CHAPTER5
DISCUSSION

DISCUSSION

In the present investigation an attempt was made to optimise a regeneration protocol, metabolite content from the in vitro shoot and the effect of chemical mutagen EMS on in vitro shoot formation and its metabolite content on the two species of *Portulaca.* Each aspect of the investigation provided novel information with future implications.

5.1 PLANT REGENERATION STUDIES

The regeneration within the species of *Portulaca* was initiated in presence of PGR's such as BA, Kin, IAA, NAA, 2,4D and IBA. Cytokinins and auxins are responsible for growth and morphogenesis and interplay of these PGR's lead to varied morphogenic responses (George and Sherington 1984). The classical experiment of Skoogs and Miller (1957) on tobacco callus elaborated the importance of cytokinin and auxin interaction and their ratio in cellular differentiation and organogenesis.

Success in plant tissue culture is largely dependent on the explant and a number of reports indicate the importance of the selection of the explant type which governs morphogenesis (SivaKumar and Krishnamurthy 2004, Bhatia et al. 2009, Song et al. 2011) In the present investigation leaf was utilized as a source of explants because of its easy availability, and is known to differentiate shoots causing little disturbance to the population in wild. Within the herbaceous species regeneration from leaf explants have been documented in a number of plants such as *Solanum nigrum* (Sridhar and Naidu 2011); *Oenothera biennes* (Sattar et al. 2016); *Abutilon indicum* (Shankarsingh et al. 2016); *Saussurea involucrate* (Guo et al. 2007) etc. The leaf explants was cut and the morphogenic responses started with swelling and differentiated shoot buds from these cut margins. Similar pattern of morphogenic response was noted by Becerra et al. (2004) in *Passiflora edulis* and De Klerk (1997) opined that these cut regions induce cell division and the internal environment of the explants comes in direct contact to PGR's facilitating its uptake.

Regeneration studies have been carried out on a number of plant species in presence of different mediums such as White's medium (1962) on *Carica papaya* (Litz and Conover 1983), Nitsch and Nitsch medium (1967) on *Brassica compestris* (Sato et al. 1989), Gamborg's B5 medium in Barley (Goldstein and Kronstad 1985), Woody Plant Medium on *Stereospermum suaveolens*(Trivedi and Joshi 2014) etc. however Murashige and Skoog's medium is the most preferred medium. The experiments of regeneration for the two species of *Portulaca (P oleracea* and*P grandiflora)* under investigation were initiated in presence of

MS medium only and their mode of regeneration and response towards different PGR's was evaluated. It was observed that both the species responded differently to the PGR's for which Brown et al (1995) opined that genotype is an important factor which can influence morphogenic response in vitro within species, cultivars or even individual. Fiuk and Rybczynski (2008) reported genotype and PGR's dependent response in five species of *Gentiana* cultures. Kallak et al. (1997) described the differential manifestation of callus and rhizogenesis under the influence of genotype in carnations thus emphasising that it plays an important role in response to different PGR's.

For regeneration studies a control devoid of PGR's was maintained which formed a single shoot (*P oleracea*) or root (*P grandiflora*). Thong and Madhialagan (2014) also reported a similar pattern of suppressed growth in *Cardamine hirsuta* for which Werner et al. (2001) suggested that the single shoot is formed due to the presence of endogeneous cytokinin occurring naturally in the plant. Since the medium does not contain exogeneous cytokinin and the excised explant requires time to synthesise its own endogeneous cytokinin therefore shoot induction is suppressed in the control medium.

5.1.1 *P oleracea*

5.1.1.1 Individual cytokinins induced shoot buds and in vitro shoots

In the present study it was revealed that *P oleracea* could serve as a model plant where the whole spectrum of morphogenesis was displayed by the leaf explant. Cytokinins played a pivotal role as regeneration was induced in presence of this PGR. Direct shoot regeneration was obtained through leaves in the plant which is a preferable mode as it allows large scale multiplication of the plantlet without clonal variations as indirect organogenesis from calli leads to somaclonal variants (Reddy et al. 2010). In presence of single concentration of BA or Kin and a synergistic combination of both were able to differentiate shoot buds from the cut ends which gradually progressed and covered the entire explants. A similar pattern of shoot bud progression was observed in *Bigonia tuberhybrida* (Nada et al. 2011). The type and concentration of cytokinin had a determining effect on the establishment of regeneration system (Nada et al. 2011) as it suppresses apical dominance leading to the development of lateral shoots in *Sophora* spp. (Jana et al. 2013).

It was observed that BA individually formed in vitro shoots which has also been observed in a number of plants such as *Bacopa monnieri* (Joshi et al. 2010), *Citrus mitis* (Sim et al. 1989) etc. Kin regenerated shoot buds at higher concentrations which failed to elongate into shoots. Erdag and Emek (2009) have reported the superiority of BA over Kin in *Anthemis xylopoda*.

Similarly plants such as *Coleus forskohlii* (Krishna et al. 2010) and *Physalis peruviana* (Otroshy et al. 2013) have also documented the effectiveness of BA to induce multiple shoots as compared to Kin. Chauhan et al. in 2012 have suggested that BA is the suitable cytokinin for multiplication and micropropogation of plants as they reported highest number of shoot/explants in *Garcinia indica* in presence of this hormone. The number of shoots increased with increase in concentration of BA and decreased at higher level and similar findings are also reported in *Alstroemeria* (Pierik et al. 1988; Pedraza-Santos et al. 2006).

5.1.1.2 Individual auxins induced roots and callus

Generally auxins are the PGR's which induce rhizogenesis and callogenesis within the explants. In the present study it was observed that auxins IAA and NAA formed roots and roots with shoots in some explants. Root forming capacity of IAA/NAA has been reported in a number of plants such as *Lathyrus sativus* (Barpete et al. 2014), *Pityopsis ruthii* (Wadl et al. 2011). IAA formed shoots which were healthy and robust at low concentration (0.5-5µM) along with fragile roots while NAA formed shoots at 0.5µM beyond which shoot forming capacity was hindered and the organogenetic pathway shifted to root formation. This suggested that the explants had a certain level of endogenous cytokinin whose activity was triggered by the uptake of low concentration of auxin and this cytokinin auxin ratio lead to the development of shoots. Thus in the present investigation IAA/NAA adopted an organogenetic pathway leading to the formation of root or shoot however 2,4D induced callus. The formation of callus in 2,4D has also been reported in *Andrographis paniculata* (Sharmila et al. 2013) and *Croton urucurana* (Lima et al. 2008). 2,4D has the capacity to stimulate callus formation even at low level as it affects the RNA metabolism which effects the transcription of messenger RNA's. The transcription leads to coding of proteins, required for growth which stimulates callus induction (George 1996).

