2.1 Medicinal plants review

Numerous medicinal plants possess very high content of phyto-constituents and antioxidant compounds viz., phenol, flavonoids, tannins, terpenoids, vitamin C and E, carotenoids, etc., which can prevent free radicals induced disorders viz- atherosclerosis, cancer, retinopathy, liver disorders, diabetes, ulcerative colitis, hypertension, allergy, arthritis, cirrhosis, inflammation etc. (Alam et al., 2013) (Anderson et al., 2001). Expanding upon these for example, it was reported by several studies that the polyphenols are directly associated with hepatoprotective activities (Wu et al., 2017). Another report of ethanolic extracts of *Psidium guajava, Carica papaya, Vernonia amygdalina,* stem bark of *Mangifera indica* found some important phenolic, flavonoids, saponins, terpenoids, reducing sugar, and tannins reported (Ayoola et al., 2008). Again, beside of the antioxidant properties the *Ennicostemma littorale,* and *Datura stramonium* bears steroid, glycosides and alkaloids in it. While the IC_{50} values in DPPH scavenging activity of the *P. guajava, C. papaya, M. indica, V amigdalina was* 0.04, 0.58, 0.313, and 2.3 respectively. In a study of antioxidant activity of *in vitro* cultured extracts of hairy roots and shoots *of Salvia officinalis* L., using DPPH free radical scavenging assay, phosphomolybdate assay, total phenol content, and rosmarinic acid concentration, diterpenoids (carnosic acid and carnosol). The methanolic extract of root and hairy root of tissue cultured plants possessed strongest DPPH free radical scavenging activity and reducing power in activity. Wherein, acetone extracts of shoots obtained in the *in vitro* cultures best effect was observed against linoleic acid oxidation than in the plant grown in field (Grzegorczyk et al., 2007). In an experiment of antioxidant activity and total phenol content of *in vitro* propagated plants of *Ballota nigra* L. from *in vitro* nodal explants and wild plant. From the experiment it was observed that the tissue cultured *B. nigra* extracts exhibit higher free radical scavenging activity (DPPH) and metal reducing activity (FRAP) but less effective in prevention of linoleic acid peroxidation (LPO). *In vitro* propagated plants showed higher antioxidant properties than in wild plant. The shoot extracts collected during flowering period showed higher content of phenolic and flavonoids than in immature plants. From the result it was suggested to use of *in vitro* derived *B. nigra* plant is for pharmaceutical purposes for better medicinal contents (Makowczynska et al., 2015). A similar study of *in vitro* propagation and evaluation of antioxidant properties in *Scrophularia kakudensis* , the adventitious shoot achieved from the nodal explants in the MS media supplemented with 2mg/L BAP and 0.5mg/L IAA and rooting on half strength MS media supplemented with 0.5 mg/L IAA carried higher content of acacetin than in the root extracts. Also, the phenol and flavonoid content was higher in the shoot extracts than in the root and callus extracts, concluding that germplasm conservation and commercial cultivation of *S. kakudensi* could be highly beneficial for therapeutic purposes (Manivannan et al., 2015).

While comparing flavonoid content and antioxidant properties in wild and tissue cultured *Nardostachys jatamansi,* the tissue cultured roots showed greater antioxidant capacity than in the wild plant. The IC_{50} value of *in vitro* propagated plant was 29.55 µg/ml and wild plant was 24.18 µg/ml. In the HPLC analysis for the detection of quercetin content the tissue cultured *N. jatamansi* extract showed 1.95 mg/ml quercetin and the wild plant showed 1.83 mg/ml quercetin (Pant et al., 2023). Antioxidant compounds from tissue cultured *Artemisia annua,* the *in vitro* callus was generated in the MS media supplemented with different combinations of plant growth regulators. Highest DPPH radical scavenging activity and phenolic content was obtained in the MS media supplemented with 0.5 mg/L NAA + 0.5 mg/L BAP. In the GC MS analysis showed highest caffeic acid content in that PGR combination. The callus produced in this media combination increased the caffeic acid content and antioxidant activity compared to leaf extract of *A. annua.* This study concludes with that the tissue cultured *A. annua* plant enhance the pharmaceutical properties, but the callus extracts are more effective for production of bioactive compounds (Prihantini et al., 2018). The demonstration of anticancer and antioxidant activity of Tannins isolated from callus of *Achyranthes aspera L.* it was observed that the callus extract of callus grown in the MSDN medium showed highest antioxidant activity. The leaf extract showed significant anticancer activity against the Jurket cell line. The callus culture extracts contain higher anticancer activity as compared to the control. From the experiment it was concluded that the *in vitro* biomass production may be an alternate and effective approach for production of bioactive compounds such as tannins from *A. aspera* and in some medicinal plants (Ambreen et al., 2024). In an experiment for production of bioactive compounds in root culture of *Oplopanax elatus*, adventitious root was developed in the culture using a balloon type airlift bioreactor system. Maximum adventitious root and highest bioactive compound was obtained in the culture supplemented with 50 g/L sucrose and

