CHAPTER 5

Discussion

DISCUSSION

In the present work tissue culture studies in tree species of family bignoniaceae ie. *Oroxylum indicum* and *Stereospermum suaveolens* were carried out. An attempt was made to develop a protocol for germination of seeds of these two species for generating large number of seedlings which were utilised for regeneration studies. After optimising the protocol for regeneration of these species studies for synthetic seeds were also done.

5.1 Seed germination studies

The initial experiments were carried out to germinate seeds of these two species as they possess low seed germination under natural conditions. Poor seed germination in tree species is a common problem which can be overcome by giving suitable treatment (Swaminathan and Revathy, 2013). Therefore the seeds of *O. indicum* and *S. suaveolens* were germinated in different substrates for the selection of a suitable substrate which allowed maximum germination and develop large number of seedlings. It has been known that the substrate selection are species specific and must be considered for seed germination for raising the seedlings. (Jaiswal and Chaudhary, 2005). There are several reports on effect of substrates on germination on plants like *Jatropha curcas* (Gairola *et al*, 2011*), Gonystylus bancanus* (Utami *et al*, 2006) and *Calendula officinalis* (Ming *et al*, 1999).

In the present studies cocopeat proved to be suitable substrate for seed germination of *O.indicum* and *S.suaveolens* as it develop maximum number of healthy seedlings within three weeks. Similar observations were recorded for *Gonystylus bancanus* (Utami *et al*, 2006) which had the highest percent of seed germination in cocopeat. Seedlings growth in cocopeat has been reported in a number of species like *Pterocarpus macrocarpus* (Kijkar, 1991), *Eucalyptus tereticornis* (Kumar amd Marimuthu, 1997) and *Swietenia macrophylla* (Woods *et al*, 1998). A combination of sand and soil substrate were not able to favour germination of *O.indicum* and *S.suaveolens* seeds, as sand has high permeability and soil has a very high tendency of compression, resulting in poor rate of germination (Ming *et al*, 1999).

The another substrate tried for seed germination was filter paper placed in petridish with distilled water. As it is known that filter paper can hold sufficient amount of water and dissipate water evenly all over the surface quickly and thus has effect on germination of seeds (Jaiswal and Chaudhary, 2005). Therefore seeds of both the species were germinated in petridish containing filter paper but the germination was less as compared to cocopeat and there was development of seedlings with curled leaves. The reason could be capillary rise is very important attribute which means that capacity of the substrate to circulate water throughout the surface, filter paper is the one which is having the moderate water holding capacity is considered good for germination (Jaiswal and Chaudhary, 2005).MS and WPM medium have different mineral salt concentrations which greatly affects *in vitro* germination and therefore the same were utilised for germination of seeds of both the tree species. In presence of MS and WPM medium poor germination was observed which indicated that macro and microelements were unnecessary for germination in *O.indicum* and similar reports are documented for *Vigna subterranean*(Kone *et al,* 2015).While in *S. suaveolens* the MS basal medium was equally effective as cocopeat for seed germination while the WPM medium failed to germinate the seeds. Similarly MS medium in *Pterocarpus marsupium* (Mishra *et al,* 2013) was effective for seed germination whereas in contrast WPM medium was effective for seeds germination of *Senna macranthera* (Faria *et al,* 2012).

Seed treatment can ensure both success in seed germination and germination speed to be quick and homogeneous (Azad *et al,* 2011a; 2011b). The seeds of two species under study when soaked in distilled water resulted in germination, similar observations are reported for seeds of *Strychnous spinosa* (Prins and Maghembe, 1994). Soaking in water prior to sowing is known to enhance rate and percent germination in species like *Cedrus deodara, Hardwickia binata, Pongamia pinnata, Cinnamomum camphora, Melia azadarach, Terminalia chebula* and *Terminalia tomentosa* (Bedell 1998).There are many reports which suggest that presowing treatments prove effective for seed germination in tropical forest tree species (Matin and Rashid, 1992, Koirala *et al,* 2000,Khan *et al,* 2001,Matin *et al,* 2006).

In *O. indicum* the cocopeat: sand combination played a synergistic role in the overall growth of seedlings in terms of length, collar diameter and biomass. Cocopeat helped in retaining moisture, providing organic nutrients while sand improved the texture in terms of porosity and aeration. The importance of sand along with other substrates in potting medium has been

observed which helped in seedling growth of sandalwood (Fox *et al,* 1990). The findings by Annapurna (2002) in sandalwood has shown that a mixture of planting substrates like compost, sand and soil can generate good quality seedlings. Whereas in papaya vermicompost:soil:sand (1:1:1) was optimum for seedling growth like length, biomass etc. (Bhardwaj, 2014).

In *S.suaveolens* the germination rate (5.4) of seeds was maximum in cocopeat as the seedling emerged within 10 days whereas in other substrate the rate of seedling emergence was slow. The mean daily germination was similar in cocopeat and MS medium(2.2).The germination index when calculated it was maximum in MS medium(9.9) and nearly same in cocopeat (8.9).Thus from the overall results it was observed that the cocopeat substrate was ideal substrate in developing many healthy seedlings at a faster rate. Cocopeat is considered as a good growing media component (Abad *et al,* 2002) and has been suitable for roses(Blom, 1999) and many potted plants(Treder and Nowak, 2002).Similarly the cocopeat has given higher parameters of germination in papaya seedling(Bhardwaj, 2014).The MS basal medium was also equally effective for mean daily germination and germination index. Similar findings where MS medium has proved to be effective are reported for seed germination in *Vigna subterranean* (Kone *et al*, 2015) and *Salvia sclarea* (Ghanbari *et al,* 2012).

Thus from the studies it is revealed that the seeds of *O.indicum* and *S.suaveolens* which possess less germination under natural conditions they can be germinated in cocopeat with development of large number of seedlings, as cocopeat is an ideal medium for seed raising applications and is commonly used as natural planting substrate (Yau and. Murphy, 2000).

5.2 Regeneration studies

In vitro clonal propagation has been extensively used for large scale multiplication of many important forest tree species (Bonga and von Aderkas, 1992;Ahuja, 1993) and therefore, in the present studies *in vitro* regeneration of two medicinally important forest trees *O.indicum* and *S.suaveolens* was carried out to develop a regeneration protocol.

5.2.1 Regeneration utilising different explants

In *O. indicum* and *S. suaveolens* different explants like cotyledonary leaf, hypocotyl, cotyledonary node and nodal were utilised and suitable one were selected for establishing

shoot cultures. Reports says that success of in vitro shoot formation depends on the type of explants used (Koroch *et al,* 2002; Zobayed and Saxena, 2003; Leng *et al,* 2004; Hong *et al,* 2004). The explant type governs the morphogenesis in tissue culture and they are known to affect multiple shoot induction in a number of tree species like *Dalbergia sissoo* (Pradhan *et al,* 1998),*Pterocarpus marsupium* (Anis *et al,* 2005),*Albizia lebbeck* (Mamun *et al,* 2004) and *Albizia odoratissima* (Rajeswari and Paliwal, 2006).Sharma and Rajam (1995) have stated that also reports that different explants resulted in different morphogenic responses in *Solanum melongena.*

5.2.1.1 Establishment and multiplication of shoot cultures

The shoot cultures of *O.indicum* and *S.suaveolens* were established and multiplied in MS and WPM medium fortified with PGRs.