5.1.1.3 Synergistic cytokinins enhanced the rate of regeneration

Individual concentration of auxins was insufficient to induce a high rate of regeneration of shoots. BA was effective in inducing shoot buds which elongated into shoots whereas Kin induced shoot buds, therefore a combination of BA with Kin was studied to evaluate the synergistic effect of the two. A number of reports have suggested that single concentration of BA is effective in inducing higher number of shoots as compared to Kin but the effect is enhanced when the two cytokinins are coupled together as in *Chlorophytum borivilianum* (Ashraf et al. 2014); *Lagenaria siceraria* (Saha et al. 2007); *Primulina tabacum* (Ma et al.

2010); *Stevia rebaudiana* (Sreedhar et al. 2008); *Physalis peruviana* (Otroshy et al. 2013) etc. Within the synergistic concentrations it was observed that low concentration of BA and Kin (0.5 and 2.5 µM) induced shoot buds only. The study proposes that the level of exogeneous cytokinin is insufficient to induce the proliferation of shoot buds to in vitro shoots. Low concentration of BA (2.5 µM) and high concentration of Kin (15 µM) induced shoots and callus both. Studies on *Tylophora indica* (Rathinavel and Sellathurai 2010) have reported that high concentration of cytokinins induces shoot and callus. Direct organogenesis was achieved at moderate concentration of BA (5 µM) and high Kin level (10 µM). This mechanism of "de novo organogenesis" can be explained in three steps. The plant cells first acquire competence and then are induced by phytohormone signal to form a population of developmentally determined cells. This leads to morphogenesis within the explant (Sugiyama 1999). Thus the regeneration of the plant via organogenesis takes place under optimal conditions of phytohormone which in the present study was cytokinin. The shoots formed were healthy and stout at this optimal concentration of 5 µM BA and 10 µM Kin however at high concentrations of cytokinins the in vitro shoots remained stunted with pale yellow leaves and the number of in vitro shoots decreased. The negative effect of high concentration of cytokinin on plant growth and development has been documented in *Cardamine hirsuta*(Thong and Madhialagan 2014) and *Cleistanthus collinus* (Elangomathavan et al. 2017). This stunted and abnormal morphology of the shoots was explained by Ramage and Williams (2004) where they proved that these abnormalities were due to the changes in expression of knotted 1-type homeobox gene (TobH1) in the studies on *Nicotiana tabacum*. Thus it was deduced from these experiments that not only the type of cytokinin but also the concentration plays a crucial role in determining the morphological response which was also confirmed by the studies of *Chrysanthemum morifolium*(Song et al. 2011).

It has been established in a number of plants and also as discussed that cytokinins are responsible for differentiation and morphogenesis and several theories for its mode of action have been proposed. They regulate shoot formation and multiplication by cell division and expansion of cells. The hormone binds to a high receptor protein resulting in conformation changes. This activates a signal transduction cascade through *his/asp*phosphorylation/dephosphorylation or by guanidine nucleotide binding/hydrolysis leading to changes in transcription of the gene (Mok and Mok 2001).

5.1.1.4 Synergistic cytokinin and auxins formed callus

In the present investigation cytokinins induced in vitro shoots, individual auxins induced roots and callus and synergistic cytokinins formed enhanced number of shoots. For determining an optimum concentration for callus induction a synergistic effect of cytokinin (BA/Kin) and auxins (IAA,NAA,2,4D) was employed. Shah et al. (2003) noted that induction of callus is controlled by a combined influence of auxin and cytokinin. Charriere et al.(1999) indicate that callus induction depends on specific concentration of plant growth regulator which again varies from species to species. Thus for callus induction BA/Kin + IAA, BA/Kin+ NAA and BA/Kin +2,4D were employed.

Synergistic combination of BA and IAA induced green-yellow callus and similar results were observed by Singh et al.(2009) observed in *Rauvolfia serpentina* that leaf segments cultured on MS media with BAP and IAA formed green calli. Rooting was obtained in lower concentration of BAP and IAA. Similar trends have been observed in a number of plants such as *Alpinia galangal* (Singh et al. 2014), *Plumbago zeylanica* (Rout 1999).Hopkins and Hüner (2004) have reported the importance of concentrations of IAA in rooting and noted that high level of auxin causes significant inhibition of root growth. Similar results were obtained in our study where higher level of IAA along with BAP suppressed rooting and promoted callus proliferation (10µM BA and 10µM IAA).

The synergistic effect of Kin and IAA was also studied and the response was similar to BAP and IAA. Roots differentiated at lower concentration of Kin and IAA and at higher levels callus was obtained. Studies on *Sisymbrium* (Pareek and Chandra 1978) *Gentiana* spp. (Fiuk and Rybczynski 2008) and *Beta vulgaris* (Welander 1976) have also reported similar trends in the formation of callus and root in presence of Kin and IAA. Safdari and Kazemitabar (2009) have reported optimum callus formation in *P oleracea* under the influence of BAP and IBA which is contrary to the present studies.

The next cytokinin and auxin combination tried for the study was BA/Kin with NAA. The combination of BA and NAA induced a large amount of callus with roots as compared to Kin and NAA indicating that BA was efficient in inducing a response from *P oleracea* as compared to Kin. The term “Rooty callus” can be used where the callus is accompanied with root formation (Ikeunchi et al. 2013) and it was also noted that low concentration of BA (0.5µM) with NAA induced prolific roots and little callus while high concentration of BA (5-10µM) and NAA formed profuse callus. It was inferred that low concentration of BA when combined with auxin induced roots but at high concentration only callus formation was noted. Otroshy et al. (2013) suggested that appropriate concentration of auxins in absence or

lower concentration of cytokinins are crucial for root and callus induction.The combination of Kin and NAA also induced callus and roots which is similar to the studies of SivaKumar and Krishnamurthy (2004) in *Gloriosa superba.*

Since the auxins IAA/NAA along with cytokinins formed roots and callus therefore another auxin 2,4D was employed along with BA/Kin to induce profuse callus. Generally cytokinins and 2,4D induce callus due to their role in DNA synthesis and mitosis (Skoog and Miller 1957). In the present studies the callus induced was white and friable which is similar to the reports on *Allium* (Monemi et al. 2014). It was observed that low concentration of BA and 2,4D produced callus and when the concentration of BA was increased to 10µM with 0.5µM 2,4D in vitro shoots also differentiated along with callus. This suggested that low concentration of auxin 2,4D was potent enough to induce callus along with low concentration of BA and when the concentration of BA was increased to 10µM it could overcome the effect of auxin and simultaneously induced in vitro shoot and callus. The optimum amount of callus was quantified by fresh and dry weight of the callus. The role of BA and 2,4D to induce optimum callus has been reported in a number of plant species such as *Simmondsia. chinensis* (Bala et al. 2015) *Andrographis paniculata* (Sharmila et al. 2013), *Hymenocallis littoratis* (Sundarasekar et al. 2012), *Scoparia dulcis* (Sakthi and Mohan 2012) and *Tecomella undulate* (Patel and Patel 2013). A similar pattern of callus induction was noted in presence of Kin and 2,4D however the amount of callus quantified was less than BA and 2,4D. It was thus inferred from the experiment that the coupling effect of BA and 2,4D was more effective in inducing callus in leaf explants of *P oleracea* as compared to Kin and 2,4D. Studies on *Gymnema sylvestris* (Roy et al. 2008) have reported that maximum callus induction was obtained in presence of Kin and 2,4D which is contrary to the present studies. It was also observed that old callus cultures turned brown even after transfer to a fresh medium. Tamilselvan and Rajeswari (2014) have suggested that it could be due to high phenol content and oxidation of phenolic compounds in the callus.

