2^{bc}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	33	0	0	33	0.3 ± 0.2^{ab}	0.3 ± 0.2^{ab}	0.0 ± 0.0^{a}	0.3 ± 0.2^{b}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
NAA(2µM)	0	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	7	100	50	0	0	1.0 ± 0.0^{c}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	15	0	50	0	0	0.0 ± 0.0^{a}	0.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	30	0	0	0	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Values represent mean ± S.E of six replicates in each experiment. Means values followed by different superscript letters within a column are significantly different at $p\leq 0.05$ according to Duncan's Multiple range test.

The conclusion of synthetic seed studies in *S.suaveolens* is stated as:

- The half strength and full strength MS media were able to germinate synseeds in terms of shoot emergence and shoot and root emergence but compared to *O.indicum* in *S.suaveolens* full strength medium with 3% sucrose was able to germinate synseeds into shoots and roots.
- The maximum germination in terms of shoot emergence (67%) with an average of was observed from *in vitro* nodes encapsulated in half strength medium fortified with Kn(8µM) matrix when placed in regenerative medium fortified with BAP(4µM)+Kn(8µM) after 15 days of storage. It resulted in maximum number of shoots 1.3 ± 0.4 and 1.0 ± 0.4 after 7 and 15 days of storage respectively.
- The maximum percent germination into shoot and root (33%) was obtained when *in vitro* nodes were encapsulated in full strength MS medium matrix and transferred to NAA (2µM) regenerative medium and the encapsulated *in vitro* nodes in full strength MS medium fortified with Kn(8µM) transferred to NAA(2 µM) medium after 15 days of storage but the later one resulted in more number of roots(1.5 ± 0.7).
- The germination frequency of synseeds was mostly observed after 15 days of storage and it decreased after 30 days of storage in all the regenerative media except and half strength Kn(8µM) regenerative medium (half strength MS+BAP(20µM) matrix) the synseeds resulted in 33% germination in terms of shoots after 30 days of storage.

4.4.6.2 Effect of substrates on synseed germination

To compare the effect of substrates on synseed germination the matrix and regenerative medium which were suitable in terms of germination into shoot and root were selected from filter paper substrate and the same were taken for agar substrate. The MS medium fortified with Kn(8µM) matrix and MS regenerative medium fortified with NAA(2µM) were proved to effective for retaining the regenerative ability of synseeds into shoot and root after 15 days of storage and hence were selected.

The *in vitro* nodes encapsulated in full strength MS medium fortified with Kn (8µM) matrix were stored in petridish containing full strength static basal MS medium respectively. The synseeds were stored at 4ºC and harvested after 7, 15 and 30 days storage and observations were recorded after 4 weeks of transfer to each time interval.

4.4.6.2.1 A comparison of filter paper and agar substrate on germination of *S.suaveolens* synseed

The *in vitro* nodes were encapsulated in MS medium fortified with Kn(8µM) matrix stored in agar substrate were placed on NAA (2µM) regenerative medium. It was observed that the synseeds stored in agar substrate obtained better response compared to filter paper. The synseeds stored in agar substrate resulted in germination into shoots and shoot and root in both liquid and static medium after 7days (Fig.98c, d) and 15 days of storage. The percent germination was maximum in static after 7 days of storage (50%) and (33%) in liquid NAA (2µM) medium after 15 days storage compared to filter paper (Fig.97).