5.2.1.1.1 Effect of individual cytokinins on shoot induction

In *O.indicum* and *S. suaveolens* all the explants varied in their morphogenic response when placed in MS and WPM medium fortified with individual cytokinins, (BAP/Kn/TDZ) as they are known to influence the induction of shoot/s (De Paiva Neto *et al,* 2003;Chakravarthi *et al,* 2010;Satapathy *et al,* 2014). Similar reports with a differential response of explants in presence of cytokinins had been observed in *Carthamus tinctorious* (Mahadevappa *et al,* 2014). According to Devi *et al* (2011) the explants from younger plants had more regeneration capacity than the explants procured from adult trees. This could be due to less amount of differentiation and more number of juvenile tissues present in seedling explants.

In the present studies when cotyledonary leaf explants of *O.indicum* and *S.suaveolens* were placed in MS and WPM medium fortified with individual cytokinins it failed to regenerate into shoots as there was only callus induction. Similarly in *Ricinus communis* friable callus was induced from cotyledon explant (Elaleem *et al,* 2015). The reason for failure in shoot formation and formation of callus in cotyledonary leaf segment explants could be the age of the explant, and its orientation in culture as reported in *Jatropha curcas* where the youngest explants induced the highest regeneration response as compared to one and two-week-old explants. They obtained best regeneration response in explants, prepared from cotyledonary leaf of freshly germinated seeds with 87.5% of the explants forming callus, out of which 94%

of the callus regenerated into shoots (Mazumdar *et al,* 2010). Famiani *et al* (1994) proposed that explants from young leaves show more regeneration potential than older leaves as the younger leaves have less differentiated and more metabolically active cells, and therefore, under suitable hormonal and nutritional conditions show improved plant regeneration. Furthermore, explants of different age may have different levels of endogenous hormones and, therefore, the age of explants would have a critical impact on the regeneration efficiency. There are other reports with similar results in plants like *Platanus occidentalis* (Sun *et al,* 2009), *Morus alba* (Thomas, 2003), *Cajanus cajan* (Dayal *et al,* 2003), *Rosa hybrida* (Ibrahim and Debergh, 2001), *Aegle marmelos* (Islam *et al,* 1993).

In *O. indicum* the hypocotyl explants were excised from seedlings below the cotyledonary node and placed on medium but it resulted in poor response as there was only callus induction in presence of individual cytokinins. A similar response with induction of callus from hypocotyls explants has been reported in *Vernicia fordii* (Lin *et al,* 2016).In *S.suaveolens* also the hypocotyl explant resulted in poor morphogenic response. There was formation of shoots in only 20% of cultures in the presence of Kn at 4µM which failed to survive after 4 weeks. Similar results were observed in *Bixa orellana* (De paiva neto *et al,* 2003) were only 10% hypocotyl explants produced adventitious shoots. The poor response may be because of the position of hypocotyl on the seedling and age of seedlings to differentiate into adventitious buds as reported in *Eucommia ulmoides* .The hypocotyl segments taken from 3-week-old seedlings and nearer to cotyledonary nodes formed less number of adventitious buds.In general, this may be related to explant position, polarity or age effects. (Chen *et al,* 2008).

In comparison to cotyledonary leaf and hypocotyl explants, the cotyledonary node and nodal explants proved to effective for regeneration of *S.suaveolens* and *O.indicum* as the shoot cultures were successfully established in MS and WPM medium fortified with individual or combination of PGRs. Mathur and Batra (2014) have also reported that cotyledonary nodes and nodal segments containing axillary buds have the potential for developing into complete plantlets.

There are many reports documenting successful establishment of cultures utilizing cotyledonary nodes like *Dalbergia sissoo* (Pradhan *et al,* 1998), *Quercus floribunda* (Purohit *et al,* 2002) and *Butea monosperma* (Ratnaprabha *et al,* 2017).The nodal explants also have a potential for rapid shoot regeneration as reported in tree species like *Psidium gujava*

(Amin and Jaiswal, 1987), *Sterculia urens* (Devi *et al,* 2011) and *Balanites aegyptiaca* (Siddique and Anis, 2009).

In the present studies 20 day old seedlings of both the species were utilised for regeneration studies. Cotyledonary nodes obtained from 21 days(young) seedlings have shown to respond early and differentiate large number of shoots in *Cassia sophera* (Parveen and Shahzad, 2010).Age of explants also plays a major role in inducing multiple shoot regeneration which has been reported in number of plant species including *Vicia faba* (Khalafalla and Hattiori 1999)*Morus alba* (Thomus, 2003) and *Pterocarpus marsupium* (Husain *et al,* 2007) whereas cotyledonary nodes obtained from 30 day seedlings of *Wrightia tomentosa* were used as explants and cultured on MS medium with BAP(5 mg/l)(Joshi *et al,* 2009).

In both the species different concentrations of the three individual cytokinins (BAP/Kn/TDZ) resulted in varied response. The type of cytokinins and its concentration depends on plant species for micropropagation of woody plants and various cytokinins differ in their activity (Nikolic *et al,* 2006).In *O.indicum* out of the three individual cytokinins (BAP/Kn/TDZ) used BAP at 8µM proved to be effective in inducing 83% response with 1.2 ± 0.4 number of shoots from cotyledonary node explants after 4 weeks whereas in WPM medium fortified with BAP (16µM) concentration was effective in establishing cultures. In *S.suaveolens* MS medium fortified with BAP (20µM), was effective in inducing shoot cultures from cotyledonary node explants whereas in WPM medium all the three cytokinins were effective at lower concentrations in establishing shoot cultures of which BAP at 8µM resulted in maximum response. In both the species the number of shoot decreased as the BAP concentration increased. Similarly in *Sapindus emarginatus* the number of shoots decreased as the concentration of BAP increased (Srinivas *et al,* 2015).This inhibitory effect of higher concentrations of BAP on shoot formation has also been reported in *Albizia chinesis* (Anis *et al,* 2005). In both the species MS medium fortified with Kn and TDZ resulted in poor response.

When nodal explants were utilised there was development of 1.7 ± 0.4 number of healthy shoots in MS medium fortified with BAP (20µM) and WPM medium fortified with BAP (16µM) resulted in inducing maximum shoots (1.3 ± 0.4) while Kn and TDZ resulted in poor response in *O.indicum*. Similarly BAP alone was sufficient to trigger the growth of the shoots in comparison to Kn in *Soymida febrifuga* (Chiruvella *et al,* 2013), *Salix tetrasperma* (Khan

et al, 2011) and in many woody plant species like *Balanites aegyptiaca* (Anis *et al,* 2010), *Vitex negundo* (Ahmad *et al,* 2008).

In comparison to cotyledonary node, the nodal explants of *S.suaveolens* TDZ proved to be the effective cytokinin followed by Kn and then BAP. MS medium fortified with TDZ (1 µM) and WPM medium fortified with TDZ (0.2µM) evoked a maximum of 83% and 100% response respectively for formation of shoot. Similarly when TDZ was added singly in medium it was effective for shoot induction in *Andrographis neesiana* (Karuppusamy and Kalimathu, 2010), *Sterculia urens* (Devi *et al,* 2011),*Sapindus emarginatus* (Srinivas *et al,* 2015).

In *O.indicum* the *in vitro* nodes were excised from shoots (after 4 weeks) which developed from cotyledonary node and nodal explants and subcultured on the same medium but the shoot number were only one or two per node in presence of individual cytokinins. There was formation of only 1.7 ± 0.2 shoots in WPM medium fortified with BAP(16µM) after 8 weeks and in WPM medium with BAP(8µM) an average of 2.0 ± 0.9 shoots were developed after 12 weeks from the *in vitro* nodes of cotyledonary node explants. Similarly in *Quercus floribunda* multiple shoots were induced in WPM medium with BA (22.19µM) which was much more effective (Purohit *et al,* 2002).