5.1.1.5 Multiplication and elongation of in vitro shoots

It was deduced from the results that cytokinins differentiated shoots and stunted shoot cluster while synergistic cytokinins and auxins formed callus and roots. The cluster of invitro shoots were sustained on the cytokinin rich medium which was insufficient to induce appreciable elongation of the shoots. Mok and Mok (1982) have opined that cytokinin rich medium hinders the elongation of shoots in culture. Siddique et al. (2015) encountered a similar problem in *Cassia angustifolia* where the shoots were stimulated but inhibition in elongation

was noted in presence of cytokinins. They suggested that this phenomenon is due to high activity of cytokinins and could be overcome by transferring the shoot cultures on cytokinin free medium.

Elongation of the in vitro shoots can be achieved by transferring the shoot cultures on a number of media fortified with coconut water, deoiled rice barn, GA_3 IAA, increased sucrose concentration etc. (Geetha et al. 1998; Chen et al. 2003; Siddique and Anis 2006; Anand et al. 2011; Gbadamosi and Sulaiman 2012;Thakur et al. 2014). In the present investigation the elongation of the shoot was achieved when it was transferred from cytokinin to a medium enriched with different concentrations of coconut water. Coconut water is an undefined mixture useful in culturing different plant species (Arditti and Ernst 1993; Suttle 1996) because it is a source of many PGR's, vitamins and inorganic ions (Yong et al. 2009).The positive effect of coconut water on the growth of developing tissues has been suggested due to the presence of fatty acids which is utilized as a source of energy to form phospholipids and glycolipids in the formation of cell membrane (Turnham and Northcote 1984, Lopez-Villalobos et al. 2001). The other components like cytokinins and absissic acid are also present in coconut water which too facilitates growth of the plants (Hoad and Gaskin 1980) and this explains the prolific rate of shoot development in *P oleracea*. Similar results in shoot multiplication and elongation have been reported in *Asparagu.s racemosus* (Thakur et al. 2014).

5.1.1.6 Regeneration from in vitro leaf explants

In vitro regeneration of *P oleracea* was achieved utilizing in vivo leaf explants and the juvenile in vitro leaves were also evaluated for its regenerative potency. Chaudhari et al. (2008) have remarked that in vitro leaves are an excellent source of explants as they do not pose a threat to the natural bio resources, low risk of contamination and can be used for genetic manipulations. In the present studies the regeneration potential of in vitro leaf explants was tested because they had application in further set of experiments. These explants can be easily established and healthy shoot can be obtained. In comparison to in vivo leaves, in vitro leaves were able to differentiate less number of shoots. The reason can be the smaller size of the in vitro leaves. Becerra et al. (2004) have also reported less number of shoots from in vitro leaf explants as compared to leaves obtained from green house plant in *Passiflora edulis*.

Thus the regenerative capacity of both the in vivo and in vitro leaves was firmly established in the study.

5.1.1.7 Rooting of in vitro shoots

Once the in vitro shoots were formed the next step was to root them and form an independent plant. The strength of the medium for root induction was noted to be crucial factor and many studies indicated that half strength MS medium is conducive for root formation as compared to full strength (Ahmed and Anis 2005; Faisal and Anis 2005;Ceasar 2010). This is in conjecture to our studies where half strength was superior to full strength medium. Auxins like IAA, IBA and NAA are generally employed for the rooting of in vitro shoots. IBA is noted to be a very potent root inducing auxin and rhizogenesis was achieved in a number of studies such as *Dendrocalamus hamiltonii*(Zang et al.2016); *Cassia angustifolia* (Siddique et al. 2015);*Gloriosa superba* (SivaKumar and Krishnamurthy 2004);*Catharanthus* spp.(Verma and Mathur 2011); *Astragalus nezaketae* (Erisen et al.2010); *Ophiorrhiza japonica* (Kai et al. 2008); *Eryngium foetidum* (Arockiasamy 2002). The effectiveness of IBA in root induction could be because the rate of translocation and degradation of IBA is slow which facilitates its localization near the point of application hence increasing its efficiency to induce roots (Kai et al. 2008). Other auxins can also induce rooting in shoots such as rooting from IAA *Cajanus cajan*, *Murraya konini* and *Trifolium glomeratum* (Rout 2005; Kaushal et al. 2006; Fleck et al. 2009) and NAA in *Garcinia indica*, *Vigna unguiculata* (Brar 1996; Chauhan et al. 2012).

Thus in the present investigation the in vitro plants were induced in presence of cytokinin BA and Kin, elongated in coconut water and rooted in half strength MS medium.

5.1.1.8 Optimization of callus biomass and callus growth index

The biomass of the callus was optimised at 2.5 µM BA and 2.5 µM 2,4D and a number of studies have optimised the callus biomass as in *Mucuna prurians* (Janarthanam and Sumathi 2015), *Tecomella undulata* (Patel and Patel 2013).The callus growth index was calculated to determine the time duration when optimum callus growth. It was recorded to be 7 week after which there was a decline in callus weight and the harvesting of the callus was done at this time duration for the metabolite studies. The duration for callus index growth was dependent on plant species. In *Celosia argentea* (Abu Baker 2014) the callus induction started from the 5^{th} day and reached its lag phase at 6 weeks while in *Toddalia asiatica* (Rajkumar et al. 2010) in 20 weeks.

5.1.2 *P grandiflora*

P grandiflora is another succulent species belonging to the family Portulacaceae. Studies for regeneration were tried with leaf and nodal explants in MS medium

5.1.2.1 Individual cytokinins or auxins induced low response

The leaf explants of *P oleracea* gave encouraging results for regeneration from leaf explants therefore regeneration studies of *P grandiflora* was also initiated with a similar explant. It was observed that single concentration of cytokinin (BA/Kin) and auxins (IAA, NAA, 2,4D) failed to induce morphogeneic response depicting that individually these cytokinin and auxins were insufficient to induce the cells to divide and induce an organogenetic response. Song et al. (2011) in their studies on six cultivars of *Chrysanthemum morifolium* noted that it had low regeneration capacity in the influence of BA, Kin and IAA similar to the present study. Waseem et al. (2009) concluded that a high concentration of endogeneous auxin in the plant prevents shoot regeneration.

5.1.2.2 Cytokinins differentiated shoot buds at high concentration

Individually cytokinin failed to induce in vitro shoots so a synergistic combination of BA and Kin was employed. It was observed that at low concentrations of cytokinin failed to induce shoot buds and the explants turned brown after 6 week. Studies on *Lagenaria siceraria* (Saha et al. 2007) reported the browning of the explants in presence of cytokinin BA and Kin.

