Chapter – V

Discussion

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Low cost tissue culture medium

The need for low-cost plant tissue culture systems, applicable for micropropagation and *in vitro* conservation of plant genetic resources, has been emphasized to allow the large-scale application and adaptability of such technology in developing countries (IAEA, 2004). Low cost option should lower the cost of production without compromising the quality of the micropropagation and plants (Stephan and Rajavel, 2014). This problem has been addressed by inventing reliable cost effective tissue culture methods without compromising on quality of plants. Cost of chemicals, media, energy, labor and capital affects the production cost. Low cost technology means an advanced generation technology in which cost reduction is achieved by improving process efficiency and better utilization of resources (Savangikar *et al.*, 2002).

Tissue culture refers to growing and multiplication of cells, tissues and organs on defined nutrient medium under aseptic and controlled environmental conditions. The technology is used for the production of doubled haploids, cryopreservation, propagating new plant varieties, conserving rare and endangered plants, difficult-to-propagate plants, production of secondary metabolites and transgenic plants (Ahloowalia *et al.*, 2002).

Recurring costs of micropropagation and *in vitro* conservation include those for chemicals that are used in culture media, i.e., carbon sources, gelling agents, inorganic and organic supplements, and growth regulators. In plant production through micropropagation, media chemicals cost a little less than 15% of the total cost Prakash *et al*., 2004. Out of all components used in a media, gelling agents such as agar contribute 70% to the total cost of media George *et al.*, 2008.

Effect of low cost Macro Nutrient

In this study the cost of medium reduced when low cost macronutrients source were used (Table-3.2). This is in agreement with the findings by Gitonga *et al.*, 2010, in which the cost was reduced by 96.2%, 93.1% and 95.0% respectively when low cost macronutrients (Ammonium fertilizer, Epsom salt and potassium fertilizer) were used in the in the initiation and multiplication of banana plants. According to Ogero *et al.*, 2012, the use of ammonium quarry salt, Epsom salt and Potassium fertilizer in the regeneration of cassava reduced the cost of macronutrients by 96.2%, 93.0% and 94.9% respectively.

Similar results achieved in *Tylophora indica* had the regeneration response of the shoots formed (Tables 4.1). All the media containing low cost macronutrient was compared to the media with conventional macronutrients. Commercially available macro nutrients occurring in the form of hydro Agri's

fertilizer has also been used successfully as an alternative resource for *in vitro* micropropagation *Tylophora indica*.

In the present study conventional MS medium replaced the different types of low cost macro nutrients Ammonium nitrate fertilizer, Calcium chloride fertilizer, Potassium Nitrate fertilizer, Magnesium Sulphate fertilizer and Potassium dihydrogen phosphate are used for *in vitro* culture. Similar studies were conducted with locally available agrochemicals as low-cost sources of tissue culture nutrients. This is in agreement with work done by Santana *et al*., 2009, who used different concentrations of Hydro® Agri´s fertilizer for *in vitro* regeneration. They reported that higher rates of fertilizer were toxic to the tissues and that intermediate fertilizer use at 2 g/l seemed to be optimal for *in vitro* propagation. Similarly Escobar *et al*., 2006, also tried different kinds of fertilizers at different rates and reduced the cost reduction of 24.4%.

Anoop and Chauhan 2011, investigated on cost effective and economically cheaper alternatives to MS salts, agar and sucrose. In that low cost media, tapioca was used as a substitute for agar and cane sugar in place of sucrose due to easy availability. Calcium ammonium nitrate (6.6 gm/l) and sugar (30 g/l) were used in place of MS salts. The results indicated that low cost media was consistently better for shooting and proliferation in cultivar of *Kufrihi malini*.

Effect of Low cost Micro Nutrient

Low cost micro nutrients were standardized to conventional MS micro nutrient low-cost macro nutrients by adding Potassium Iodide (LR), Power B-boran (Boric Oxide), Manganese Sulphate fertilizer, Zinc Sulphate fertilizer, Adbor powder (Sodium Molybdate fertilizer), Chelated fertilizer (Copper Sulphate), Grandular / powder (Cobalt chloride) using *in vitro* culture. Similar results obtained from Gitonga *et al*., 2010, developed a low-cost protocol for micropropagation in banana and they used locally available fertilizers as a substitute for micronutrients. Savangikar *et al.*, 2002, developed a low-cost medium by replacing the conventional sources of Murashige and Skoog (MS) salts with Easy grow vegetative fertilizer containing both macro and micronutrients. A low cost medium was developed for *in vitro* microprogation of C*assava* by Santana *et al*., 2009. Hydro Agri's fertilizer was used as a substitute for Murashige and Skoog macro and micronutrients.