3 mg/L IBA. The biomass and bioactive compound productivity were highest in the 40days old culture. The polysaccharide, flavonoid and phenolic content was higher in the Ars than the field grown plants. The *in vitro* cultured AR exhibit higher antioxidant activity in the DPPH radical scavenging activity than the field grown plant. The AR extract also exhibits anticancer activities against human gastric cancer cell line AGS. Concluding that bioreactors can be used for efficient production of bioactive compounds of *O. elatus* (Jiang et al., 2015). The production of withaferin A through hairy root culture of *Physalis minima* L., the hairy root production was carried out using wild-type A4 strain of Agrobacterium rhizogenes to enhance production of withaferin A. 200 µM acetosyringone was used in the culture media for transformation. The withaferin A accumulation was enhanced through elicitation to the high-yielding root lines, biotic and abiotic elicitors were used in the high-yielding root lines. As a result, 21-fold increase in production of withaferin A accumulation was observed in the elicited cultures as compared to non-elicited culture after 4 days of elicitation. This experiment concludes use of the elicitors can be effective and alternative aspect for the biosynthesis of withaferin A (Halder & Ghosh 2023).

A report for production of polyphenols in *Salvia bulleyana* Diels through inducing hairy root cultures, the hairy root culture was obtained in the YBM medium supplemented with acetosyringone. The four selected transformed roots were cultured in WP medium for 5 weeks. 10 polyphenolic compounds including caffeic acid and its derivatives, rosmarinic acid and its derivatives, salvianolic acids: K, E and F were found in the hydromethanolic extracts in UPLC-PDA-ESI-MS/MS test. The total polyphenol content was varied in different cultures, but significantly higher than the plants root extracts grown in wild (Wojciechowska et al., 2020). Likewise, the production of flavonoids in the callus cultures of *Sophora flavescens* Aiton for enhancing maackiain (Maac) production, the calli induced using leaf explant in the MS media supplemented with 0.5mg/L BAP and 2 mg/L picloram possessed highest accumulation of Maac. The *in vitro* callus was transformed into shoots in the MS media supplemented with TDZ and NAA (Park et al., 2020). A comparative study was conducted to produce phenolic compounds, antioxidant activity in wild and tissue cultured *Ageratina pichichensis* was conducted in the wild plant, *in vitro* plant, callus culture, cell suspension culture using total phenol, flavonoid, DPPH, ABTS, TBARS assay and the compound identification by HPLC analysis using methanolic extract. Comparative study of phenolic compound the callus culture showed significantly higher (2.7 folds) flavonoid and phenolic content than the wild and *in vitro* plants. In the comparative antioxidant study using ABTS, DPPH and TBARPS study the *in vitro* plant lowest antioxidant activity while the wild plant showed highest antioxidant activity. In the HPLC study for compound identification the cell suspension culture produced higher epicatechin, and caffeic acid than callus culture, but no compounds like p-coumaric acid, epicatechin, and caffeic acid were identified in the wild plant. Concluded that both wild and *in vitro* plant of *A. pichichensis* produce phenolic compounds and possesses antioxidant activity, the callus and cell suspension cultures of *A. pichichensis* are proved to be an effective alternative for obtaining bioactive compounds (Motolinia-Alcántara et al., 2023).

Whereas accumulation of secondary metabolites in tissue cultured *Eryngium alpinum* L., *in vitro* shoot multiplication was obtained in the MS media. In the quantitative analysis of phenolic acid test and flavonoid of methanolic extracts of *in vitro* grown shoots, 19.59 to 32.95 folds phenolic acid and 3.02 to 4.43 folds more flavonoids were obtained in *in vitro* grown shoots than the basal leaves from the intact plant depending on the culture system. Seven different phenolic acids were obtained in the *in vitro* shoot culture- caftaric, caffeic, 3,4-dihydroxyphenylacetic, neochlorogenic, chlorogenic, rosmarinic acids, and isochlorogenic, and three flavonoids- quercitrin, isoquercetin, and robinin. Highest biomass growth, phenolic acid and flavonoids accumulation was in the MS media supplemented with BAP, IAA, and GA_3, (1mg/L each) (Kikowska et al., 2020). In an experiment *in vitro* propagation and comparative phenolic, flavonoid, and antioxidant activity of callus and other parts of *Phytolacca americana* L., the shoot cultures were obtained using leaf explants in the MS media fortified with 2 mg/L 2,4-D, and nodal explants on 2 mg/L kinetin. The leaves of *P. americana* contain higher phenol and flavonoid content while the callus contains more phenolic than flavonoids (Trunjaruen et al., 2022). In the analysis of total phenol, flavonoid and antioxidant activity of callus, rhizome, and callus treated with elicitors, the extracts were obtained in the petroleum ether and chloroform: methanol (1:1 v/v). the total phenol content was maximum in the rhizome extracts while the callus extract showed lower phenolic content (gallic acid). Flavonoid was only detected in the rhizome extract. Total antioxidant activity was highest in the petroleum extracts of rhizome while the callus extract exhibited lower antioxidant activity. The callus treated with elicitors (100 mg/L yeast extract and 50 mg/L salicylic acid) exhibited significant increase in the phenolic and antioxidant activity in DPPH assay. Concluding that the use of elicitors in the ginger tissue culture could cause significant effect in enhancing antioxidant capacity and pharmacological industries (Ali et al., 2018).