5.1.2.3 Combination of cytokinins and auxins induced shoot bud differentiation

Since the single and synergistic combination of cytokinin failed to induce in vitro shoots therefore synergistic combination of cytokinin and auxin was employed. The chain of events leading to the development of adventitious shoot bud is complex yet the ratio of auxin and cytokinin affects the pattern of morphogenesis in petiole and leaf explants (Thorpe 2004). Thus a combination of BA/Kin + IAA, BA/Kin + NAA and BA/Kin + 2,4D was tried for regeneration of in vitro shoots.

It was observed that a combination of BA/Kin and IAA induced callus and shoot buds. The number of shoot buds produced on BA and IAA were more as compared to Kin and IAA. Thus superiority of BA to induce shoot buds was again established with these PGR's. A number of studies have reported shoot bud differentiation in BA and IAA such as *Solanum virginianum* (Shete et al. 2017), *Basella alba* (Shekhawat and Manokari 2016), *Coleus forskohlii* (Krishna et al. 2010).

A combination of cytokinin and NAA was also tried for regeneration. The explants induced callus only in presence of 2.5 µM BA and 0.5 µM NAA however when the concentration of NAA was increased to 2.5-10 µM along with BA (2.5-10 µM) induction of red-green compact callus which differentiated shoot buds was obtained. These results again proved that for shoot bud differentiation auxin concentration is important and the coupled interaction of both cytokinin and auxin is conducive for shoot formation. Differentiating callus and shoot bud from leaf explants in presence of BA and NAA has been reported in *Ruta graveolens* (Ahmed et al. 2010)**,** *Dianthus.chinensis* (Kantia and Kothari 2002) and in Roman Chamomile (Echeverrigaray et al. 2000). The induction of red to green callus is also reported in *Celosia argentea* (Abu Baker et al. 2014) in presence of BA and NAA.

Kin and NAA also induced callus however its growth was less as compared to BA and NAA. This enhanced capability of BA and NAA to induce callus and shoot bud as compared to Kin and NAA is also reported in *Anthurium andreanum* (Raad et al. 2012) and *Crepis novana* (Corral et al. 2011).

The conclusion of the studies is that it was observed that BA/Kin and NAA produced yellow, red to green callus along with shoot buds only which did not proliferate into shoots. Low regenerative capacity along with green callus has also been noted in *Asystasia gangetica* (Tamilselvan and Rajeswari 2014) in presence of BA and NAA.

Combinations of BA/Kin and 2,4D were also evaluated to determine their morphogenic response and whether the combination could enhance in vitro shoot formation. The combinations of BA+2,4D and Kin +2,4D induced white to green friable callus of varying rate of growth. Concentrations of BA and 2,4D were again efficient to induce high growth of callus as compared to Kin and 2,4D. This implied that cytokinin (BA/Kin) were the determining factor controlling growth of the callus as 2,4D was constant in both type of combinations. Fast callus induction in presence of BA and 2,4D was also noted on *Achyranthes aspera* (Sen et al. 2014), *Allium ampeloprasum* (Monemi 2014), *Citrus acida* (Chakravarty and Goswami 1999).

Thus in the present investigation it was observed that the cytokinins (BA/Kin) and auxins (NAA, IAA and 2,4D) singly or in combination could induce shoot buds only and at a combination of 10 µM BA and 5 µM IAA in vitro shoots were obtained. Similar to the present study is the report on *Uraria picta* (Ahire et al. 2011) where the leaf explants when cultured on cytokinins BA/Kin/TDZ and auxins IAA/NAA/2,4D induced yellow to green callus without shoot formation. Studies on *Triticum monococcum* (Miroshnicenko 2017) reported that conventional use of auxins for regeneration lead to poor regeneration which

could be due to genetic components prevailing over exogeneous auxin in controlling the invitro response and the use of auxin with PGRs like Daminozide and phenyl urea cytokinins could improve the rate of regeneration. The studies therefore conclude that the rate of regeneration of *P grandiflora* is low probably due to the presence of some endogeneous components which interferes with the general activity of cytokinin or auxin or genetic factors which control the normal expression of the PGRs.

5.1.2.4 Young leaf explants had a high regenerative potential

It was observed that a high concentration of BA (10 µM) and IAA (5 µM) could induce 1 or 2 shoots therefore it was envisioned that age of the explants may enhance the rate of regeneration as it plays a significant role in organogenesis. Liu et al. 2006 opined that physiological stage of the explants is an important factor which governs the regeneration and callus induction. Studies on *Ophiorrhiza japonica* (Kai 2006) *Passiflora edulis* (Beccera et al. 2004) further stated that younger explants exhibited greater morphogenic potential as compared to older explants.

In the present study the number of shoot bud and in vitro shoots increased with young leaf explants while in the older leaves 1or 2 shoots were induced. Other studies have also shown the similar effect such as bottle gourd (Hans et al. 2004), cucumber (Mohiuddin et al. 1997), Squash (Lee et al. 2003).

5.1.2.5 Multiplication of in vitro shoots on high concentration of BA

The in vitro shoots induced on young leaves of *P grandiflora* were further multiplied at a high concentration of BA. Similar results were obtained in *Spilanthes* (Pandey and Agarwal 2009) where the shoot buds induced in a ratio of cytokinin and auxin (BA and NAA) were multiplied on higher concentration of BA. High number of in vitro shoots was achieved in the studies of Lee (2003) and Verma and Mathur (2011) under the effect of BA.

5.1.2.6 Elongation of shoots with GA_3

The elongation of in vitro shoots was achieved on a medium supplemented with GA_3as it is known to increase the shoot length of the plant as reported in *Jatropha curcas* (Deore and Johnson 2008), *Capsicum annuum* (Golegaokar and Kantharajah 2006), *Citrus jambhiri* (Saini et al. 2010) *Pothomorphe umbellate* (Pereira et al. 2000) etc. De la Guardia and Benlloch (1980) have proposed that growth activity of GA_3 is due to increase transport of potassium ion. Studies on *Pinus sylvestris* and *Picea glauca* suggested that elongation of the

stem in presence of GA_3 is due to stimulation of sub apical meristem (Little and McDonald 2003)

5.1.2.7 In vitro leaf explants had less regenerative capacity

Thus the complete in vitro shoots were obtained from young leaves in presence of BA and IAA which was multiplied on high concentration of BA and elongated on GA_3. When the same protocol was applied for the regeneration from in vitro leaves the number of shoots induced was less as compared to young in vivo leaves. Becerra et al. (2004) during the studies of *Passiflora edulis* have reported a reduced response in terms of in vitro shoots and callus from in vitro leaf explant. They advocated that this decline in response is due to high humidity, low light intensity and medium composition in the culture vessels. In vitro leaves have reduced number of xylem and phloem tissues and a thin cuticle on the epidermal cells (Cozza et al. 1997). These factors thus affect the morphological response of the in vitro leaf explants.