In *S.suaveolens* also the *in vitro* nodes were excised from shoots (after 4 weeks) and subcultured in fresh medium fortified with individual cytokinins for shoot multiplication. Similarly in *Pistachia vera* enhanced shoot induction was achieved from excised nodes on MS medium (Benmahioul *et al,* 2016).In *S.suaveolens* it was observed that the MS medium fortified with TDZ (0.1 and 0.2µM) and WPM medium fortified with Kn (8µM) were effective in the multiplication of shoots at every passages from cotyledonary node and nodal explants. But compared to cotyledonary node the *in vitro* shoots developed from nodal explants resulted in maximum shoot number upto 32 weeks. In MS medium supplemented with TDZ (0.1, 0.2µM) there was development of an average of one or two shoots which were healthy with normal leaves in every passage. Increase in shoot number at lower concentration of TDZ in *S.suaveolens* is in agreement with similar results in *Cassia angustifolia* (Siddique and Anis, 2007) and *Pterocarpus marsupium* (Husain *et al,* 2007), *Embelia ribes* (Raghu *et al,* 2006). Huetteman and Preece (1993), Murthy *et al* (1998) and

Faisal and Anis (2006) have also reported that the TDZ induces proliferation of axillary buds and produces multiple shoots in woody species. In *S.suaveolens* there was increase in shoot length after 16,24 and 32 weeks in MS medium fortified with TDZ (0.1µM, 0.2µM).This is in contrast to the findings reported in *Pyrus malus* (Van Nieuwkerk *et al*, 1986) and *Rhododendron* (Preece and Imel, 1991) where TDZ induced stunted shoots.

Compared to MS medium the WPM medium fortified with Kn (8µM) was optimum in inducing multiples in subsequent passages reaching to an average of 3.8 ± 0.6 shoots after 12 weeks. These shoots were strong and healthy with well-developed leaves as compared to cotyledonary node. Similar observations are reported for *Ficus carica*, the best shoot regeneration was reported on Woody Plant Medium (WPM) supplemented with Kn (Fra´guas *et al*, 2004) and also in *Vigna* and *Gerbera jamesonii* (Sen and Mukherjee, 1998, Tyagi and Kothari, 2004).

It has been known that fortification of culture media with different plant growth regulators i.e. auxins and cytokinins is not sufficient to regenerate large number of shoots. This cultures are improved by incorporating additives in the media which promotes their growth and development (Bansal and Gokhale, 2012).Therefore in *S.suaveolens* the effect of different additives like PVP, $AgNO_3$, Calcium pantothenate and Casein hydrolysate was studied for enhancement of shoots they were added in the following combinations like MS+TDZ(0.1 µM,0.2µM) and WPM+Kn(8µM). Observations revealed that MS medium fortified with TDZ (0.2µM) and $AgNO_3$ (20mg/l) resulted in maximum shoot number (3.0 ± 1.0) while medium fortified with TDZ (0.2µM) and Calcium pantothenate (0.5mg/l) resulted in maximum shoot length (7.8 ± 0.8cms). Similarly in potato maximum growth of plantlet was obtained on MS medium fortified with GA_3 (0.25mg/l) and calcium pantothenate (2mg/l) (Moeinil *et al*, 2011). Silver nitrate in MS media enhanced multiple shoot regeneration and *in vitro* growth of *Punica granatum* (L.) cv 'Bhagwa' (Chikkalaki *et al*, 2017). Supplementing media with silver nitrate has been well established in tissue culture as it is known to enhance shoot multiplication and somatic embryogenesis (Idei and Kondo 1998; Ramage and Williams 2002).Whereas addition of PVP (100mg/l) along with TDZ (0.2µM) in MS medium resulted in healthy single shoot formation, similar results are documented by Shukla *et al* (2009) in *Stereospermum personatum* where addition of PVP (250mg/l) induced healthy shoots. Incorporation of casein hyrolysate resulted in poor response in *S.suaveolens* as there was

formation of friable callus at base which resulted in weak shoots in all the concentrations tried. Similarly addition of CH (0.025%), induced basal callus which in turn reduced the shoot regeneration frequency, shoot number and length considerably in *Sterculia urens* (Hussain *et al,* 2008).

5.2.1.1.2 Synergistic effect of two cytokinins on cotyledonary node and nodal explants

The individual cytokinin was able to induce only one or two shoots a combination of two cytokinins (BAP+Kn, BAP+TDZ, Kn+TDZ) was tried in *O.indicum* and *S.suaveolens* to evaluate its effect on enhancing the number of shoots.

In *O. indicum* from cotyledonary node explants the MS medium supplemented with BAP (8 µM) with TDZ (0.2µM) resulted in maximum number of shoots (1.0 ± 0.4) after 4 weeks.

Similar observations are reported in *Stevia rebaudiana* (Ghauri *et al,* 2013) where BAP and TDZ combinations evoked an optimum response. Whereas the WPM medium fortified with BAP (8µM) and Kn (8µM) proved to be effective with shoot number reached to 1.2 ± 0.3 which was more compared to MS medium. Similarly in optimum shoot initiation was obtained in WPM medium fortified with BAP (4.43µM) and Kn(4.64µM) in *Mesua ferrea* (Jadhav and Deodhar, 2015).

In *S.suaveolens* the cotyledonary node explants obtained maximum number of shoots (2.0 ± 1.2) in MS medium fortified with BAP (8µM) and Kn (4µM) amongst all the combinations tried whereas in WPM medium fortified with Kn (2µM) and TDZ (0.2µM) resulted in 1.0 ± 0.0 shoots and 2.8 ± 0.5 nodes with 100% response which was less compared to MS medium. Similar findings have been reported in *Wedella calendulacea* (Emmanuel *et al*, 2000) where MS medium supplemented with BAP and Kn resulted in forming highest number of shoot.

When nodal explants of *O.indicum* were placed in combinations of cytokinins ,out of all, MS medium fortified with BAP(20µM) with Kn(8µM) resulted in average of 2.0 ± 0.6 number of shoots whereas in WPM medium fortified with BAP(16µM) with Kn(4µM) resulted in formation of 100% cultures with 2.0 ± 0.4 shoots. A synergistic effect of BA and Kn in promoting shoot initiation has been reported in *Acacia catechu* (Kaur and Kant, 2000;Thakur *et al,* 2002) and *Bauhinia vahlii* (Bhatt and Dhar, 2000).It was observed that in *O. indicum* Kn alone was ineffective in inducing shoots but along with BAP it was effective in formation of shoots. Similarly, Bhat *et al* 1995 reported the ineffectiveness of Kn alone for multiple shoot induction in *Piper sps*. The synergistic effect of BAP and Kn in promoting the shoot has also been reported by Emmanuel *et al* (2000) in *Wedella calendulacea*. In *S.suaveolens*

nodal explants placed in MS medium fortified with BAP (4µM) with Kn (4µM) resulted in forming 1.5 ± 0.3 number of shoots with 100% response, similar results with MS medium fortified with BAP and Kn was proved to be optimum in rose cultivars (Kumud *et al*, 2015).Whereas in WPM medium fortified with BAP (4µM)+Kn(8µM)resulted in maximum 1.3 ± 0.2 number of shoots this results are in accordance to that obtained in *Cinnamomum camphora* where WPM with BA(13.32µM) and Kinetin(4.65µM) gave best results in terms of shoot formation (Nirmal Babu *et al,* 2003).