A conventional plant culture medium usually contains a basal solution with major and minor mineral elements, a source of carbon (that is, sucrose), vitamins, growth regulators, a gelling agent for semi-solid media and water Trigiano and Gray, 2000; Roca *et al.*, 1980, Roca, 1984, established the conditions for *Tylophora indica* propagation using conventional tissue-culture techniques.

Similarly, in the present study the cost of medium reduced when low cost micronutrients source was used (Table-3.3). In this study, *Tylophora*

indica regeneration response of shoots developments were studied (Table-4.2). In these media containing low cost micronutrient was compared to the conventional micronutrients. Low cost macronutrients such as Potassium Iodide (LR), Power B-boran, Boric powder, Manganese fertilizer, Zinc Sulphate fertilizer, Adbor powder, Chelated fertilizer, Grandular/ powder and they were used in the present study.

Likewise, MS Iron source (Control) was replaced with different concentration of low cost Iron source (Table-3.4). The low-cost iron source morphogenic response of *Tylophora indica* was studied (Table-4.3). Similarly, on *Tylophora indica* the effect ofconventional MS vitamins and different concentration of low cost vitamins was also observed (Table-3.5). The selected different concentration of low cost vitamins and their morphogenic response of *Tylophora indica* shoot tip and node explant was recorded (Table-4.4). In these different concentration of B-complexvitamin tablets (0.5 to 2.0 mg/l), B-Fort vitamin tablets (0.5 to 2.0 mg/l) was used.

This might be due to nutrients composition, considered to be major sources of variation in plant tissue culture Gitonga *et al.*, 2010 and higher nitrate, Sulphate and relatively lower phosphate content of MS. Composition and quantity of nutrients in MS medium have significant effects on rate of cell growth, differentiation and cell totipotency. MS used in media culture represented the optimum quantity and this result was approved by Ahloowalia *et al.*, 2002 when comparing with full strength of MS.

Effect of Low Cost Carbon source

Even though carbohydrates are of prime importance for cell growth, maintenance and differentiation *in vitro*, the fundamental aspects of carbon utilization and metabolism in cell and tissue cultures have yet to be fully understood Ganapathy *et al.*, 1995. In the present study also, the growth of *Tylophora indica* is greatly influenced by different carbon sources supplemented in the media. In plant tissue culture, AR grade sucrose serves as a carbohydrate supply to provide energy for cell. In order to reduce the cost of the culture medium, commercially available white refined sugar (table sugar), unrefined brown sugar, sugarcane juice and jaggery at different levels were used. But in the present study, high frequency, maximum number of shoots was induced on white refined sugar supplemented medium. The results obtained are in line with the earlier observations in *Mulbury* by Vijaya Chitra and Padmaja, 2001, where addition of white refined sugar instead of sucrose in the multiplication medium increased the shoot number and also growth of the shoots. In contrast to our study many authors have reported sucrose as a better source for shoot proliferation than other carbon sources in micropropagation of several plant species such as Patchouli *Pogostemon cablin* Berth (Kumaraswamy *et al.,* 2009; *Centellea asiatica* Anwar *et al*., 2005; Peach root Tauquer *et al*., 2007). Sucrose has been reported to be the best source of carbon and energy Bridgen, 1994. However in the present study, the use of white refined sugar has shown best results than the use of sucrose. This suggests that

sucrose can be replaced by white refined sugar for *Tylophora indica* tissue culture. Similar study in Many laboratories have reported the use of table sugar in plant propagation medium Ganapathi *et al.,* 1995; Kaur *et al.,* 2005; Zapata 2001 has successfully reduced the cost of banana tissue culture by 90% by replacing the tissue culture AR grade sucrose with a commercial sugar. Besides, utilization of locally available table sugar can reduce the cost of potato tissue culture by 34-51% without any quality problems of tissue cultured plants (Demo *et al.,* 2008). It is therefore recommended that white refined sugar can be considered as low cost substitute for *Tylophora indica* micropropagation.

This has also been used in previous studies as a low cost of carbon source in the conservation of turmeric Tyagi *et al.,* 2007 and micropropagation of banana Gitonga *et al.,* 2010. According to Kubota *et al.,* 2001, Demo *et al.,* 2008, the supply of sugar to the culture medium promotes plant growth *in vitro* and compensates for the low or negative net photosynthetic rate as a result of poor photosynthetic ability thus increasing the survival rates of tissue sections cultured *in vitro*. Therefore, plantlets require an initial source of carbon and hence energy from the medium until they are capable of using carbon dioxide as their main source of carbon.