Furthermore, the production of flavonoids in tissue cultured *Momordica charantia* L., the *in vitro* plants and callus were generated in the MS media fortified with different concentrations of auxins and cytokinin. The phenol and flavonoid were analysed using thin layer chromatography and spectrophotometric analysis. In the 6-week-old callus culture 2.90mg/g of flavonoid were detected, while in the multiples shoots of *in vitro* plants the flavonoid content was 2.96 mg/g dry wt. (Mala & Kamal, 2007). In a study for enhancing production of flavonoids by elicitation in *Hypericum perforatum,* methyl jasmonate was used in the *in vitro* cell suspension was culture of *H. perforatum.* The elicitation treatment with 100 µmol/L MeJA in the suspension cultures, after 15 days highest flavonoid content was obtained in the culture which was almost 2.7 folds of the control culture. In this process the activities of phenylalanine ammonia lyase was increased and catalase (CAT) were inhibited after MeJA treatment in the culture, leading to the enhancement in the production of flavonoid. Concluding that MeJA elicitation is a significant elicitation method for the production of flavonoids in the *H. perforatum* (Wang et al., 2015).

2.2 Review of *in vitro* propagation of medicinal plants

Plant tissue culture as an alternative and emerging technology for plant propagation and production of secondary metabolites combined with transgenic technologies and genetic stability study are frequently used in plant tissue culture. While elicitor studies have been evolved in signal molecules, induction mechanism, functional genes, e.g.- yeast and *A. niger* are used to improve bioactive compounds in tissue cultured plants. Plant tissue culture is used not only for production of bioactive compounds but also to study biosynthesis of the active ingredients. Gene silencing, gene interference, overexpression, chemical elicitors, and site directed mutation, enzymes could be used in the medicinal plants for improving bioactive compounds in the plants (Wang et al., 2017). Various works of plant tissue culture have been reported over the years, these include the tissue culture of rootstocks explants of different varieties of peach (Okinawa, Nemared, and Garnem peach), the in vitro proliferation was varied in different genotypes of peach i.e- the shoot numbers were 3.33-7.44, and 3.77- 7.44 in Garnem peach, Nemared, and Okinawa respectively the BAP concentration used (3 to 5mg/L). subsequently when 0.2mg/L IBA was mixed with 5mg/L BAP the shoot proliferation was increased to 7.48 average number of shoots. From this result it was observed that the adventitious shoot was increased in the culture media combination with BAP and IBA (Eliwa et al., 2024). While an efficient *in vitro* propagation technique developed for Strychonus potatorum an endangered medicinal plant using leaf explant for conservation and commercial production of the species, they reported that the explants were formed callus in the MS medium supplemented with 0.4mg/L KIN and 3mg/L 2,4-D (Chaudhary et al., 2013). Micropropagation or *in vitro* explant cultures are also most useful tool for preservation of endangered plant species through germplasm conservation, large scale propagation, and enhancing the production of secondary metabolites. Khan et al., 2021 developed *in vitro* propagation method and investigated phytochemicals of wild and micropropagated *Saussurea costus* (Falc.) Lipsch. Best suitable bud initiation media was carried in MS media supplemented with 2mg/L BAP, 1mg/L NAA and 0.25mg/L gibberellic acid, maximum shoot length was formed in the MS media bearing 1.5mg/L BAP and 0.25mg/L NAA, and 0.5mg/L kinetin. Maximum rooting was observed in the media supplemented with 0.5mg/L BAP, 0.5mg/L IAA, and 0.5mg/L IBA. The *in vitro* propagated *Saussurea costus* contains higher flavonoids and anthocyanin in compared to wild plant. Also, the secondary metabolite content was varied in the wild and tissue cultured plant. Anis & Faisal 2005 developed an effective protocol for plant regeneration of *Psoralea corylifolia* using shoot tip culture. Reported highest frequency of shoot regeneration in the MS media supplemented with 5 µM BAP and 0.5 µM NAA using nodal explants of *Psoralea corylifolia* (Anis & Faisal, 2005). Dakah et al., 2014 developed *in vitro* propagation protocol and studied antioxidant activity of wild and tissue cultured *Ziziphora tenuior* L. In this study, the explant cultured in the MS media supplemented with 1.5 mg/L of NAA formed 70% callus in dark condition after 45 days. These calluses formed shoots and multiplied in the MS medium supplemented with 2 mg/L BA after a month. The shoot length was 6.1cm each shoot and shoot number was 4.21 when MS media supplemented with 1 mg/L Kin + 0.1 mg/L NAA. The comparison of antioxidant activity, the methanolic extracts of *in vitro* grown plants supplemented with 1.5mg mg/L and 1mg/L kin + 0.1 mg/L NAA showed stronger DPPH activity (IC_{50} was 0.307 and 0.369 mg/ml) than wild grown *Z. tenuior* (0.516 and 9.229 mg/ml) (Dakah et al., 2014). Meena et al., 2010 developed and efficient rapid *in vitro* propagation protocol of *Citrullus colocynthis* from shoot tip. Highest number of shoot formation was observed in the MS media containing 0.5mg/L BAP and 0.5mg/L NAA. The rooting of the explant was stimulated in the media containing 4mg/L IBA with 0.2% activated charcoal (Meena et al., 2010). Priyadarshini et al., 2014 standardized effective *in vitro* propagation protocol for *Heliotropium indicum* using leaf, node and internodes. Highest callus formation of the explants was obtained in the MS media containing 2.0 mg/l BAP + 3.0mg/l NAA. Shoot and bud proliferation was obtained in the MS media supplemented with 1.0 mg/l BAP. The response of the callus was best in the MS media fortified with 0.5mg/L GA3 resulting 14.8 average shoot number. Rooting of the explants was best in the MS media containing 0.2 mg/l IBA resulting 14.6 number of roots per plantlet (Priyadarshni et al., 2014). Ramesh, S. et al., 2013 standardized effective protocol for *in vitro* propagation of *Sarcosa asoca* (Roxb.) De Wilde using shoot tip and nodal explants. Reported highest callus formation in the MS media fortified with 2.4-D, highest shoot formation on the MS media supplemented with 0.5mg/L BAP and rooting on 4mg/L IBA (RameshS et al., 2013). Seyyedyousefi et al., 2013 reported effect of BAP and NAA on plant regeneration of Alstroemeria (Alstroemeriaceae) using apical and lateral buds on rhizome. Highest shoot length was obtained in the MS media supplemented with 1.0 mg l-1 NAA and maximum root formation. Highest shoot formation was observed in the media with 0.20 and 0.50 mg l-1 NAA (Seyyedyousefi et al., 2013). Gurnani et al., 2012 developed *in vitro* propagation protocol for medicinal plant *Bacopa monneiri* and reported highest rate of shoot multiplication in the MS media with 3mg/l BAP. Highest root formation was observed in the MS media supplemented with 1mg/L BAP and 3mg/L IAA (Gurnani et al., 2012).