The *in vitro* nodes excised from shoots which developed (after 4 weeks) from cotyledonary node were utilised for further multiplication of shoots but failed to enhance the shoot number in further passages. Similarly the *in vitro* nodes of shoots developed (after 4 weeks) from nodal explants were also subcultured in MS and WPM medium fortified with respective concentrations. After 8 weeks it was observed that WPM medium fortified with BAP (8µM) with Kn (8µM) resulted in maximum number of shoots (4.0 ± 0.7) followed by MS medium fortified with BAP (8µM) +TDZ (0.25µM) with only 1.7 ± 1.1 shoots whereas after repeated subculturing in the BAP (8µM) +TDZ (0.25µM) combination, it resulted in maximum shoot number (5.3 ± 1.5) by the end of 16 weeks but on further subculture after 24 and 32 weeks there was reduction in shoot number. In desert teak also multiplication rate of shoots was obtained during first four passage and the multiplication rate got stabilized at fifth passage and thereafter, a decline in number was observed (Varshney and Anis, 2012). Similar results has been documented in several plant species viz., *Syzygium alternifolium* (Sha Valli Khan *et al,* 1999), *Nyctanthes arbortristis* (Siddique *et al,* 2006), *Aegle marmelos* (Raghu *et al,* 2007) after repeated subculturing.

In *S.suaveolens* the *in vitro* nodes of shoots which developed from cotyledonary node obtained were less responsive in terms of multiple shoot formation when subcultured in respective combinations of MS and WPM medium. There was maximum of 2.0 ± 0.6 shoots with 100% response in WPM medium fortified with BAP (2µM) with TDZ (0.2µM) combination. Similarly in *Calophyllum inophyllum* multiple shoot formation was achieved on WPM supplemented with BAP (2.22–44.00µM) and TDZ (0.91–4.54µM) from the decapitated seedling explants (Thengane *et al,* 2006).

When *in vitro* nodes of shoots developed from nodal explants (after 4 weeks) were subcultured there was maximum of 2.2 ± 0.8 number of shoots in MS medium fortified with BAP(8µM) and TDZ(0.2µM) followed by Kn(8µM) with TDZ(0.2µM) with 2.0 ± 0.4 shoots

after 8 weeks. Zimmerman and Scorza (1992) and Jonoubi *et al* (2004) reported that medium supplemented with TDZ and another cytokinin could effectively improve shoot regeneration. In subsequent passages after repeated subculturing in BAP(8µM) with TDZ(0.2µM) combination maximum of 10.2 ± 2.2 number of multiple shoots were formed by the end of 32 weeks whereas MS+ Kn (8µM)+TDZ (0.2µM) resulted in maximum shoot length reaching (8.1 ± 1.0) cms by the end of 24 weeks. Similarly in *Boucerosia diffusa* BAP (8.87µM) and TDZ (2.27µM) showed better proliferation. The medium enriched with BAP and TDZ had an affect on the shoot growth in peach (Zimmerman and Scorza, 1992), bread fruit (*Arthocarpus communis* Forst) (Mariska *et al*, 2004) and star fruit (*Averrhoa carambola* L.) (Supriati *et al*, 2006). Whereas in *Bauhinia vahlii* combined effect of Kn and TDZ in MS medium was observed on shoot regeneration (Bhatt and Dhar, 2000).

5.2.1.1.3 Synergistic effect of cytokinins and auxins on cotyledonary node and nodal explants

Cytokinins and auxins play a key role for *in vitro* regeneration as cytokinin affects the formation of shoots Mahadevappa *et al* (2014) and its interaction with auxin controls the cellular differentiation and organogenesis. Therefore in the present studies cytokinin were added with low concentration of auxins in *O.indicum* and *S.suaveolens*. The substantial role played by a low concentration of auxin in conjunction with cytokinin on shoot regeneration is well documented in several trees (Siril and Dhar, 1997).

Addition of low levels of auxin along with cytokinin is known to increase shoot numbers in *Tectona grandis* (Tiwari *et al*, 2002) as well as in other plant species like *Wrightia tinctoria* (Purohit and Kukda, 1994) and *Gmelina arborea* (Tiwari *et al*, 1997).But in *O.indicum* and *S.suaveolens* addition of auxin in medium with cytokinin resulted in poor response in MS and WPM medium after 4 weeks from both the explants.

In *O.indicum* the cotyledonary node explants when placed in MS medium in presence of BAP (20µM) and NAA (0.1µM) were able to form maximum number of shoots(1.7 ± 1.0) which was slightly more compared to individual BAP concentration. Similar results where highest percentage of response was found in MS medium fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l) that was followed by BAP (1.5 mg/l) alone in *Ocimum sanctum* (Thakur *et al*, 2017), whereas in WPM medium the response was poor in terms of shoot formation in all the concentrations, similar reports were observed in *Pterocarpus marsupium* by Anis *et al* (2005) who reported the addition of IAA and NAA with optimal concentration of BA significantly

reduced the frequency of shoot formation. In case of nodal explants 83% response with 1.2 ± 0.3 number of shoots were obtained in MS medium fortified with BAP (20µM) and IBA (1µM) after 4 weeks On the medium with BAP (5µM) and IBA (0.5µM), which gave the highest shoot formation in *Prunus avium* (Ruzic and Vujovic, 2008).Whereas in WPM medium the response improved with 100% developing 2.3 ± 0.7 number of shoots in BAP(16 µM) and IAA(1µM). Similarly in *Salvadora pesica* IAA proved to be potent amongst all the auxins tried.(Mathur and Batra, 2014) Highest percent response was also observed at (3.0 mg/L) BAP and (0.5 mg/L) IAA in *Sapindus emarginatus* (Srinivas *et al*, 2015).

The *in vitro* nodes excised from shoots developed from cotyledonary node explants (after 4 weeks) failed to form shoots after 8 weeks. Whereas when in vitro nodes from shoots developed from the nodal explants were subcultured in MS and WPM medium with respective concentrations. There was a maximum of 3.5 ±0.7 shoots developed in WPM medium fortified with BAP (16µM) and IBA (0.1µM) after 8 weeks. In subsequent passages the MS medium fortified with BAP (20µM) and IBA (0.5µM) there was an increase in number of shoots to 3.7 ± 1.1 after 12 weeks. Similarly the highest shoot induction was observed on MS media supplemented with BAP (0.5 mg/l) in combination with IBA (0.1 mg/l) with an average number of 3.1 ± 0.6 shoots per explants in *Prunus domestica* (Wolella, 2017). The number was slightly higher in WPM medium fortified with BAP (16µM) +IAA (1µM) with an average of 4.0 ± 1.7 after 12 weeks. Similarly maximum number of shoots (13.9) were obtained on WPM medium fortified with BAP (1mg/l) and IAA (0.5mg/l) in *Ficus religiosa* (Siwach and Gill, 2011).

In *S.suaveolens* when explants were placed on combinations of cytokinin and auxins it also resulted in forming single shoot from both cotyledonary and nodal explants after four weeks as the addition of auxins failed to enhance the number. Husain *et al* 2005 has also reported similar observations in *Pterocarpus marsupium* where the addition of auxins IAA/NAA with 0.5-1 µM concentrations and individual cytokinins like BAP or Kn were not beneficial as they reduced the frequency of shoot formation. In MS medium fortified with TDZ (0.2µM) and NAA(0.1µM) a 83% response was obtained with only 0.8 ± 0.2 shoots whereas in WPM medium fortified with Kn(2µM) and IAA(0.1µM) the response was improved to 100% with 1.0 ± 0.0 shoots from cotyledonary node explants. Similarly in *Tulipa edulis* MS medium with TDZ (2.0mg/l) with NAA (2.0, 4.0mg/l) resulted in maximum bud induction (79.2, 72.9%) (Zhu *et al*, 2014).When nodal explants were placed in the MS medium fortified with

Kn(2µM) and NAA(0.5µM) and WPM medium fortified with Kn(2µM) and IAA(1µM) resulted in maximum of 100% response with 1.0 ± 0.0 shoots. Similarly maximum shoot formation was achieved when the media was supplemented with Kn (2.0 mg/1) and NAA (0.1mg/1) in *Asparagus racemosus* (Vijay and Kumar, 2009) and Nodal explants of *Stevia rebaudiana* when cultured on WPM medium with Kn and IAA but resulted in less proliferation than BAP and NAA(Okuyucu *et al*, 2016).