Effect of low cost growth regulators

Among the different low-cost growth regulators (Table-3.7), the low-cost growth regulators and their morphogenic response of *Tylophora indica*

was studied (Table-4.6). In these different concentration of Auxin IAA (AR), NAA (AR), IBA (AR) Cytokinin, BAP (AR), Kinetin (AR), and GA_3 (AR) alternative using growth hormones were used.

Effect of Low Cost Solidifying agent

From our experiments carried out, it clearly indicated that organic and supporting substitutes are easily available and there were a big different in cost as stated in the control media in (Table-3.1). Low price alternative gelling agent can be used as substitute in (Table-3.8) in plant tissue culture. In this study, *Tylophora indica* regeneration response of shoots developments for different solidifying agent were also observed (Table-4.7). Likewise Maliro and Lameck, 2004, showed that Cassava flour has been used as a substitute for agar. It has been reported that the combination of starch, semolina and potato powder or combination of starch and agar can be a low-cost option for shoot induction in African violet by Sharifi *et al*., 2010.

As reported by Deb and Pongener 2010, agar substance is often used in plant tissue culture as supporting agent, but because of the relatively high material costs caused them to do research for alternative materials with lower cost. Instead of high price of pure grade agar, there are some doubts about its nontoxic nature that influenced researcher to find alternatives material (Sharifi *et al.*, 2010). Pierik 1989 and Nagamori and Kobayashi, 2001 stated cheaper agar alternatives include various types of starch and gums have been investigated

in commercial micro propagation. Commercial starch or flour are used as a supporting agent containing a high amount of starch, vitamin C and carbon sources (carbohydrates), and a low amount of other minerals.

Low cost instruments and glassware's

Media chemicals cost less than 15% of micro-plant production (Prakash, 1993). In some cases the cost may be as low as 5%. Of the medium components, the gelling agents such as agar contribute 70% of the costs. Other ingredients in the media - salts, sugar and growth regulators - have minimal influence on production cost and are reasonably cheap. According to Nagamori and Kobayashi, 2001 and Savangikar *et al.,* 2004, plant cultures can be maintained in rooms with air conditioners and tube lights instead of highly priced plant growth chambers. The conventional method of downward illumination can be replaced by sidewise lighting systems which not only reduce the number of lights but also provide more uniform illumination to the cultures. According to FAO, in small scale laboratories, the autoclaves can be replaced by large sized pressure cookers which are much cheaper. Escobar *et al.,* 2005, stated that instead of having one or two huge horizontal autoclaves which generate hot air pockets in the sterilizing room, it is better to have more of smaller vertical autoclaves which keep the air cool. The normal practice to use costly aluminum foil for wrapping instruments for sterilization can be replaced by stainless steel containers which are autoclavable. Prakash *et al.,* 2004, have stated that the

expensive imported vessels can be replaced with reusable glass jars and lids which can reduce the cost of production. Savangikar *et al*., 2004, have reported that disposable plastic bags eliminate the cost of washing of culture vessels and can reduce the labor cost per propagule by 60%. Kodym and Francisco, 2004, have reported the use of glass bottles and baby-food jars with polypropylene caps as economic when compared to conical flasks which are more expensive.

It is advisable to have a separate area for storage of chemicals, apparatus and equipment. It would not only facilitate constant availability but also save cost from bulk purchase. Chemicals required in small amounts should not be purchased in large quantities as they may lose their activity, pick up moisture or get contaminated George *et al.,* 2008. *In vitro* growth of plants is largely determined by the composition of the culture medium. The main components of most plant tissue culture media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators, a gelling agent, Gamborg *et al*., 1968; Gamborg and Phillips, 1995.

Direct Organogenesis

The effectiveness of 2,4-D and BAP on bud sprouting and shoot multiplication of *Tylophora indica* several Kalimuthu and Jayaraman, 2012 in other regulators were treated in the present study. Micropropagation of *Tylophora indica* spp. have been reported previously on MS solid media in contrast our study Krishna Reddy *et al*., 2010. The use of low cost media for

micropropagation has several advantages. Besides the saving in the cost of the culture medium shoots or plantlets growing on low cost media are easier to handle during cultures and transfer to soil. Traces of agar will also remain on the roots of solid media. This will encourage microbial infection and mortality of the plantlets. Shoot cultures in low cost media have been employed successfully in several plant species FAO/IAEA, 2002. Although organic additives form a constituent of most plant tissue culture media. It is believed that this is not a limiting factor since green tissues are partly autotrophic and can produce the essential vitamins. Cultures in media prepared in tap water also appeared to be normal. Tap water will contain most of the microelements in significant quantities but the actual concentration will vary with location and has to be tested critically before culture media are routinely prepared with it.