5.1.2.8 Cytokinins induced regeneration from nodal explants

Regeneration of shoots from the leaf explants was moderate and therefore nodal explants were tried for regeneration in presence of cytokinin. It was observed that axillary shoots branched and multiples were induced without callus phase. BA was more effective in inducing in vitro shoots as compared to Kin while the efficiency increased in presence of synergistic combination of the two. Sahoo and Chand (1998) suggest that the development of shoot from nodal explants is the function of cytokinins. The stimulatory activity of BA is because it helps overcome apical dominance and release lateral bud from dormancy to induce shoot formation (George 1993). Superiority of BA over Kin to induce axillary bud differentiation has been reported in a number of species such as *Cypripedium flavum* (Yan 2006); *Ceropegia sahyadrica* (Nikam and Savant 2007); *Andrographis paniculata* (Purkayastha 2008). It was also noted that synergistic combinations gave better results which is supported by studies such as *Solanum virginianum* (Shete et al. 2017).

Regeneration from both the leaf and nodal explants revealed that both the explants have moderate rate of regeneration.

5.1.2.9 Shoot tip necrosis is reduced in presence of high level of Ca^{2+}

The shoots induced from nodal explants were affected with STN an anomaly which caused browning of the tips with severe loss to the cultures. In a multiple cluster the central shoot

showed symptoms for necrosis and lower axillary buds produced shoot. Similar observations were made on *Vitis vinifera* L. (Thomas 2000) where axillary buds produced fresh sprouts whenever the shoot tip was affected and necrotic plants showed a lower level of Ca++ and Mg++. It is due to the deficiency of Ca (Sha et al. 1985; McCown and Sellmer1987) and Boron (Mason and Gutteridge 1974). The, calcium deficiency could result in disturbance of metabolic activities of growing tissues, which in turn result in growth abnormalities such as shoot necrosis (Bairu et al., 2009).

Mirabbasi and Hosseinpour (2014) in their studies on *Ulmas glabra* have reported that STN can be controlled by incorporating high concentrations of Ca and Boron. Therefore experiments were performed in elevated concentrations of $CaCl_2$, Ca-gluconate and Boron to sustain the cultures. It was observed that the number of shoots decreased with increase in concentration of B but STN remained the same. This implied that the increased concentration of Boron was not effective in controlling STN and was toxic to the plant thus affecting the growth rate.

Two sources of Ca ($CaCl_2$ and Ca-gluconate) were employed for the study and both were reported to be effective in controlling STN however shoots derived from higher concentrations of $CaCl_2$ were healthy as compared to Ca-gluconate. Effectiveness of $CaCl_2$ to alleviate STN has been reported in a number of plants such as banana (Martin et al. 2007), *Soymida febrifuga* (Chiruvella et al. 2011),*Pyruspyrifolia* (Thakur and Kanwar 2008). Ca-gluconate also reduced STN appreciably but the shoots developed had long internode and pale green leaves therefore this source of Ca was not preferred to alleviate STN.

5.2 METABOLITE STUDIES

In the present investigation the metabolite content of the two species of *Portulaca* were analysed and the results obtained are discussed in the present section. *P oleracea* has the presence of primary metabolite fatty acids which are a constituent of phospho lipid structures of membranes of different organelles. Betalains on the other hand were analysed in *P grandiflora* which are secondary metabolites produced by the plants. The secondary metabolites are synthesised as a mechanism of defence, not utilized directly by the plants and include flavours, oil, fragrances and pigments (Demain and Fang 2004). Betalains are one such pigment produced by the members of Caryophyllales in higher plants (Brookington et al. 2011) utilized extensively in food industry (Stintzing and Carle 2005; 2007).

5.2.1 *P oleracea*

P oleracea is rich in omega fatty acids which has a number of nutritional benefits. Omega fatty acids are PUFA's which determine the composition of cell membrane and physiological roles such as enzyme activity, membrane transport and receptor functions. It helps in the prevention and treatment of coronary artery disease, arthritis, hypertension, inflammatory autoimmune disorder and cancer therefore these fatty acids should be included in the regular diet (Simopolous 1991).

5.2.1.1 Fat content in the in vivo and invitro samples

In the present investigation the total fats of in vivo and in vitro samples and the relative fatty acid content and constituent of the fats was determined. The first part of the study was to determine qualitatively the presence or absence of the fats in the different samples followed by analysis of the constituent fatty acids in the samples which responded positively to the tests. Sudan III test is a common test to determine the presence of lipids and has been extensively utilized to detect lipids in plant extracts (Martin 1969; Read et al. 2003). All the samples (in vivo and in vitro) except roots tested positive for the presence of fats. Studies on roots of *Borassus flabellifer* (Sahni et al. 2014) have reported negligible fat content by GCMS analysis. The studies conclude that fats may be present in the roots of *P oleracea* in negligible content which was not detected by physical observation by Sudan III test. Further analysis of fats and their constituent fatty acids was initiated on in vivo plant, stem, leaves, in vitro shoots and callus.

Analysis of the fat content revealed that within the in vivo samples, leaves had the highest fat content followed by whole plant and stem. This implied that leaves are the primary site for the synthesis of these fatty acids. Rawsthorne (2002) and Chuman and Brody (1989) suggest that fatty acids are synthesised in the plastids such as chloroplasts which supports the results as leaves are the sites where chloroplasts are present in abundance. Conversely Shintani and Ohlrogge (1994) suggest that mitochondria could be synthesising fatty acids. Plants are an abundant source of fats as high fat content have been reported in a number of species such as *Gongronema latifolium* (Atangwho et al. 2009), *Castanopsis* spp. *Machilus edulis*, *Cinnamomum* species of the Sikkim Himalayas (Sundriyal and Sundriyal 2000)and *Piper nigrum* (Maisuthisaku et al. 2008)

Within the in vitro samples the fat content of callus was slightly higher than the in vitro shoots. The content of metabolites in callus cultures has been reported to be higher than in vitro plants in a number of studies such as phenols and flavonoids in *Stevia rebaudiana*

(Tadhani et al. 2007); Oleanolic acid, rutin, chlorogenic acid and scopoletin content in *Fabiana imbricate*(2004).Radwan et al.(1975) studies on fatty acids of higher plants indicate that the lipids ofcallus culture and suspension culture constitute 5-8% of the dry weight.

Another important finding of the present study was that the fat content of the in vivo plant and in vitro shoots was similar. This implied that in vitro shoots can be a substitute for in vivo shoots for different genetical and biochemical manipulations. Studies of Paul et al. (2010) on *Pogostemon cablin* have also reported similar oil content in the in vitro raised plants and in vivo plant. The studies report fat content of callus higher than in vivo shoot. Similarly a high content of oil was noted in callus cultures of *Jatropha* as compared to the natural plant part i.e. seeds indicating that PGR's can promote the biosynthesis of fatty acids (Hernandez et al. 2015).