Similarly, establishment of *in vitro* propagation protocol for *Atropa acuminate* using the leaf and root explants. MS media supplemented with 0.5mg/L BAP and 1mg/L NAA showed most efficient *in vitro* callus development from root explant and MS media supplemented with 1mg/L BAP and 1mg/L NAA from leaf explant of *Atropa acuminate.* Reported an average weight of 33.13mg in the callus derived from root explant and an average fresh weight of 22.14mg per explant in the callus derived from leaf explant (Dar et al., 2021). While the effect of BAP concentrations on *in vitro* shoot multiplication of bamboo, 0-9mg/L BAP was used in the MS medium for establishment and multiplication of bamboo shoots using nodal explants. Reported that MS medium supplemented with 4mg/L BAP and 5mg/L BAP was best for shoot initiation. 4mg/L BAP was observed best for shoot inducing shoot multiplication (Chavan et al., 2021). While other reports of effect of growth media and plant growth regulators on rooting and shoot proliferation of Moroccan Native Almond (*Prunus dulcis* Mill.) Genotypes using internodal explants, the explants cultured on MS and Heller medium with different concentrations of BAP, IBA, TDZ, KIN. Reported that most effective shoot formation was observed in the MS medium supplemented with 1mg/L TDZ (Kodad et al., 2021). In another demonstration, the effect of different concentration of BAP and NAA on *Orthosiphon stamineus* plant using petiole explant, reported that highest number (4.33) of shoot proliferation and maximum callus fresh weight (avg of 3.2g) was observed on the MS media supplemented with 1.0 mg/L BAP and 0.2 mg/L NAA. In comparison with petiole and leaf explant of *O. stamineus* in tissue culture, the petiole explants were most suitable for efficient plant regeneration (Yoong et al., 2019). Whereas effect of different concentration ratio of BAP and NAA on *in vitro* propagation of *Caladium bicolor* (Aiton) Vent., all the hormone combinations induced callus formation. Callus formation on controlled MS medium was very low, most effective callus formation was observed in the media 4mg/L BAP and 0.5mg/L NAA, highest shoot multiplication in 1mg/L BAP and 0.5mg/L NAA and rooting at 3mg/L BAP and 0.5mg/L NAA (Seydi et al., 2016). Likewise effect of auxin and cytokinin on *in vitro* shoot regeneration and rooting of *Valeriana jatamansi* Jones using rhizome, reported that highest shoot elongation and multiplication was in the MS media fortified with 2mg/L BAP and 1mg/L NAA, highest rooting on the MS media with 1.5mg/L BAP and 1.5mg/L NAA. Higher concentration of PGR like BAP(2-3mg/L) and NAA(1-1.5mg/L) was not favourable for root length but increases higher root proliferation (Nazir et al., 2022).