Similar findings of *Tylophora indica* from most of the studies reported mass propagation through indirect regeneration except for studies conducted by Bera and Roy, 1993 and Faisal *et al.*, 2007 in contrary to our results to direct regeneration through leaf and nodal explants in of *Tylophora indica* and it was recorded through compact tissue formation at the basal cut end. In *Tylophora indica*, explants showing direct organogenesis on cytokinin reverted to indirect mode of regeneration when cultured on cytokinin-auxin combination. Interestingly, in both the plant species 2,4-D (2.0 mg/l) + BAP (1.0mg) combination treatment was most responsive for optimal regeneration irrespective of the

explant type wherein the preformed shoots were encouraged to elongate under the influence of NAA and the regenerative tissue started multiplying and differentiating new buds as a result of the synergistic effect of cytokinin-auxin medium. Synergistic effect of cytokinin-auxin combination has also been demonstrated earlier, Bilkey and Cocking 1981. In present study NAA at lower concentrations enhanced the shoot induction in the presence of optimal cytokinin concentration which is in agreement with the results obtained in *Tylophora indica*, Sharma and Chandel, 1992.

In corresponding to our study cytokinin alone or in combination with NAA and IBA has been to exhibit maximum shoot organogenesis in *Glycine max* Kothari *et al.*, 1991 and Sharma and Kothari, 1991, *Piper logum*, Sarasan *et al.*, 1993, In *Eryngium foetidum* BAP alone was sufficient for obtaining organogenic callus Arokiasamy and Iganicimuthu, 1998. These results are coherent with our reports that the Kin or BAP (0.5-3.0 mg/l) are needed for shoot bud differentiation of many plants from various explants (Franklin *et al.*, 1991; Dan and Reichert, 1998 and Venkatachalam *et al.*, 1998).

Earlier reports demonstrated that plant regeneration was possible through apical shoot bud, node, internodes, leaf and nucellar tissues explants (Pati *et al.*, 2008; Arumugam *et al.*, 2003; Ajithkumar & Seeni, 1998; Hossain *et al.*, 1993). Successful regeneration of plantlets using mature nodal explants nucellar tissue Hossain *et al.*, 1993, cotyledon Hossain *et al.*, 1994), hypocotyls

Hossain *et al*., 1995, embryonic tissue Islam *et al.,* 1995 and cotyledonary node Arumugam & Rao, 1996, of *Aegle marmelos* has been reported. As observed in the present study, Ajithkumar and Seeni, 1998 reported that nodal segments were more responsive than apical shoot tips. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of a stem.

The shoots had elongated and grew well and developed many leaves. For shoot, proliferation cytokines are one of the major important factors affecting the response, Bhojwani, 1980. A wide range of cytokinins like BAP, 2ip and kinetin have been employed in shoot proliferation Bhojwani and Razdan, 1982. However, a wide survey of literature suggests that BAP is the most reliable and effective cytokinins. The shoot proliferation effect of BAP observed in the present study is in consonance with other reports Rathore *et al.,* 2008.

The result is in accordance with different reports from the literature where cytokinins have been regarded as one of the most important factors affecting the response of shoot proliferation Lane, 1979; Bhojwani, 1980; Garland and Stolz, 1981. A wide range of cytokinins like kinetin, BAP, Auxinhave been employed in shoot proliferation. Similar results were also reported for direct shoot bud formation from root explants of *Tylophora indica* on MS supplemented with different concentrations of BAP (10.72 to 26.80 µm) Chaudhuri *et al.,* 2004.

Rooting

The regenerated shoot required IAA, IBA, NAA concentration for rooting. On transfer to media supplemented either with IBA, IAA and NAA in the growth of each isolated plantlet was enhanced with simultaneous adventitious rooting from the basal part. Similar observation have also been reported in *Glycine max*, Widolm and Rick, 1982 and Write *et al.*, 1986, chick pea Arokiasamy *et al.*, 2000.

In the present study, IAA was found to be more effective for higher frequency of root initiation. The highest frequency of rooting was recorded on medium containing 0.4 mg/l IAA in concentration, where all shoots formed roots within 10-15 days of culture. Similar observation was also made *in vitro* rooting induction reports in different plants by Punitha and Yogendra Kumar, 1992, *Cajanus cajan* by Siva Prakash *et al.*, 1994, *Morus alba* Patnaik and Chand, 1996 and in *Centellea asiatica* by Patra *et al.*, 1998.