It has been reported that auxins inhibits the proliferation of axillary buds, it works antagonistically to cytokinin limiting the availability of cytokinin to lateral buds (Muller and Leyser, 2011). The differential response between IAA and NAA is attributed to the instability of IAA under light conditions (George, 1993). These could be the reason in *O.indicum* and *S.suaveolens* the cytokinin and auxin combinations failed in enhancing number of shoots.

5.2.2 Indirect organogenesis

In *O.indicum* and *S.suaveolens* shoots get differentiated from basal callus of nodes. Similarly indirect multiple shoot formation was noticed in many plants (George *et al*, 2000). Efficient plant regeneration was observed from shoot segment derived callus in *Holarrhena antidydentrica* (Raha and Roy, 2003). The formation of callus at the base of *in vitro* explants was studied by Saini and Jaiwal (2000) on *Peganum harmala*, Martin (2000) on *Holostemma adakodien* , Ndoye *et al* (2003) on *Balanites aegyptica* and Ayisire *et al* (2009) *in Piliostigma thonningii*.

In *O.indicum* from cotyledonary node explants morphogenic callus was formed below shoots in MS medium fortified with BAP (4µM) which when transferred to BAP (4µM) and GA_3 (1 µM) differentiated maximum number of shoots (19.3 ± 2.4) after 16 weeks. *In vitro* organogenesis from cotyledonary node derived callus cultured in MS medium has been observed in *Trapa japonica* (Hoque and Arima, 2002).Addition of GA_3 (1µM) in the medium helped in elongation of shoots also in *O.indicum*. Similarly 2.25 fold elongation of shoots in 96% of shoot cultures was achieved on MS basal medium supplemented with GA_3 (1µM) within 2 weeks (Karuppusamy and Kalimathu, 2010). The morphogenic callus from cotyledonary node explants when transferred to MS medium fortified with BAP (20µM) and NAA (1µM) resulted in maximum of 12.0 ± 0.0 number of shoots after 12 weeks. In *Albizia*

amara also BAP (1mg/l) with NAA (1mg/l) were effective in inducing multiple shoots from cotyledonary node explants (Indravathi and Pullaiah, 2013).

In *O.indicum* the nodal explants also differentiated multiple shoots from morphogenic basal callus. Similar results have been documented in *Coccinia grandis* where basal nodal callus formed large number of shoots (Thiripurasundari and Rao, 2012). There was formation of 11.0 ± 0.6 number of shoots after 12 weeks in BAP (20µM) with NAA (0.5µM) combination. BAP and NAA is known to favor multiple shoot regeneration via callus redifferentiation as reported in reported in *Artemisia dracunculus* and *Artemisia Absinthium* (Mackay and Kitto, 1988; Nin *et al*, 1996).

In *S.suaveolens* there was formation of morphogenic callus with shoot buds at the base of cotyledonary node explants as observed in *O.indicum*. This callus was induced in WPM medium fortified with BAP (16µM) and when it was subcultured in medium fortified with BAP(16µM) along with $AgNO_3$(20mg/l) it failed to enhance the shoot number and instead a large amount of stunted shoots were formed after 12 weeks. Similarly in *Helicteres isora* the shoot buds and meristemoids developing on SMI (BAP 1mg/1) showed an increase in sprouting of new shoot buds and development of shoots when supplemented with $AgNO_3$ (2mg/1) (Chawla and Bansal, 2014). Hence in order to elongate them the shoots of were transferred to WPM medium fortified with coconut water (10%).There was a significant increase in shoot number (22.5 ± 3.9) as well as shoot length (8.5 ± 3.5cms) by the end of 32 weeks. Addition of coconut water (10%) in WPM medium along with BA (1mg/l) resulted in multiple shoots in *Aquilaria crassna* (Van Minh, 2005).

5.2.3 *In vitro* rooting of *O.indicum* and *S.suaveolens* microshoots

The elongated shoots of *O.indicum* and *S.suaveolens* were placed in half and full strength (liquid and static) MS and WPM medium for *in vitro* rooting.

In both the species the liquid media proved to be effective in terms of root number and root length as compared to static media. In *O.indicum* static media failed to induce roots whereas in *S.suaveolens* the root induction was observed in static medium with a maximum of 8.8 ± 3.8 roots in half strength MS medium fortified with IBA (1µM). There are reports which documents that rooting can be obtained in solid but better results are obtained in liquid media as observed in blueberry (Zimmerman and Brome, 1980). Other reports with similar results

are reported in *Wrightia tomentosa* where the length of roots and shoots was significantly higher in liquid rooting medium (Joshi *et al,* 2009). Use of liquid medium increased the rate of rooting to 80% and generated a notable reduction of development of callus (Sambe *et al,* 2010).This could be explained by the fact that liquid media are known to be effective for rooting as the nutritive elements are easily available to explants (Hammerschlag, 1982) and in static agar can create a critical pressure of turgescence which puts the cells in situation of stress.

In *O.indicum* the half strength MS basal medium failed to induce roots after 4 weeks. Similarly in *Quercus floribunda* (Purohit *et al,* 2002) and in *Dalbergia sisso* (Pradhan *et al,* 1998) half strength PGR free medium failed to induce roots. When the shoots were rooted in WPM medium half strength liquid basal medium resulted in 100% response with only 2.0±0.0 number of roots. Whereas in *S. suaveolens* roots were induced in MS and WPM basal medium with less response except in half strength WPM liquid medium root length was maximum with 4.9±3.2cms. Root induction in basal WPM has been reported earlier in *Salix* by Gebhardt (1992) and Park *et al* (2008). The production of adventitious roots in medium without auxin may be due to endogenous level of salicylic acid playing important role in plant growth development (Raskin, 1992) and *in vitro* rooting (Khalafalla and Hattori, 2000) or due the endogeneous level of hormones in the regenerated microshoots (Minocha, 1987).
When medium was fortified with different concentrations of IBA and NAA the root induction was observed in both the species. Similar reports are documented in *Sterculia urens* (Devi *et al,* 2011) and *Cassia sophera* (Parveeen and Shahzad, 2010) where addition of IBA and NAA to half strength MS medium facilitated better rhizogenesis.
In *O.indicum* the half strength and full strength MS medium fortified IBA resulted in less response while in WPM liquid medium improved response was observed in half strength medium fortified with IBA(10µM) with 7.0 ±1.3 number of roots with 100% response after 4 weeks whereas at 5µM root length was maximum with 3.3 ± 0.0cm. Similarly the WPM medium with IBA(2.46µM) obtained highest number of roots in *Cinnamomum camphora* (Nirmal Babu *et al,* 2003) and also in *Salix tetrasperma* also where half strength was superior to full strength WPM medium (Khan *et al,* 2011).

In *S.suaveolens* also half and full strength MS and WPM medium were able to induce roots but half strength was superior to full strength. As in half strength MS liquid medium fortified

with IBA developing maximum of 9.3 ± 0.8 number of roots in liquid IBA(2.5µM) with 100% response and half strength WPM medium fortified with IBA(2µM) resulted in 100% response with 8.5 ± 3.1 roots which was less compared to MS medium. Half-strength MS containing IBA (9.9µM) induced rooting in 45% of the shoots (Pradhan *et al*, 1998). Altaf (2006) in Kinnow tree, Gokhale and Bansal (2009) in *Oroxylum indicum* also obtained the rooting of shoots in half strength MS medium fortified with IBA(2mg/l). Others reports includes *Cassia sophera* (Parveen and Shahzad, 2010), *Gardenia latifolia* (Reddy and Saritha, 2013) and *Terminalia bellirica* (Phulwaria *et al*, 2012), *Holarrhena antidysentrica* (Kumar *et al*, 2005) where half strength MS medium proved to be optimum in induction of roots.