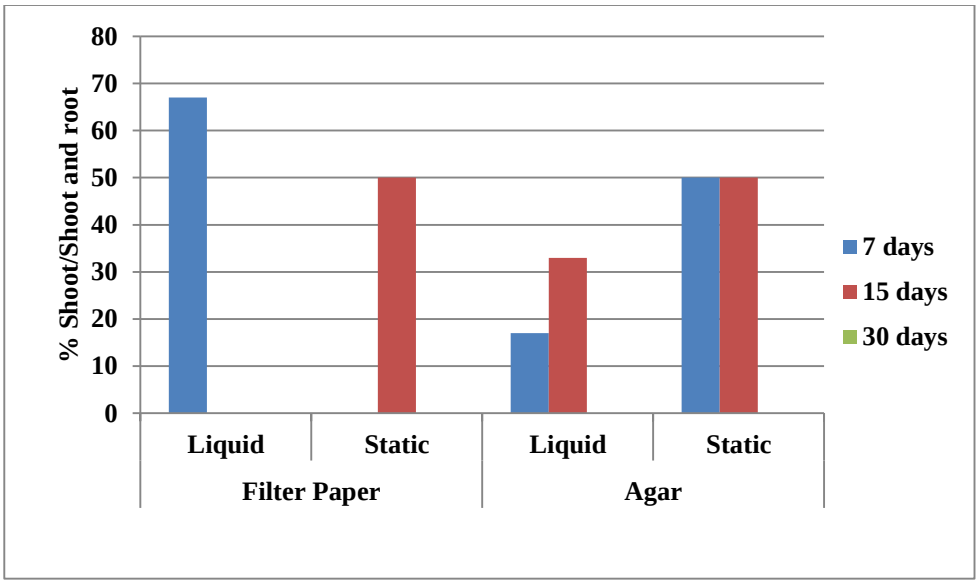

Fig.97:Effect of agar substrate in MS+Kn(8µM)matrix and MS+NAA(2µM) regenerative medium in *S.suaveolens*

Hence it can be concluded that in *S.suaveolens* the agar substrate could also be helpful in storing and retaining the regenerative ability of synseeds as that of filter paper.

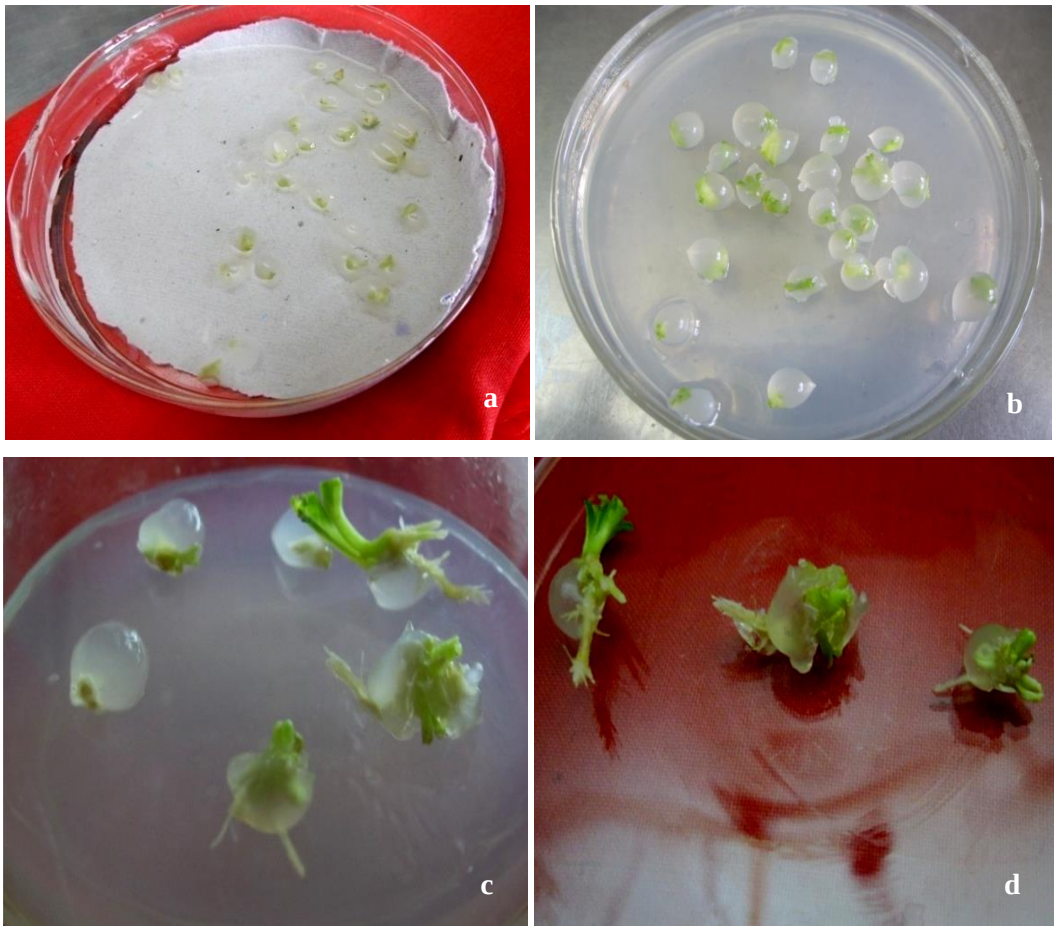

Fig.98: Effect of agar substrate on *S.suaveolens* synseed germination after 4 weeks
a. Synseeds placed in filter paper substrate for storage
b. Synseeds placed in agar substrate for storage
c. and d. Shoot and root emergence in NAA(2µM) medium (MS+Kn8µMmatrix) from synseeds stored for 7 day