Hardening

The use of organic matter such as animal manures, human waste, food wastes, yard wastes, sewage sludges and composts has long been recognized in agriculture as beneficial for plant growth and yield and the maintenance of soil fertility. Reddy and Reddy, 1999 reported significant increases in micronutrients in field soils after vermicomposting applications compared to those in soils treated with animal manures.

Indirect organogenesis

The callus induction of *Tylophora indica* was successfully initiated and established in the laboratory by using 0.2 mg/l of BAP and 2 mg/l of NAA in the MS medium supplemented with 3% of sucrose and 0.8% of agar Faisal and Anis, 2003, Faisal and Anis, 2005. Nodal explants of *Tylophora indica* produced maximum callusing potential in the medium supplemented with 2,4-D at 5 mg/l. Various factorial combination of hormones elucidated different morphogenetic potential of leaf and node explants. Morphogenetic potential of tissue explants is also altered by genetic and physiological age of the mother plant Chaudhuri *et al*., 2004; Thomas and Philip, 2005; Sivakumar *et al*., 2006.

The callus was fast growing, green, compact and capable of sustained growth even on repeated sub culturing. In earlier studies on *Tylophora indica*, Faisal and Anis, 2003 have reported the production of highly proliferative callus from the leaf explants on 2,4,5-trichlorophenoxy acetic acid, whereas Thomas and Philip, 2005 obtained optimum callusing on 2,4-dichlorophenoxy acetic acid and benzyladenine supplemented medium. The stimulatory role of benzyladenine for shoot organogenesis from leaf callus was also advocated by Faisal and Anis, 2003. In earlier reports, either indole 3 butyric acid or indole 3 acetic acid has been reported to be optimum for rooting in regenerated shoots of *Tylophora indica* Bera and Roy, 1993.

Callus induction is necessary, as the first step, in many tissue culture experiments. Callus and cell suspension can be used for long-term cell cultures

maintenance. Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. Due to the limited availability and complexity of chemical synthesis, plant cell culture becomes an alternative route for large-scale production of this desired compound Thomas and Philip, 2005.

In vitro flowering

Flowering is considered as a complex process regulated by environment and internal plant factors, and occurrence *in vitro* is relatively rare. However, *in vitro* flowering has been reported for several species in spinach Al-khayri *et al.*, 1991, in soybean (Dickens and Van Staden, 1985. Initiation of flowering and complete seed formation in vitro may become a valuable research tool for plant breeders ensuring seed purity Kumar *et al.*, 1995.

The induction of *in vitro* flowering could be an option for attempting *in vitro* improvement through selective breeding method as well as to study the reproductive biology of *Tylophora indica*. *In vitro* studies could play an important role in attempting group improvement. Though this approach has not been exploited, the possibility cannot be virtually ruled out. The present study describes the influence of various concentrations of gibberellin (GA_3) in combination with various auxins (IBA, IAA, 2,4-D, and NAA), on flowering from shoot tip and nodal segments under *in vitro* condition.

In the present study, initiation of flowering in vitro was observed very earlier compared to that of in vivo. This result is in correlation with the studies of Kumar *et al.,* 1995 and Patil *et al.,* 1993. The present study showed that *in vitro* flowering in *Tylophora indica* is influenced by different hormonal regimes, which are GA_3 in combination with IBA, IAA, 2-4-D and NAA in low cost medium.

This is in accordance with the report on cauliflower (Kumar *et al.,* 1995). In correlation with our present study it was shown that 2,4-D induced flower from *in vitro* shoots of *Murraya paniculata* by Jumin and Ahamed, 1999. Stated that exogenous cytokin in stimulates flowering by activation of endogenous cytokinin in ascending xylem sap. Exogenous cytokinin application to apical meristems of the mitotic cycle that commonly precedes flowering (Bernier *et al*., 1977). Jumin and Nito, 1996(a), Jumin and Nito, 1996(b), found that cytokinin applied to branch internodes of flowering plants of *Fortunella hindsii* and *Murraya paniculata,* could induce flowering *in vitro.*

In general, the plantlets obtained from shoot tip and cotyledon cultures resulted in precocious flowering *in vitro* within 22 to 25 days after culturing. Merc and Palmer, 1978, Merc and Palmer, 1982 and Patil *et al*., 1993 noticed *in vitro* flowering in 3-5 weeks of culture in sun flower under various situation like light/dark, pH, various combination of auxins plus GA_3 or along or even in hormone free media. Likewise, in coherence with this result in the present

study *in vitro* flowering was observed under various variable parameters like pH, photoperiod and also the impact of various auxin in combination with GA_3. Patil *et al.,* 1993, observed that wide range of plant growth regulators, inhibitors and environmental conditions tested in sun flower have no effect on the early initiation of flowering *in vitro.* In coherence with this result we also observed early initiation of *in vitro* flowering in our present study. *In vitro* flowering and seed formation were obtained from cultured shoot tips of 'super Cayene' and Zippy peppers grown in liquid medium. In contrast to this we obtained *in vitro* flowering and seed formation in solid medium. Observed flowering from *Capsicum frutescens* regenerated shoots tips in 60-90 days. In contrast we observed *in vitro* flowering in *Coriandrum sativum* results clearly showed growth regulators combination influence successful flower induction, Stephan and Jayabalan, 2000.