The fat or lipid content of tissue culture mediate plants or callus cultures is dependent on a number of factors. Spener et al. 1974 studies on *Hydnocarpus anthelminthica* demonstrated that the lipid content decreases with increase in age of the cultures. Radwan and Mangold (1975) studies on *Glycine soja* and *Brassica napus* recommended that the environmental conditions during the growth of the cultures is important as they effect the lipid content as well as its profile demonstrating that aerated cultures had high content of saturated long chain fatty acids. The temperature inversely affected the lipid content of *Brassica napus* and *Tropaeolum majus* and PUFA was less at a high temperature of 30ºC as compared to 5ºC (Radwan et al. 1978). The present studies on the in vitro raised plants and callus depicted high lipid content as they were initiated in a well aerated environment (mouth of the vessel was plugged with non-absorbant cotton) and at a temperature of 25^{o}C.

5.2.1.2 Fatty acid fingerprinting of in vivo and in vitro samples

The fats obtained from the in vivo and in vitro samples were subjected to the analysis of constituent fatty acids. GC studies revealed that Palmitic acid, Linoleic acid and Linolenic acid are the major fatty acids present in the in vivo shoot, stem and leaves. Lem and Stumpf (1983) suggest that Palmitic acid, Palmitoleic acid, Oleic acid and Linolenic acid are the abundant fatty acids in *Anabaena variabilis* while the leaves of higher plants are rich in Linolenic acid .Linolenic acid are reported to be the major fatty acid in the leaves of a number of plants such as *Punica granatum* Ercisli et al.(2007), Radwan et al. (1975), *Petroselinum crispum* (Lopez et al. 1999) however the studies on *Perilla frutescens* reported that seeds are a better source of Linolenic acid while the leaves accounted to only 0.2%. Palaniswamy et al.(2001) analysed the fatty acid content of *P oleracea* at 6, 10 and 14 leaf

stage and suggested that the content varied with the stage of development. Ezekwe et al.(1999) studies on eight accessions of *Portulaca* depicted that Linoleic and Linolenic acid are the major fatty acids while Omar-Alwara (2001) suggested that besides these fatty acids long chain fatty acids such as EPA, DPA and DHA were also present. The present investigation also reports the presence of Linolenic and Linoleic acid as the major fatty acids but EPA, DPA and DHA were absent in the samples. Studies of Liu et al. (2000) also did not detect the presence of these very long chain fatty acids.

The analysis of in vivo fatty acid content in different samples was followed by the fatty acid analysis of in vitro cultures. Aly et al. (2008) emphasised that the quality and quantity of fatty acid synthesised in vitro needs to be studied and compared with the fatty acids produced in natural plants. The fatty acids synthesised in vitro are generally influenced by the PGR's as they determine the mode of organogenesis and in turn may affect the type of fatty acids produced in culture. Stearns Jr and Mortan (1975) have reported that PGRs play an important role in the synthesis of fatty acids as in soybean suspension cultures it was noted that auxin and gibbrellins formed higher amount of palmitic acid and low amount of PUFA while cytokinin and gibbrellin promoted the formation of unsaturated fatty acids such as Linoleic and Linolenic acid. The effect of PGR's in lipid composition and lipid metabolism in terms of its addition and alteration have been elucidated in a number of studies (Eugene et al. 1975; Banibrata and Gadgil 1984; Marta 1993; Wennuan et al. 1995; Matsuda et al. 2001).

In the present studies it was noted that in vitro shoots synthesised a higher content of Linoleic and Linolenic acids as compared to callus cultures. A possible explanation is that in vitro shoots were induced in cytokinins while callus cultures were formed in presence of BA and 2,4D and these PGR's were determining the fate of fatty acid synthesised and their pathways. It is also suggested that BA modify the fatty acid content as it influences the synthesis of pyruvate kinase leading to the formation of carbonated chains in the synthesis of fatty acids (Ireland 1979). Machado et al.(2006) in the studies of *Cereus peruvianus* opined that the difference in fatty acid synthesis of callus and shoots is due to differential metabolic influx in callus tissues. Schaeffer and Sharpe (1971) have reported that cytokinin effect the synthesis of Linolenic acids in leaves. The PGR's not only govern the biosynthesis of fatty acids but also their relative concentration. A recent studies of Hernandez et al. (2015) in *Jatropha* have reported that the concentration of PGR's also play an important role in fatty acid synthesis as fatty acid was absent in less concentration of BA and NAA while at high concentration of BA eight different types of fatty acids particularly Linoleic and Linolenic acid was synthesised.

It was also noted in the present studies that the content of Linoleic and Linolenic acid was slightly less with in vitro shoots as compared to in vivo shoots. Studies of *Petroselinum crispum* (Lopez et al. 1999), *Ajuga multiflora* (Sivanesan 2016) have also reported lesser fatty acid content in the in vitro shoot cultures as compared to the in vivo plants.

Callus induction is mainly required for the large scale production of the secondary metabolites and for genetic engineering (Jose et al. 2012). In the present studies the fatty acid content of callus cultures depicted Palmitic acid as the major fatty acid and Oleic acid, Linoleic acid, Linolenic acid and Docosanoic acid were also present. Studies of Haldar and Gadgill (1984) in *Cucumis melo* have also reported Palmitic acid to be the prominent fatty acid callus cultures obtained from cotyledon explants. Similar results were reported in callus cultures of *Wedelia prostrata*(Abd El-Mawla et al. 2011), and *Petroselinum crispum* (Lopez et al. 1999).

5.2.1.3 Occurrence of long chain fatty acid in cultures

Long chain fatty acids have been reported in the present studies where the carbon chain length was both odd and even. Odd chain fatty acids were of rare occurrence in plants but with the advent of analytical techniques traces have been reported in a number of plants and their function in the plant system needs to be studied in detail (Rezanka et al. 2009). In the present studies callus cultures synthesised Heptadecanoic acid and Tricosanoic acid which are odd chain fatty acids. Such odd chain fatty acids have also been detected in the callus cultures of *Cereus peruvianus* (Machado et al. 2006), *Sonneratia alba* (Patil et al. 2011). GCMS studies revealed the presence of long chain fatty acids such as Arachidic acid, Docosanoic acid, Tetracosanoic acid and Hexacosanoic acid in different samples and similar studies are reported in *Petroselinum crispum* (Lopez et al. 1999).Thus the fatty acid fingerprinting in both in vivo and in vitro samples reported the presence of Omega fatty acids like Linoleic and Linolenic acid and long chain fatty acids up to the range of C26

5.2.2 *P grandiflora*

Another species of *Portulaca* i.e. *P grandiflora* was analysed for betalain which is an important secondary metabolite extensively used in colouring neutral and low acidic food (Stintzing and Carle 2007). This food pigments is soluble in water having a nitrogeneous moiety, present in the members of Caryophyllales and some fungi consisting of red pigment betacyanin and yellow betaxanthin (Harris et al. 2012). Beet root is a rich source of betalain but since it is an underground modification of root so the rate of contamination is high,

unpleasant flavour due to pyrazines and disagreeable smell due to geosmins making it unfit for food industry (Acree et al. 1976). Therefore alternative source of this pigment are being explored (Swarna et al. 2013). *P grandiflora* was consequently analysed for the presence of betalains in different parts of the plant in the present investigation.