Other experiments for standardization of *in vitro* large-scale production protocol of *Eryngium maritimum* L., MS media with macro salt modifications and different concentration of PGR ratios were used for axillary bud propagation and rooting. The axillary bud proliferation and rooting were enhanced in the media where lower concentration of nitrogen and PGR were added. From the experiment it was observed that the survival of the *in vitro* plantlets was dependent on potting media size when grown *ex* *vitro*. Concluding that the lower nitrogen content in the media with plant growth regulators enhanced the micropropagation rate and rooting, in the *ex-vitro* plantation the substrate containing lower nitrogen content at larger pot size enhanced plantlet survival (Mežaka et al., 2023). While for standardization of *in vitro* propagation protocol for *Prunus africana* using nodal explants, successful axillary shoot initiation was obtained in the woody plant medium (WPM) supplemented with 15m/L sucrose, 1.0 mg/L BAP. Rooting was successfully induced in the WP media supplemented with 15g/L sucrose and 1.5mg/L IAA. After rooting *in vitro* plants were successfully transplanted in the sterilized soil containing pertile (2:1 v/v) (Komakech et al., 2020). In another study of *in vitro* propagation of *Clitoria ternatea* using nodal explant, shoot tip, and cotyledonary node explants in MS media supplemented with BAP and GA_3. In this experiment highest number of shoot were obtained by nodal explants in the MS media supplemented with 2mg/L BAP and shoot elongation in the MS media supplemented with 0.5 mg/L GA_3. *Ex vitro* rooting of the *in vitro* cultured *C. ternatea* shoots was obtained by dipping the *in vitro* developed shoots in the solution containing 250mg/L IBA for half an hour and transplanted in sterile soilrite (Pandeya et al., 2010). In another experiment for establishment of *in vitro* propagation of *Origanum scabrum* (Boiss. & Heldr.) for commercial exploitation in the horticulture and pharmaceutical industries, in this study effect of explant collection on several season and position of explant stem (shoot apex, 1^{st} to 5^{th} node) and effect of temperature were studied. From the result the optimum season for the collection of explants was during the vegetative growth of the plant (April to May), for shoot explant the 1^{st} node was found most suitable explant of the *O. scabrum*. However, the temperature did not cause any effect on the microshoot number and rooting, but the length of the microshoot was higher at 25ºC (Alexopoulos et al., 2023). In a study of effect of plant growth promoters and culture methods on micropropagation of *Brassavola nodosa* (L.) Lindl. hybrid, for development of efficient micropropagation and large-scale production protocol of *B. nodosa* six different concentrations plant growth regulators (BAP with or without adenine sulfate) were used, also effect of NAA and IBA concentrations in the explant of *B. nodosa* were used. In the experiment, higher multiplication rate was observed in the modified MS medium supplemented with 2.0 mg/L BAP with 30.0 mg/L adenine sulfate. Rooting of the *in vitro* plantlets were obtained 0.5mg/L NAA or 1mg/L IBA (Xu et al., 2022). Moreover, the effect of different plant growth promoters on micropropagation of *Paris polyphylla* for standardization of mass propagation protocol using rhizome, the rhizome explants (50% explants were treated with 0.5 µg/mL activated charcoal) were inoculated on the half strength and full-strength MS media supplemented with different concentrations of BAP and auxin. 80% of the explants successfully formed shoots in the MS media supplemented with 0.5 µg/mL BAP and 0.2 µg/mL NAA containing activated charcoal. MS media containing activate charcoal induced shoots more effective than in medium with non-activated charcoal (Puwein & Thomas, 2022). Carrying on, the effect of growth regulators on micropropagation of *Stahlianthus thorelii* Gagnep using tubers, the explants grown in the MS media 5mg/L BAP and 4mg/L kinetin formed 5.55 ± 0.59 and 5.48 ± 0.87 shoots/explants, respectively. The *in vitro* grown explants were again inoculated into MS media supplemented with different concentrations of BAP and NAA. Highest shoots per explant (7.54 ± 0.79) were obtained in the MS media supplemented with 3mg/L BAP and 0.5mg/L NAA after 8 weeks. Highest root (26.17 ± 1.5) induction was observed in the MS media fortified with 0.5mg/L NAA and 0.5mg/L IBA. 100% of the acclimatized plantlets were survived in the mixture of soil: sand: compost (1:1:1) (Van Yen & Li, 2022). Subsequent demonstrations alike study on effect of growth regulators on micropropagation of Ornamental Plant Rosa hybrida L., nodal explants were initiated in the MS medium fortified with 2.5 µM BAP. Shoot proliferation experiment was conducted on the MS media supplemented with kinetin, BAP in combination with GA_3. BA was found to be better option for shoot proliferation and elongation as compared to KIN. Highest shoot proliferation was observed in the MS medium fortified with 10.0 µM BA in combination with 0.5 µM GA_3. Root induction was obtained highest on the $1/4^{th}$ MS media supplemented with 5.0 µM of IBA. 85% of the micropropagated plants were survived in the polyhouse and greenhouse condition (Aggarwal et al., 2020).