Somatic embryogenesis

Efficiency of all the studied explants was also assessed for indirect organogenesis in *Tylophora indica*. *In vitro* regeneration via callus culture may be quicker and easier than conventional breeding technique to induce morphological and chemical variations, Vasil and Vasil, 1981. Interestingly, it has been observed that in the same plant, different explants showed differential response for callus induction which may be explained on the basis that different

explants were at different biochemical status at the time of inoculation Mc Nicol and Graham, 1990.

In *Tylophora indica* during the present study four types of treatments were involved in callus induction viz., IAA, NAA and 2,4-D + BAP medium which showed either organogenesis or embryogenesis or both simultaneously depending on the explant type. While in previous reports of indirect organogenesis in *Tylophora indica* through various explants different hormones like 2,4,5-T Faisal and Anis, 2003, Faisal and Anis, 2005, 2,4-D in combination with BA or TDZ Faisal *et al.,* 2005, Thomas and Philip 2005 were found responsive for optimal callus induction through various explants. However, in contrast to this Faisal and Anis, 2003, reported organogenesis from yellow, friable leaf callus induced on 2,4,5-T. When subcultured separately on the same medium, exhibited organogenic and embryogenic response respectively Sharma Chendal 1992, also reported nodular callus formation from matured leaf explants of *Tylophora indica* on BA containing medium. Their study also showed occurrence of adventitious shoot production and indirect organogenesis with in the same culture depending on the presence or absence of auxin in the medium. While on 2,4-D/BAP containing medium large number of shoots produced from leaf explant, but on incorporation of low concentrations of IAA callus formation occurred which turned nodular on subculture to IBA containing medium.

While in *Tylophora indica* callus mediated organogenic/embryogenic response was obtained in presence of BAP except shoot tip explants where direct organogenesis was recorded on BAP containing medium. Callus induced on BAP containing medium exhibited only organogenesis in case of nodal explants while through *in vivo* and *in vitro* derived leaf explants BAP induced calli showed both organogenesis and embryogenesis simultaneously on the same medium. Optimum response was recorded at 1.0 mg/l of BAP for maximum shoot and embryoid production in all the explants tested. In contrast, Faisal and Anis, 2003, Faisal and Anis, 2005 advocated the advantageous role of 5.0 mg/l Kn for satisfactory shoot bud organogenesis and plantlet production in *Tylophora indica*. In their studies they were unable to induce high frequency shoot production from leaf and stem derived calli while embryogenesis was completely absent. However, studies in other species report the efficiency of 5.0 mg/l BAP for organogenesis, Anis and Faisal, 2005.

This is in accordance with the observations of Faisal *et al.*, 2005, and Thomas and Philip, 2005, in *Tylophora indica* the Kin was found superior to NAA, IBA and Kn for obtaining shoot regeneration. However, none of the studies in *Tylophora indica* reported so far has shown simultaneous occurrence of organogenesis and embryogenesis within the same culture as evident from present investigation, Chandrasekar *et al.*, 2006. In present investigation *in vivo* derived leaf explants were found more organogenic in nature while there *in vitro* counterparts had more of embryogenic tendency. This might be

attributed to increase in embryogenic competency of *in vitro* leaf explants due to higher levels of endogenous hormones.

Nodal segments of *Tylophora indica* cultured on NAA and IBA + Kin containing medium exhibited callus mediated shoot regeneration. Optimum callus formed from the axil on 5.0 mg BA and BA (5.0 mg) + NAA (0.1 mg), regenerated well with repeated subculture on the same medium. Explants inoculated on cytokinin-auxin combination medium showed almost equal or lesser productivity than on single cytokinin concentration. In *Tylophora indica*, combination medium induced indirect regeneration in all the explants evaluated. While nodal and shoot tip explants were involved in organogenesis only the leaf and root explants exhibited both organogenic and embryogenic capacity.