When NAA was used in medium, in *O.indicum* both half strength and full strength MS and WPM medium induce roots. Half strength MS medium fortified with NAA (5µM) resulted in maximum number of roots 7.0 ± 2.2 and maximum root length was observed at 4.5 ± 0.2 cm at NAA (10µM) and full strength WPM medium fortified with NAA (2.5µM) resulted in 100% response with an average of 5.7± 0.6 roots. In *S.suaveolens* also half and full strength MS and WPM medium fortified with NAA were able to induce roots but the response was less compared to IBA. There was a maximum of 4.5 ± 0.8 roots were developed in half strength MS medium fortified with NAA(5µM) and maximum root length in NAA(1µM) ie. 4.3 ± 0.6 cm whereas in WPM full strength medium fortified with NAA (5µM) resulted in maximum of 5.5 ± 1.6 number of roots. NAA was favourable for rooting of *in vitro* plants of *Acacia Senegal* (Badji *et al*, 1991) and in teak shoots (Shirin *et al*, 2005).

Therefore in both the species IBA proved to be the effective auxin for *in vitro* rooting in terms of number of roots. Superiority of IBA over other auxins in root formation has also been reported in *Cassia siamea* (Parveen *et al*, 2010). Sane *et al* (2001) had obtained 80% rooting on *Acacia tortilis* after a treatment with IBA compared to NAA. This stimulatory effects of IBA on the root development may be due to several factors such as its preferential uptake, transport, and stability over other auxins (Ludwig-Muller, 2000). In *Parkia globosa* also IBA seems to be better than NAA to induce rooting (Sambe *et al*, 2010).This hormone has always been a potential auxin that induces rooting in *in vitro* regenerated shoots (Iriondo *et al*, 1995; Mansor *et al*, 2003;Rajore and Batra, 2005;Rajeshwari and Paliwal, 2008) and in

woody trees usually low level of this growth regulator is effective for rooting of shoots (Rai *et al,* 2010).

5.2.4 Hardening of plantlets

The ultimate success of the micropropagation protocol depends on the successful rooting and survival of the plantlets in field conditions (Martin, 2003).Hardening is a crucial step prior to transplantation of plants to the soil. Type of potting mixture used during acclimatisation is one of the important factors determining the survival percentage of the plants (Kaur *et al,* 2011). There are different potting substrates which have been employed for hardening of *in vitro* raised plants by various workers like soilrite for *Carica papaya* (Agnihotri *et al,* 2004),soaked cotton for *Saccharum offinarum*(Gill *et al,* 2004),cocopeat and sand for *Garcinia indica* (Chabukshwar and Deodhar, 2005).Soil:vermicompost for *Tylophora indica* (Kaur *et al,* 2011).

Hence in the present studies in *O.indicum* and *S.suaveolens* different natural planting substrates were utilised for hardening of plantlets under lab conditions and greenhouse conditions. In both the species the growth and survival of plants was successfully done under greenhouse conditions. Similarly in *Cassia sophera* regenerated plantlets were successfully transferred to greenhouse conditions with 90% survival rate (Parveen and Shahzad, 2010) ,also in *Santalum album* the rooted plantlets were successfully hardened in plastic cups containing coco-peat, sand and soil in the ratio of 1:1:1 in the controlled environment under greenhouse conditions.(Singh *et al,* 2016).Shukla *et al* (2009) have also achieved more than 80 % of plants survival when tissue culture raised plantlets of *Stereospermum personatum* were transferred to net pots containing coco-peat in green house.

In *O.indicum* sand and Cocopeat:Sand:Soil substrate was optimum in terms of % survival (67%) whereas in *S.suaveolens* out of different substrates tried for hardening the plantlets cocopeat:soil proved to be optimum as there was 100 % survival. In contrast in *Garcinia indica* out of the three potting mixtures utilised for hardening procedure the survival was better in Cocopeat, Cocopeat:Sand as compared to Cocopeat:Sand:Soil.(Chabuleshwar and Deodhar, 2005)

At the time of hardening different parameters like shoot and root length, total plant height, number of new leaves and leaf length were studied for different substrates. There was an increase in plant height, number of leaves, and their length in each substrate after one month

in *O.indicum* and *S.suaveolens*. Results are in accordance to *Quercus floribunda* where the plant height, the average number of leaves, shoot diameter and number of nodes also increased significantly with time and tissue culture raised plantlets could be successfully established(Purohit *et al,* 2002).In *O.indicum* out of different substrates the maximum new shoot length (6.4 ± 0.5)cms was observed in sand substrate while new root length (22.8 ± 0.0)cms and final plant height(42.5 ± 0.0)cms was maximum in Cocopeat:Sand substrate while leaf number(4.0±0.0) and leaf length(2.6 ± 0.4)cms was maximum in sand substrate. Whereas in *Tylophora indica* shoot height and number of leaves was maximum in soil:vermicompost:Azotobacter:Pseudomonas (Kaur *et al,* 2011).In *S.suaveolens* highest new shoot length was observed in Sand:Soil (8.2 ± 1.2) cms and Cocopeat:Soil (8.2 ± 1.0)cms while new root length (11.3 ± 1.3)cms and final plant height (19.2±2.3)cms was maximum in Sand:Soil substrate. The leaf number was maximum in Cocopeat:Sand (5.0 ± 0.6) and Sand:Soil (5.0 ± 0.5) while leaf length was maximum in Cocopeat:Soil (3.6 ± 0.4)cms. In contrast maximum plant height and number of leaves were resulted in Cocopeat: Perlite and minimum in Sand:Soil in gloxinia and saintpaulia (Kashyap and Dhiman, 2011).

Thus the from studies on regeneration of *O.indicum* and *S.suaveolens* revealed that there was establishment of shoots in both the MS and WPM medium, regeneration in both the media has been achieved in *Cinnamomum camphora* (Nirmal Babu *et al,* 2003) but in *S.suaveolens* MS medium proved to be effective for establishing, multiplication and rooting of shoots while in *O.indicum* WPM medium was efficient for its regeneration. Similarly MS medium has been proved best medium for shoot induction, proliferation, elongation and rooting in *Bauhinia racemosa*(Rajanna *et al,* 2011) and WPM medium for *Salix tetrasperma* (Khan *et al,* 2011).

The cotyledonary node and nodal explants of both the species has the ability to regenerate but the healthy and maximum direct multiplication of shoots was achieved through nodal explants. Nodal explants have been effective for regeneration in *Vitex negundo* L. (Ahmad and Anis, 2007).The overall efficacy of PGRs proves that out of the three cytokinins BAP was effective for formation of shoots individually and in combination with other cytokinin in *O.indicum*. These results are similar to those reported by Dalal and Rai (2004) and Gokhale and Bansal (2009) where BAP was potent enough for maximum induction of shoots in *O.indicum*. Whereas in *S.suaveolens* TDZ was potent cytokinin which worked individually and in combination with cytokinin for forming multiple shoots. The stimulating effect of

TDZ on multiple shoot formation has also been reported for several medicinal and aromatic plant species (Faisal *et al,* 2006).

The rooting of microshoots of both the species were also achieved on both the medium but optimum number of roots were induced in half strength WPM medium fortified with IBA (10µM) with 7.0 ± 1.3 roots in 100% cultures in *O.indicum* and half strength MS medium fortified with IBA (2.5µM) with 9.3 ± 0.8 roots in 100% cultures in *S.suaveolens*. Similarly IBA has been used and has stimulatory effect on *in vitro* root induction in *Tecomella undulata* (Varshney and Anis, 2012) and *Parkia biglobosa* (Sambe *et al,* 2010). Whereas hardening of plantlets were best achieved on sand substrate in *O.indicum* and Cocopeat:Soil in *S.suaveolens*.