5.2.2.1 Beetroot is utilized as a standard for analysis of betalains

Extraction of betalain was performed in methanol with sodium ascorbate as a source of ascorbic acid. Moreno et al.(2008) recommends that the extraction of betalains is performed with organic solvent methanol and ascorbic acid is added to lower the pH which will stabilize the betalain pigment and prevent oxidation by polyphenoloxidases (PPO). Similarly Strack et al.(2003) have proposed the addition of ascorbic acid as it prevents the degradation of betalains and appearance of residues in the mixture. Studies on *Hylocereus polyrhizus* (Woo et al. 2011) suggested that 0.1% concentration of ascorbic acid is optimum to maintain the stability of the pigment. In the present investigation sodium ascorbate was the source of ascorbic acid. Studies on *Opuntia* spp. (Stintzing et al. 2005); *Chenopodium quinoa* (Escribano et al. 2017); *Parakeelya mirabilis* (Chung et al. 2015) etc. have also utilized sodium ascorbate as a source of ascorbic acid.

5.2.2.2 Betalain content was highest in the stem of the plant

Beet root was selected as a standard because it is a well known source of betalains (Georgiev et al. 2008; Pavokovic and Krsnik- Rasol 2011). The red food dye obtained from beetroot “Redibeet E 162” has been used as a standard by Trejo-Tapia et al.(2008) and crude betalain extract of beetroot separated by HPLC have been used as standard by Lee et al. (2013). The qualitative confirmation for the presence of betalain was carried out by paper chromatography with isopropanol: ethanol: water: acetic acid as mobile phase. A similar mobile phase for the separation of betalain pigment was utilized in thin layer chromatography of callus cultures of *Beta vulgaris* var ‘Dark Detroit’ (Trejo-Tapia et al. 2008); *Hylocereus polyrhizus* (Rodriguez et al. 2016) etc.

The betalain content of various in vivo samples were analysed and it was observed that stem has maximum betacyanin content and betaxanthin was absent. Rink and Bohm (1985) reported that betaxanthins were absent in the plants of *P grandiflora* however when fed with L-DOPA the plant could synthesise both betacyanin and betaxanthins in petals. Betalains are reported to be absent in rootsand young leaves and negligible in old leaves**.** Maximum betalain content was present in the purple coloured petals of *Gomphrena globosa* with traces

of betaxanthin (Kuglar et al. 2007). Similarly highest betacyanin content was recorded in flower followed by stem and leaves in *Talinum triangulare* (Swarna et al. 2013). These reports suggest that flowers are a good source of betalains followed by stem.

5.2.2.3 Sodium ascorbate in the samples stabilizes betalain content

The experiments revealed that the stem sample without sodium acerbate had better betalain content as compared to the one with ascorbate. However the analysis of the pigments after 24 hours revealed that there was a decline in betalains in the sample without ascorbate while the sample with ascorbate could maintain its stability. This suggested that addition of ascorbate ion is essential to preserve the stability of the pigment. Reynoso (1997) in the studies of Garambullo and beetroot have reported that there was a70% decline in betalain pigment in the absence of ascorbate after 24 hours.

Thus studies on betalain content of *P grandiflora* revealed that addition of sodium ascorbate to the samples prevented the degradation of betalain. Betacyanin was present in the betalain content while betaxanthins were absent in the plant and maximum content of betalain was present in stem.

5.3 MUTATION STUDIES

5.3.1 *P oleracea*

Mutations are the heritable changes which alter the genotype of an individual. It is of two type's Spontaneous mutation and Induced mutation. The frequency and occurrence of spontaneous mutation is very low and therefore induced mutation is frequently used for introducing desirable traits within an organism. The green revolution of 1970s was possible mainly due to mutation breeding in plants ably guided by Prof. MS Swaminathan in India. This technique needs to be assisted by the technology of 21st century to cater to the ever increasing population of the world. With increasing demand for food and pressure on land usage no single technique can work in isolation and needs to be supported by other interventions and plant tissue culture is a technique which requires little land usage and even the recalcitrant plants can be induced to grow within the controlled environmental conditions. This technique is coupled with mutation breeding where the mutations are induced in presence of physical mutagen or chemical mutagen (Jain 2005). Plant tissue culture technique when coupled with induced mutation can accelerate the breeding program by inducing variations, selecting beneficial variants and subsequently multiplying the desired genotype (Maluszynski et al. 1995). Preil (1986) suggests that in vitro techniques can shorten the

breeding time duration and can reduce the cost of the development of new variety Thus in vitro mutagenesis can lead to incorporation of desirable traits within the in vitro plants and callus cultures within a short span.

5.3.1.1 EMS is a suitable chemical mutagen to induce mutations

In the present investigation in vitro leaf explants of *P oleracea* were treated with EMS and cultured on an optimised medium. EMS is selected as a chemical mutagen in a number of studies because of its easy usage, handling and effectiveness in inducing mutations (Jency et al. 2017). It also induces high frequency of gene mutation and low rate of chromosomal aberrations (Van Harten 1998). The mode of action of EMS is suggested as it is an alkylating agent and causes G/C to C/G or G/C to T/A transversion or A/T to G/C transition by pairing errors of 7 ethyl guanidine and 3 ethyl adenine (Greene et al. 2003; Kim et al. 2003). Mutations by EMS on the basis of codon uses is reported to cause missense and nonsense mutation however the frequency of missense mutation is more than nonsense mutation (Mc Callum 2000). Thus within the chemical mutagen EMS is frequently employed to generate genetic variability within the plants.

5.3.1.2 Determination of LC50 dose

Induction of mutation is largely dependent on the concentration/dosage of the mutagen and it is necessary to determine LC50 dose where at least 50% of the explants can survive the effect of mutagen. Jency et al. (2017) recommended that the rate of mutation, its efficiency, the number of screened plants and determination of LC50 value is important which needs to be standardised for each species as it varies with biological material, type of mutagen and the environmental condition. Rajarajan et al.(2016)studies on rice noted that concentrations below LC50 value helped in the recovery of plants after treatment. Thus determination of LC50 dose is imperative for a successful mutation program which is frequently determined by probit analysis (Kangarasu et al. 2014). In the present investigation the LC50 value of EMS treated in vitro leaf explants was 0.3% which was determined by Probit analysis. This statistical tool was useful in the determination of LD50 value in a number of plants such as Kodomillet (Jency et al. 2017), Rice varieties White Ponni and BPT 5204 (Ramchander et al. 2014), C01 and C02 varieties of Soybean(Karthika and Subba Lakshmi 2006), sugarcane varieties CP48-103 and CP57- 614 (Sadat and Hoveize 2012)*Citrus jambhiri* Lush. (Kaur 2015), *Manihot esculenta* (Kangarasu et al. 2014) etc.