In vitro propagation via direct and indirect somatic embryogenesis and multiplication of shoot from explants like leaf Bera and Roy, 1993; Manjla *et al.,* 2000; Chandrasekar *et al.,* 2006. Therefore suitable low cost *in vitro* propagation methods must be evolved to conserve this *Tylophora indica* propagation. Micro propagation of *Tylophora indica* by auxiliary shoot induction and adventitious shoot production was reported by Sharma and Chandel, 1992. The callus mediated somatic embryogenesis from leaf was reported by Jayanthi and Mandal, 2001 and inter nodal, was reported by Thomas, 2006. Explants of preliminary nature and more studies and further

refinements of techniques were absolutely necessary. Hence, an attempt has been made to study the successful development of somatic embryogenic system on Low cost medium via., Direct somatic embryogenesis using different explants of *Tylophora indica* Leaf, node and internode.

The few studies have been reported on induction of somatic embryogenesis in *Tylophora indica*, which had limited success (Chaudhuri *et al.*, 2004; Chandrasekhar *et al.*, 2006). Somatic embryos and synthetic seeds (embryos encapsulated in artificial endosperm) hold potential for large-scale clonal propagation of superior genotypes of heterogeneous plants (Redenbaugh *et al*., 1993.

Plant tissue culture plays a major role in conservation of germplasm, rapid clonal propagation, regeneration of genetically manipulated superior clones, production of secondary metabolites and *ex vitro* conservation of valuable Phyto diversity Anis, *et al.*, 2009; Anis, *et al.*, 2011. Especially, plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites.

Elictors

Recent research in the *in vitro* culture systems, reported that a wide variety of elicitors have been employed in order to modify cell metabolism. These modifications are designed to enhance the productivity of useful metabolites in the cultures of the plant cells/tissues. The cultivation period in

particular, can be reduced by the application of elicitors, although maintaining high concentrations of product Metraux *et al*., 1990, "Elicitor is a scientifically described term for stress factors that directly or indirectly triggers the inducible defense changes in a plant system that results in an activation of array of protection mechanisms, including induction or expansion of biosynthesis of fine chemicals which do have a major role in the adaptation of plants to the stressful environment" Janda *et al*., 1999.

Effect of Jasmonic acid on Total phenolics

Tylophora indica callus was subjected to Jasmonic acid for inducing phenolic substances. Many works reported that Jasmonic acid has been used to modulate the production of various secondary metabolites in plant tissue culture techniques. George *et al.,* 2008; Nojiri *et al.,* 1996 reported that the enhanced secondary metabolite production is usually associated with rapid, transient increase in the activity of key enzymes of the phenyl propanoid pathway such as phenylalanine ammonia lyase and chalcone isomerase.

Effect of salicylic acid (SA) on total phenolics

In *Tylophora indica,* callus was subjected to Salicylic acid (SA) has attained more attention because of its involvement in plant defense mechanisms, such as establishment of systemic acquired resistance (SAR), reported by Metraux *et al.,* 1990 and induction of pathogenesis related (PR) proteins was

reported by Malamy *et al.*,1990, as well as hypersensitive response. Horvath *et al.*, 2007. The protective effect of SA against abiotic stress factors such as toxic metals was reported by Strobel, and Kuc, 1995, heat stress by Dat, *et al*., 1998 and low temperature by Janda *et al.*, 1999 and oxidative damage by Kusumi *et al.*, 2006 has been demonstrated. It has also been used to enhance *in vitro* regeneration in several plant species, Quiroz-Figueroa and Mendez-Zeel, 2001, Luo JP *et al.*, 2001.

Effect of Copper Sulphate on total phenolics

Likewise, copper Sulphate also induced phenolics. Copper performs very important physiological and biochemical processes include photosynthesis, respiration, conversion of nitrogen compounds, transport of carbohydrates and also it is a constituent of the protein component of several enzymes in plants, mainly those participating in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, cell wall and cytoplasm of plant cells. Lolkema, 1985, reported that the addition of 40 µm $CuSo_4$ to the medium significantly increased the embryoids production from wheat anther cultures. Dahleen, 1995 studied the effect of different concentrations of $CuSo_4$ on callus culture of two cultivars of barley and found that medium containing 50 µm copper regenerated significantly more plants. Total and phenol content increased at high concentration of Cu and Zn.

Secondary metabolites

The primary metabolites therefore include the nucleic acids and the common amino acids and sugars and also the high molecular weight polymeric materials such as cellulose, lignin's and the protein from which the cellular structure are formed. These secondary metabolites were the molecules of our concern, which are biosynthetically restricted to a selection of *Tylophora indica* species. These appeared to have influence on the growth and development of plant Collin, 2001. Secondary metabolite unlike the primary metabolites are found to be accumulated in particular tissue at high concentrations some of them being toxic to the plant themselves if they are mislocalized Yazakik *et al*., 2008. Biosynthetic genes responsible for the formation of those secondary metabolites may be highly expressed in such tissues.