2.3 Explant surface sterilization review

In the tissue culture process contaminations in the plant cell, tissues, cultures can affect the growth and development of the plants *in vitro.* To eliminate the contaminants in the culture surface sterilization step in tissue culture process is most crucial important. Use of optimum sterilant in this step gives rise to best results in plant tissue culture to get desired plants in a large quantity without contamination (Yaswanthi, 2022). Hence, surface sterilization in plant tissue culture is a vital step for the preparation of viable explant. In an experiment for elimination of contamination in the nodal explants of *Clinacanthus nutans* intended for *in-vitro* culture 4 different sterilant were used, - Mercuric chloride, sodium hypochlorite, thiophanate-methyl, Pyroligneous acid. Out of these Pyroligneous acid showed an effective bactericidal activity but lead to fungal contamination in the culture. Therefore, Pyroligneous acid is not a considered as potential disinfectant for *C. nutans*. $HgCl_2$ in combination with thiophanate-methyl exhibit best effect against fungal contamination. $HgCl_2$ (0.2%) treatment of the nodal explants of *C. nutans* for 1 hour showed effective for *in vitro* surface sterilization and viability of the culture (Hashim et al., 2021). Eliwa., et al*.,* 2024 used 20% Sodium hypochlorite for 15 min for explant surface sterilization of peach rootstock. The Garnem genotype of peach resulted highest response (89.12%) from the tissues with 96.61% explant survival, with lowest 3.14% mortality and 0.24% contamination rate. Also, the explant response and survival depend on the explant collection dates (Eliwa et al., 2024). Another tissue culture of bulbil explant, the surface sterilization of explants was done using 250 mg/L cofotaxime solution for 30 min, followed by treating in 0.2% mercuric chloride for 7 min and 1:2 NaOCl and water solution for 10 min resulting 60% of explant survival (Irawati et al., 2023). Likewise, 99% explants of *Salacia chinensis* were survived when treated with 70% ethanol for 1 min followed by treating in 1% NaOCl and 2-3 drops of tween 20 for 15 min in the micropropagation of *Salacia chinensis* (Bagnazari, 2014). Putri et al., 2019 used 4mg/L PPM (plant preservative mixture) in the media for tissue culture of *Callophylum inophyllum,* resulting highest plant survival rate without contamination after one month of explant initiation (Putri et al., 2019). Bello et al., 2018 developed surface sterilization process for nodal culture of *Solanecio biafrae.* In this method the nodal explant was treated with 70% ethanol for 20s and 10% Calcium hypochlorite for 15 min to get contamination free explant culture (Bello et al., 2018). Stanisavljević et al., 2017 used ozone and sodium hypochlorite for surface sterilization of cherry rootstocks and reported higher survival of explants while treated with ozone in comparison with sodium hypochlorite (Stanisavljević et al., 2017). Thokchom & Maitra (2016) developed an explant surface sterilization protocol for Anthurium and reported highest explant survival rate while treated with 0.1% mercuric chloride for 3 min followed by 30s treatment with ethyl alcohol (Thokchom & Maitra, 2016). Sidhu et al., 2014 standardized callus culture protocol for *Costus pictus,* a vulnerable, threatened species of India using various concentration of hormones (Kintein, BAP, IAA, and 2,4-D) in MS media. Reported highest callus growth in half strength MS media supplemented with 1mg/L 2,4-D and 1mg/L IAA or 0.5mg/L Kinetin and 1mg/L BAP (Sidhu et al., 2014).