5.3 Synthetic seed studies

Studies on synthetic seed for both the species was also carried out with an aim to understand the effect of encapsulation matrix, storage and regenerative media on formation of plantlets.

Alginate encapsulation has become a viable technique for *in vitro* germplasm conservation and synthetic seed production from nodal segments can be used for cost-effective mass clonal propagation and delivery of tissue-cultured plants (Ara *et al,* 2000; Nyende *et al,* 2003). Therefore in the present studies encapsulation technique was utilised for the preparation of synthetic seeds of *O.indicum* and *S.suaveolens*. Encapsulation technique has been successful in sandalwood (Bapat *et al,* 1988), *Guazuma crinita* (Maruyama *et al,* 1998), *Paulownia elongate* (Ipekci and Gozukirmizi, 2003), *Vitex negundo* (Ahmad and Anis, 2010) etc.

In the *O.indicum* and *S.suaveolens in vitro* nodes were taken as explants and encapsulated for preparing synseeds. There reports which documents that synthetic seeds can often be produced more conveniently using unipolar structures such as apical or axillary buds (Ahmad and Anis, 2010; Singh *et al,* 2010; Mishra *et al,* 2011). Similarly in wide range of woody plants shoot tips or nodes, including *Eucalyptus grandis*, *Dalbergia sissoo*, *Hibiscus moscheutos* and *Olea europaea* as well as axillary buds from *in vitro* grown plantlets of *Gmelina arborea* and *Peronema canescens* has been encapsulated for preparation of synthetic seeds. (Watt *et al,* 2000, Chand and Singh, 2004; West and Preece, 2009; Ikhlaq *et al,* 2010; Sukartisingh *et al,* 2012).

5.3.1 Effect of sodium alginate and calcium chloride concentration on synseed formation

The concentration of sodium alginate and calcium chloride is one of the important factors for the successful regeneration of plants through encapsulation technology as it affects the gel matrix and capsule quality. The optimal ion exchange of sodium and calcium controls the capsule hardiness, and it varies with propagules type and plant species (Rai *et al,* 2009; Singh *et al,* 2010).Calcium alginate beads containing nodal segments differed morphologically regarding texture, shape, and transparency with different concentrations of sodium alginate and calcium chloride.

In the present study of the three different concentration of sodium alginate (2, 3 and 4%) were mixed in calcium chloride solution (50, 75 and 100mM).It was observed that the 2% sodium alginate with all concentrations of $CaCl_2$ (50,75mM and 100mM) resulted in formation of irregeular and very soft synseeds while 4% sodium alginate resulted in synseeds which were tailed and hard in texture. Whereas the 3% sodium alginate with 75mM $CaCl_2$ solution resulted in formation of round and firm beads which was the optimum gel matrix for preparation of synseed in *O.indicum* and *S.suaveolens*.

It has been known that sodium alginate preparation at low concentration becomes unsuitable for encapsulation, ie.sodium alginate (2%) resulted in formation of fragile beads without a defined shape that were too soft to handle, difficult to hold and not firm and strong enough to facilitate transfer with forceps to the culture medium. There are reports that at the lower concentration of sodium alginate after exposure to high temperature during autoclaving reduced the gelling ability (Larkin *et al,* 1988). However, at higher concentrations of sodium alginate (4–5 %), too hard beads were developed and resulted in delayed germination, shoot emergence (Faisal *et al,* 2006) (Remya *et al,* 2013;Perveen and Anis, 2014) and suppressed the emerging shoots and roots(Mathur *et al,* 1989; Daud *et al,* 2008;Cartes *et al,* 2009).

Similar findings stating sodium alginate (3%) as suitable for the formation of firm, ideal, clear and isodiametric uniform good quality beads are reported in *Balanites aegyptica* (Varshney and Anis, 2014), *Paulownia elongate* (Gozukirmizi, 2003), *Coelogyne breviscapa* (Mohanraj *et al,* 2009) and *Stevia rebaudiana* (Ali *et al,* 2012). Saiprasad (2001), Lambardi *et al* (2006) and Gantait *et al* (2012) also documented that the ideal synthetic seeds were obtained using 3% sodium alginate and 75 mM calcium chloride as the gelling matrix. This combination creates optimal ion exchange between the sodium and calcium ions, which produces firm, clear and isodiametric beads (Gantait *et al,* 2012). In Orchids also, the best conversion to plantlets of encapsulated of *Dendrobium* 'Sonia' was observed when 3% Na-

alginate drops were hardened for 30 min in 75 mM $CaCl_2$ (Wee *et al,* 2014) sodium alginate concentrations positively affected synthetic seeds shape and texture.

5.3.2 Effect of encapsulation matrix, medium strength, PGRs/regenerative medium on synseed germination

Germination of synthetic seeds varied according to encapsulation matrix, its composition and nature of encapsulated explant. Therefore in the present work different matrices were tried for encapsulation of *in vitro* nodes of *O.indicum* and *S.suaveolens*. Ganapathi *et al,* (1992), Winkelmann *et al,* (2004), Kumar *et al,* (2005), Awal *et al,* (2007) and Siong *et al,* (2012) suggested that the successful germination of the beads was probably due to the ability of microshoots to absorb the nutrients and the growth regulators in calcium alginate beads.

In our studies also development of shoot and root took place after 4 weeks of transfer from the *in vitro* nodes encapsulated in half strength WPM medium matrix fortified with BAP (16µM)/ BAP (16µM) with IBA (0.1µM) in *O.indicum* and in *S.suaveolens* full strength MS basal medium, half strength MS medium fortified with Kn (8µM), MS medium fortified with TDZ (0.2µM)/ BAP (8µM) +TDZ(0.2µM) matrix. The development of shoots and roots is simultaneous and resulted in rapid growth of plantlets within 4 weeks (Sharma *et al,* 2014).

Presence of sucrose was essential in matrix as it serves as a carbon source for encapsulated explants enables their survival during storage. In *O.indicum* and *S.suaveolens* the synseeds germination was observed in presence of 1% sucrose and 3% sucrose, but in *O.indicum* presence of 1% sucrose was effective in terms of germination into shoot or shoot and root. Similarly in *Solanum melongena* among different sucrose level tried, 1% sucrose resulted for optimum germination of synthetic seed while increased dose of sucrose delayed germination and resulted in decreased growth of shoots (Huda *et al,* 2007) while in *S.suaveolens* 3% sucrose was effective in terms of shoot or shoot and root. Similarly in *G.arborea* the MS shoot sprouting medium containing plant growth regulators and 3% sucrose appeared to be suitable for sprouting shoots (Sukartisingh, 2012). Grzegorczyk and Wysokińska (2011) also reported that shoots and roots developed only in the presence of sucrose in the gel matrix. Similarly the presence of sucrose in the alginate gel matrix also improved emergence of shoots from encapsulated shoot tips of *Camelia japonica* (Ballester *et al,* 1997) and axillary buds of *Betula pendula* (Piccioni and Standardi, 1995).

Morphogenic response of encapsulated buds are affected by factors like composition of media and the presence of growth regulators (Wysokinska *et al,* 2002). In *O.indicum* and

S.suaveolens both half strength and full strength medium were effective for germination of synseeds. Conversion of encapsulated shoot tips into plantlets greatly influenced by different growth media, medium strength, and presence or absence of a gelling agent in media (Grzegorczyk and Wysokińska, 2011).In *Aranda and Vanda coerulea* germination was also best achieved when half-strength MS media was used in the gelling matrix, which provided sufficient nutrients for the plant tissue (Gantait *et al,* 2012).While in *Salvia officinalis* among the different strengths of MS medium tried (full, half, and one-third), the presence of 1/3 strength MS medium gave a high percentage of shoot and root formation than the control (Grzegorczyk and Wysokińska, 2011).