In India large number of plant species had been screened for their pharmacological properties but still a vast wealth of endangered species are explored medicinal plants are at interest to the field of biotechnology as most of the drug industries depends in part on plants for the productions of pharmaceutical compounds Sridharan *et al.,* 2011.

The major constituent in *Tylophora indica* is the alkaloid Tylophorine. Laboratory research has shown this isolated plant extract had strong anti-inflammatory action Gopala Krishnan *et al*., 1979. Test tube studies suggest that Tylophorine is able to interfere with the action of mast cells, which

are key components in the process of inflammation Gopala Krishnan *et al*., 1980. These actions seem to support tylophora's traditional use as an antiasthmatic and antiallergic medication by Ayurvedic practitioners.

The more precise information in qualitative analysis can be obtained by gas-chromatography coupled with mass spectrometry (GC-MS) Cong *et al*., 2007. For quantitative determination, gas-chromatography with flame ionization detector (GC-FID) and GC-MS are preferred Haznagy-Radnal *et al*., 2007.

The GC-MS analysis of *Tylophora indica* leaves revealed the presence of 17 compounds. The identified compounds possess many biological properties. For instance, 14-Vitamin B_6 (R/T 16.06) had Antioxidant, Antitumor, Hypocholesterolemic properties. 17- Methyl (R/T 17.92) can be an Lubricant, used to produce dietary supplements and anti-inflammatory. Zendo, 3 exo-Bis (trichloromethyl) -5 exo, 6 exo-and -5 endo, 6 endo-epoxybicycloe [2.2.2] oetans (R/T 8.32) is an Antitumor, immunostimulant, perfumery, pesticide as antimicrobial, anticancer, anti-inflammatory and diuretic agent Praveen Kumar *et al*., 2010. 9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z,Z)-, n-Hexadecenoic acid, 1,2-Benzene dicarboxylic acid and di-is octyl ester were present in *Caesalpiniasa* ethanol extract Sarumathy *et al*., 2011. Similar types of compounds were identified in our GC MS results among the seventeen compounds of this present study.

Phytol is one among the seventeen compounds. Similarly, Maria Jancy Rani *et al*., 2011, observed the presence of phytol in the leaves of *Lantana camara* and Sridharan *et al*., 2011, observed in *Mimosa pudica* leaves. Similar result was also observed in the leaves of *Lantana cama* Sathish Kumar and Manimegalai, 2008. Phytol, Phenol, 2,4-bis (1-phenylethyl) had medicinal properties. Mangun widjaja *et al*., 2006 reported the main components of 9,12 octadecadienoic acid, Octadic- 9enoic acid and 9, 12-actadecadienoic acid present in *Croton tiglium* seed. These compounds were found to have potential antioxidant and anticancer activities.

Hexadenoic acid has earlier been reported as a component in alcohol extract of the leaves of *Kigelia pinnata* (Grace *et al*., 2002). Parasuraman *et al*., 2009 identified 17 compounds with n-Hexadecenoic acid and Octadecanoic acid as the major compounds in the leaves of *Cleistanthus collinus.* GC-MS analysis of ethyl acetate extract of *Goniothala musumbrosus* revealed the presence of n-Hexadecanoic acid (Siddig Ibrahim *et al*., 2009). n-Hexadecanoic acid, Hexadecanoic acid, Phytol, 9,12-Octadecadienoic acid, 9,12,15-Octadecatrienoic acid and Squalene were identified in the ethanol leaf extract of *Aloe vera*, (Arunkumar and Muthuselvam, 2009). Squalene is used in cosmetics as a natural moisturizer**.** Devi *et al*., 2009 reported that *Euphorbia longan* leaves mainly contained n-Hexadecanoic acid and 9,12-Octadecadienoic acid. These reports are in accordance with the result of this study.

The importance of *Tylophora indica* and its biological activity of some of these compounds were standardized, which reveals the presence of compound in *Tylophora indica* and suggest that, the contribution of these compounds on the pharmacological activity should be evaluated. Source of many plants (herbs and spices) can often be identified from the peak pattern of the chromatograms obtained directly from headspace analysis. Similarly, unique qualitative and quantitative patterns from a GC analysis will often help identify the source of many alcoholic beverages. The technique of fingerprint could really identify the false herbal products. The construction of chromatographic fingerprints aims at evaluating the quality of Herbal Medicines was reported by Yi-Zeng *et al.*, 2004.