In other reports on the effect of BAP concentrations on *in vitro* shoot multiplication of bamboo, 0-9mg/L BAP was used in the MS medium for establishment and multiplication of bamboo shoots using nodal explants. Reported that Bavistin, tween 20 along with ethanol is sufficient for explant surface sterilization of bamboo nodal explants to avoid contamination (Chavan et al., 2021). Similarly, for establishment of surface sterilization protocol for nodal explant of *Solanecio biafrae,* the nodal explants were introduced in different sterilant (Ethanol, calcium hypochlorite,) for different time duration. Highest explant survival rate was obtained after 4 weeks of culture in the nodal explant surface sterilization with 70% ethanol (20s) followed by 10% calcium hypochlorite for 15 min (Bello et al., 2018). In an experiment for surface sterilization of epical buds of different varieties of *Solanum tuberosum* (Potato) for regeneration of multiple shoots, nine treatments were used for different time interval for the sterilization of epical bud explants of ten different varieties of potato, viz- Kufri Chipsona-1, Kufri Uday, Kufri Jyoti, Kufri Surya, Kufri Pukhraj, Kufri Lalima, Kufri Khyati, Kufri Lima, Diamond, and Kufri Mohan. Tween-20, ethyl alcohol, Bavistin, and Mercuric Chloride were used as sterilizing agent. From the result highest explant survival of all the varieties after one week of the explant culture was obtained in the treatment with 0.3% Bavistin (30min) followed by 70% ethanol (30s) and 0.1% mercuric chloride (1min) (Verma et al., 2024). Another researcher performed standardization of surface sterilization protocol for different explants of tuberose cv. Arka Vaibhav, highest survival rate was achieved in the terminal stem scale explant treated in mixture of 0.1% carbendazim, 0.05% myristyl trimethyl ammonium bromide, and 0.05% chlorothalonil for overnight followed by 70% ethanol for 1 min, 4% sodium hypochlorite for 10 min, and 0.1% mercuric chloride for 15 min. in case of immature flower bud explant highest survival rate was achieved in the treatment with 1 drop tween 20 and 70% ethanol for 30 sec followed by 1% sodium hypochlorite for 3 min. And in case of tepal segment explant maximum explant survival was obtained in the treatment with 0.1% carbendazim for 30 min, 1 drop tween 20 and 70% ethanol for 30 sec and 1% sodium hypochlorite for 3 min. Among the 3 different explants, the terminal scale was found most suitable for direct organogenesis and shoot induction in the MS media supplemented with 4 mg/L BAP and 0.1mg/L IAA (Patil et al., 2023). Moreover, standardization of explant surface sterilization for induction of multiple shoots in *Phanera sirindhorniae,* most effective and highest survival of different explants survival was obtained in the treatment with 10% NaOCl for 10 min and 15 min for shoot tip explant; 10% NaOCl for 10 min and 5% NaOCl for 5 min for nodal explants. The surface sterilized explants were cultured on MS media supplemented with 1-4 mg/L BAP. Highest multiple shoots were obtained in the MS media fortified with 2mg/L BAP (Sirimat & Sakulsathaporn, 2019). Again, standardization of surface sterilization of Bitter cassava, the leaf and stem explant were treated with 70% ethanol and sodium hypochlorite. Highest explant survival rate obtained in the treatment with 70% ethanol for 5 min and 5.75% sodium hypochlorite for 10 min. The surface sterilized explants were cultured on the MS media fortified with different concentrations of 2, 4-D for callus initiation, highest callus formation was obtained in the MS fortified with 5 mg/L of 2, 4-D from the stem explants of bitter cassava (Mohamad Puad et al., 2022). Lastly, for *in vitro* propagation of *Eryngium maritimum* L. for large scale propagation, successful surface sterilization of the seeds was obtained using detergent at 40ºC for 180 min and 0.01% $KMnO_4$ for 3h and commercial bleach for 11 min. highest successful germination rate was obtained in the prolonged cold storage of *E. maritimum* seeds in sterile condition (Mežaka et al., 2023).

However, plant tissue culture also used in elicitor studies. Elicitors are frequently used in the *in vitro* cultures for inducing the production of secondary metabolites in the plants. In the analysis of bioactive compounds of *Balanites aegyptiaca* L. callus extracts, chitosan and salicylic acid were used for stimulation of secondary metabolites in the callus culture. 40mg/L of chitosan increased 2.6 folds of quercetin and 8.5 folds of kaempferol after 45 days of culture, again 0.2mg/L salicylic acid produced maximum total phenols. 40 and 80 mg/L chitosan enhanced the biosynthesis of fucose sugar which may have anticancer and antioxidant properties (Abo El-Fadl et al., 2022).

2.4 Review of somaclonal variation and detection in the *in vitro* propagated plants

Somaclonal variation is the variation in the plant or tissues which is caused at the time of tissue culture. The variation may cause due to mutations stress factors, sterilant used during explant sterilization, incomplete tissues, media components. Somaclonal variations were found to be genetic changes in the chromosomal number, karyotype changes, and point mutations among others. Though soma clonal variation cause threat to the breeders, it also provides opportunities for improvement in plant breeds, especially in the crops with a narrow genetic base (Otieno et al., 2022).