When synseeds of *O.indicum* and *S.suaveolens* were placed in different regenerative medium they varied in their response. In both the species synseeds germinated in the basal regenerative medium. There was maximum of 50% germination into shoot at 0 day from *in vitro* nodes encapsulated in half strength basal medium while after 15 days of storage maximum of 33% synseeds germinated into shoot from half strength WPM medium with BAP(16µM)+IBA(0.1µM) matrix in *O.indicum* whereas in *S.suaveolens* liquid basal medium at 0 day germinated 100% synseeds developed in full strength medium fortified with Kn(8 µM) matrix and 67% germination after 7 days of storage of synseeds developed in medium fortified with BAP(8µM) +TDZ(0.2µM) matrix, Similarly in *Oxalis traingularis* successful plant regeneration from synthetic seeds on MS basal medium was observed(Taha *et al,* 2013) and in *Ficus carica* when encapsulated nodal segments were placed on MS basal medium (control) 55.2 % shoot buds sprouted after 4 weeks of culture and to improve the bud-sprouting frequency of encapsulated nodal segments, PGRs and growth additives were added to the regenerative medium(Sharma *et al,* 2014).

In *O.indicum* and *S.suaveolens* to improve the synseed germination frequency different PGRs and an organic supplement coconut water were added in regenerative medium. The addition of growth regulators and nutrients is an essential factor for successful germination and retaining viability of the seeds (Rihan *et al,* 2017). Shoot sprouting of *P.canescens* was promimnent on medium containing only plant growth regulators indicating that a continuous supply of plant growth regulators is necessary for *P.canescens* shoot sprouting (Sukartisingh *et al,* 2012).

In both the species the supplementation of NAA in medium proved to be suitable for

germination of synseeds into shoot and root. This depicts that presence of auxin in the medium is necessary to develop balanced root and shoot system (Wysokinska *et al,* 2002).In *O.indicum in vitro* nodes encapsulated in 1/2 WPM+BAP (16µM) +IBA (0.1µM) matrix placed on half strength WPM regenerative medium fortified with NAA (5µM) resulted in maximum 33% shoot and root emergence after 15 days of storage with 0.3 ± 0.2 number of shoots and 0.7 ± 0.4 number of roots. Similarly the highest frequency of plantlet germination from encapsulated buds 70% within 4 weeks was obtained on wpm medium containing IBA 1mg/l in *Catalapa ovata* (Wysokinska *et al,* 2002).

In *S.suaveolens* MS regenerative medium fortified with NAA (2µM) (Full strength MS matrix) resulted in highest percent (50%) of shoot and root emergence after 15 days of storage with 0.5 ± 0.2 shoots and 0.5 ± 0.2 roots. Similarly alginate supplemented with MS nutrient medium has been used to improve shoot and root emergence from encapsulated explants of *Carica papaya* (Castillo *et al,* 1998) and *Chonemorpha grandiflora* (Nishitha *et al,* 2006). In *S.suaveolens* both the liquid and static MS regenerative medium helped in germination of synseed, but static medium was better in terms of shoot and plantlet emergence. Similarly in *Salvia officinalis* in comparison to liquid medium, plantlet conversion frequency was higher on agar-solidified medium, and the best result was observed on gelrite-gelled medium (Grzegorczyk and Wysokińska, 2011). Similarly few workers have attempted to use liquid medium for shoot emergence from synthetic seed and have got better results than the agar-solidified medium (Rai *et al,* 2008; Singh *et al,* 2009). In contrast agar solidified and liquid MS media with or without BA provided high frequencies of shoot regrowth from *Khaya senegalensis* encapsulated explants but liquid medium provided higher shoot numbers than agar solidified medium in the presence of BA (Hung and Trueman, 2011).

5.3.3 Effect of storage on synseed germination

The synseeds of *O.indicum* and *S.suaveolens* were stored at 4 ºC at different storage period to assess the germination frequency. As it is known that the regeneration frequency is clearly influenced by storage time (Ahmad and Anis, 2010).

In both the species *O.indicum* and *S.suaveolens* the storage of 7 days was better for synseeds germination with respect to number of emerging shoots. Similarly Taha *et al* 2013 observed

that storage period of 7 days was optimum to ensure a high plantlet conversion rate and survival percentage and the shoot number was highest from synthetic seeds stored for 7 days. In *O.indicum* WPM regenerative medium fortified with GA_3 (10µM) when utilised for placing synseeeds which were prepared in WPM basal medium matrix resulted in maximum of 0.8 ± 0.4 shoots after 4 weeks. Presence of GA_3 (0.5, 1mg/l) regenerated 70% beads into shoots and improved the morphology of shoots in *Salvia officinalis* (Grzegorczyk and Wysokinska, 2011).Whereas in *S.suaveolens* the synseeds prepared in 1/2 MS +Kn (8µM) matrix placed on half strength MS regenerative medium fortified with BAP (4µM) +Kn(8µM) resulted in highest number 1.3 ± 0.4 of shoots after 7 days of storage. Similarly maximum number of multiple shoots was regenerated from the encapsulated nodes cultured on MS medium supplemented with BAP (3µM) and Kn (0.5µM) in *Aristolochia tagala* (Remya *et al,* 2013).

In *O.indicum* and *S.suaveolens* the regenerating ability of synseeds was lost with increase in storage period and was completely nil after 30 days of storage. According to Siew *et al* (2014) generally, longer storage period will lead to slower germination rate and lower germination capability. There are other reports which are in accordance with the present findings as reported by Hung and Trueman (2011) in *Khaya senegalensis* that shoot regrowth from capsules decreased markedly with prolonged storage at 4ºC.The percentage recovery of shoots from encapsulated nodal segments decreased as the period of storage increased beyond 6 weeks(Sharma *et al,* 2014).

This decline in conversion response could be attributed to the inhibition of tissue respiration by the alginate matrix, or a loss of moisture due to partial dessication during storage as reported (Danso and Ford-Lloyd, 2003; Faisal *et al*, 2006; Faisal and Anis, 2007; Ahmad and Anis, 2010).

5.3.4 Effect of substrates on synseeds germination

In order to find suitable substrate for storage of synseeds filter paper as well as agar substrate were used to store the synseeds of *O.indicum* and *S.suaveolens*. But it was observed that there was not much variation observed in terms of germination of synseeds stored in agar substrate in *O.indicum* whereas in *S.suaveolens* there was slight increase in % germination after 7 days of storage in agar substrate in full strength MS static regenerative medium fortified with NAA(2µM) (67%) (MS basal medium matrix) and in liquid MS regenerative medium fortified with NAA (2µM) (50%) (MS+ Kn (8µM) matrix).Similarly no differences in

viability and regrowth were detected between the two different sowing substrates tested (agar-solidified medium and filter paper)(Maria *et al*,2011).

Thus, in both the species the gel matrix 3% and 75mM was optimum for formation of synseeds. Similar findings stating sodium alginate (3 %) as suitable for the formation of firm, beads are reported in *Paulownia elongate* Gozukirmizi (2003). The optimised matrix for synseeds formation of *O.indicum* was half strength WPM medium fortified with BAP (16 µM) with IBA (0.1µM) and optimised regenerative media for forming shoot and root after 15 days of storage was NAA (5µM). Whereas for *S.suaveolens* full strength MS medium fortified with Kn (8µM) matrix was optimised and NAA (2µM) as regenerative medium after 15 days of storage.