5.3.1.3 The number of EMS treated in vitro shoots per explants was less than the control

The in vitro leaf explants were treated with 0.1-1% concentration of EMS and the morphological response of the explant varied with increase in concentration of the EMS. At a low concentration (0.1-0.3%) the treated explants induced shoot formation while as the concentration increased there was a gradual shift of morphogenic from shoot induction to callus formation at 0.4-1%. Similar observation was made in *Wasabia japonica* (Hung and Johnson 2008) where callus was formed when the shoot tips were irradiated with high dosage of gamma and X-rays. The shoot cultures which were induced in presence of 0.1-0.3% EMS developed deformities in leaves and similar results are documented in the in vitro studies of *Bacopa* (Naik 2012) in presence of EMS.

In the present studies it was noted that the number of shoots decreased with increase in concentration of EMS. This decrease in the expression of regenerative capacity with increase in concentration of mutagens have been elucidated in a number of plants such as Kodomillet (Jency et al. 2017); Rice varieties White Ponni and BPT 5204 (Ramchander et al. 2014), *Saintpaulia* (Fang and Traore 2011), *Asteracantha longifolia* (Bahera et al.2012), Rice variety ADT(R) 47(Rajarajan et al. 2016). Bahera et al. (2012) have opined that EMS has a genotoxic effect on the totipotency and plasticity of cells leading to reduced expression.

EMS 0.2% is reported to be the optimum concentration for the generation of microshoots with minimum visible damage to the explant. Same can be considered for developing shoots in Peach where the shoot tip explant under the influence of 0.2% EMS developed optimum shoots (Elhiti et al. 2016). A similar stimulatory effect of EMS on shoot generation was observed in *Citrus jambhiri* (Sharma et al. 2013). Zaka et al. (2004) and Gunckel and Sparrow (1961) have suggested that this enhancing effect was due to change in rate of cell division and activation of growth hormones.

In the present studies the number of shoots formed in treated optimum concentration of EMS was less than control. Similarly Karthika and Subba lakshmi (2006) have also reported a decreased expression in gamma and EMS treated varieties of Soybean as compared to control. The number of explants of *Saintpaulia ionantha* surviving on the MNH dose (N-nitroso-N-methyl urea) also decreased as compared to control (Gaj and Gaj 1996).

5.3.1.4 Callus biomass decreased in EMS treated explants

It was observed that the fresh weight and dry weight of the callus decreased drastically after 0.2% EMS concentration. A similar reduced rate of proliferation of callus was observed in

sugarcane varieties of CP48-103and CP57-614 under the effect of EMS (Sadat and Hoveize 2012). EMS concentration of 0.2% was optimum and biomass was less than control. Studies of Krupa-Malkiewiczi (2017) on Petunia plant reported that callus viability decreased with increase in EMS concentration and the effect of EMS on callus depends on the plant genotype and concentration of mutagen.

5.3.1.5 Alteration in metabolite content in EMS treated cultures

Besides the variation in phenotype, in vitro mutation has also been utilized to study the metabolite profile of the in vitro plants. Both physical and chemical mutagens separately or in combination have been utilized for this purpose such as *Psophocarpos tetragonolobus* (Klu et al. 1997); *Artemisia annua* (Inthima et al. 2014); *Bacopa monnieri* (Naik et al. 2014). Gavidia and Perez-Bermudez 1999 isolated a mutant obtained from gamma irradiation of *Digitalis obscura*from shoot tip explants with high cardenolide content.Lee et al (2008) reported mutants in *Dendranthema grandiflorum* (Chrysanthemum) induced from gamma irradiation which had variation in flower colour and anthocyanin content 4 times lower or 6 times higher than the parent plant.

Similarly under the influence of EMS the metabolite content has been altered in a number of plants. Agarwal et al. (2017) reported enhanced level of steroid saponegins from *Trigonella foenum-graecum* and *Trigonella corniculata* when treated with EMS. Similarly the two varities of *Withania somnifera* Poshita and Jawahar 22 when treated with EMS produced plants which had a higher content of Withafarin A.

The treated cultures were subjected to the analysis of the fatty acid content both in vitro shoots and callus cultures in different doses. There was a decline in the content of Linoleic and Linolenic acid in the treated in vitro plants at 0.1-0.3% EMS. Contrary to our studies *Brassica carinata* (Barro et al. 2001) an important oil seed crop have reported a rise in erucic acid content where 6 mutant lines were developed with more than 52% of this fatty acid. (Getinet et al. 1997) have described an allele which is responsible for the change in fatty acid. (Barro et al. 2001) also suggest that a change in complex enzyme system is possible which leads to changes in expression of fatty acids. Another study on *Papaver somniferum* with gamma irradiation and EMS revealed that a variant was obtained which had a high content of the alkaloid thebaine due to macromutation in the genetic material or changes in biosynthetic pathway (Chaterjee et al. 2010). Thus further studies are required both at the molecular and biochemical level to understand the unexplained increment of the content of metabolites under the influence of mutagens.

The other fatty acids reported from the treated shoots do not show any appreciable change in the content. This is in conjunction to the studies of (Naik et al. 2012) where there is no increase in Bacoside A content of *Bacopa* in presence of EMS.GCMS studies revealed that the treated invitro shoots also synthesised cyclo propanoic acids such as Methyl 9,10 methylene hexadecanoate, Methyl 9,10 methylene octadecanoate and Octdecanoic acid 9,10,12 trimethoxy methyl ester and isomers of fatty acids such as 10,13 Octadecadienoic acid methyl ester and an isomer of Docosanoic Acid. This change in fatty acid profile implied that under the influence of EMS the biosynthetic pathway is affected which leads to the synthesis of novel fatty acids. Synthesis of long chain fatty acids such as Tetracosanoic acid and Hexacosanoic acid in the shoots also depicted that EMS has the potential to shift the biochemical pathway leading to the production of long chain fatty acids.

Fatty acid profile of treated callus depicted a high rise of Linoleic acid at 0.1% EMS and at higher concentrations the synthesis of Linoleic acid was decreased. Similarly the callus cultures were unable to synthesise Linolenic acid in all the treated concentrations. The treated concentrations had the presence of cyclo propanoic acid such as cyclo propane octanoic acid. Besides this long chain fatty acids such as Docosanoic, Tetracosanoic and Hexacosanoic acid were also synthesized. It is thus suggested that Linolenic acid and Linoleic acid were either absent or degenerated. The biochemical pathway at high concentration of EMS shifted towards the synthesis of long chain fatty acid.

A shoot culture at 0.4% EMS did not synthesise Linoleic and Linolenic acid however it has the potential to synthesise Docosanoic acid, Tetracosanoic acid and hexacosanoic acid. Similarly at 0.6% EMS shoot culture synthesised both Linoleic and Linolenic acid and odd chain fatty acids such as Tricosanoic acid. The results suggest that EMS has the potential to alter the type and content of fatty acid synthesised by the explants.

It is thus concluded from the studies that in vitro mutagenesis in presence of EMS have the potential to synthesise long chain fatty acids which is nutritionally beneficial.

In the present study the genetic determination of the mutants obtained is not done but the occurrence of variation after EMS treatment indicates changes at the genetical level for which further studies need to be conducted.