In a case study of soma clonal variation in tissue culture of Olive, it was reported that *in vitro* mass propagation of olive plants undergoes genetic variation to the parental plant in commercial production. The variation may be higher in tissue culture here same culture is used for long term. Soma clonal variation in tissue culture is a complex problem and may be detected using different approaches like RAPD. RAPD tool may be inadequate for study of genetic stability, therefore study of morphological characteristics (like growth) of tissue cultured plants may be a useful tool (Leva et al., 2012). Another study reported morphological and molecular characterization of tissue cultured banana plants, 40000 tissue cultured Grand nain banana plants were studied for soma clonal variation using morphological and molecular characterization. 25 off type plants from normal type were detected and 2 of them died. The 23 off type plants were studied using RAPD assay using 17 arbitrary primers. Cluster analysis revealed that the deformed lamina and winged petiole were related to normal plant and giant plant and weak plant were related to normal plant. From the experiment it was concluded that molecular markers can be used for detection and elimination of soma clonal variation in the tissue cultured explants (Abdellatif et al., 2012). In a study isolation of soma clonal variants for morphological and biochemical traits in *Curcuma longa.* Among 105 regenerants, five types of soma clonal variants were isolated through callus phase of culture. The variants were isolated based on the morphologic trait and conducted biochemical analysis such as oleoresin, curcumin, and volatile oil contents. Compared with the normal regenerants. The somaclonal variants produced significantly higher curcumin, oleoresin and volatile oil contents (%) compared to the normal regenerants and control plants. Reported that the somaclonal variation in turmeric plant may be new prospective for the genetic improvement of new turmeric varieties (Roopadarshini & Gayatri, 2012). While detection of soma clonal variation in the micro propagated Hibiscus sabdariffa L. using RAPD markers, the micropropagation was conducted in the MS media supplemented with different concentrations of BAP, and IBA. The randomly selected 10 micro propagated plantlets were subjected to DNA extraction using CTAB method. For RAPD assay 30 RAPD primers were used, out of 30 primers OPB-01, OPX-06 and DK-02 produced polymorphic bands. Concluding that RAPD is a useful tool for the detection of variants in tissue culture process (Govinden-Soulange et al., 2009).

In a genetic diversity study of he cultivated apple (*Malus domestica*), M7 and M9 tissue cultured plants using 20 ISSR markers. 51 randomly selected tissue cultured apple plants were studied for soma clonal variation. Reported genetic variation in each of the tissue cultured plants, significant difference in the genome size of some tissue cultured plants indicating change in genomic structure. But the degree of genetic variation was varied among the tissue cultured plants (Noormohammadi et al., 2015). While soma clonal variation in the tobacco subcultured callus (Nicotiana tabacum L.) after plant regeneration of seedlings, the whole genome DNA was extracted and RAPD was conducted using 20 RAPD primer, and 3 RAPD primers (OPC-09, OPR-12, OPA-10) showed polymorphism in the amplified products (Sedghi Ghartavol et al., 2011). In another work on somaclonal variation *Habanero Pepper* (Capsicum chinense Jacq.) using 13 ISSR Molecular Markers, reported that genetic variability was detected when cluster analysis was performed to express in the form dendrogram the relationships among the different regeneration system. Concluded that the regenerated protocol was inappropriate for *in vitro,* conservation, and genetic transformation, but may be applicable in breeding (Bello-Bello et al., 2014). *Tylophora indica* using RAPD markers. *Bacopa monnieri* leaf explants were cultured on 6µM BAP and *Tylophora indica* leaf explants were grown in MS media containing 4 µM BAP and 8 µM Kn. 10 RAPD markers were used. For RAPD assay 10 primers were used to detect soma clonal variation. From the assay an average of 62.07% and 13.19% of polymorphism were detected in the tissue cultured *B. monnieri* and *T. indica*, concluding that RAPD is a useful tool for detection of soma clonal variation in tissue cultured *B. monnieri* and *T. indica* (Pathak et al., 2013). Study of ISSR analysis in the micro propagated *Artemisia amygdalina* Decne. plant using two ISSR primers (CAC)3 GC and (ACTG)5. Two additional amplicons were observed in the tissue cultured *A. amygdalina.* Concluding that the plant *A. amygdalina* regenerated *in vitro* in the MS media supplemented with 10µM BAP and 10µM NAA possesses somaclonal variation (Khan et al., 2013).

2.5 Bioactive compounds identified in *in vitro* propagated plants using GC MS review

GC MS is a technique in combination of quantitative and qualitative separation (GC; Gas Chromatography) and identification (MS; Mass Spectrometry) for volatile and semi-volatile compounds (Iordache et al., 2009). Therefore, Gas Chromatography and mass spectroscopy (GC MS) is most efficient and popular technique for identification of structure, detection of phytoconstituents of plants and biological samples. This technique is most reliable for detection and identification of phyto-constituents of volatile substances, alcohols, long chain hydrocarbons, estters, acids, etc. Retention time, peak area, and molecular weight are used for the detection and confirmation of compounds (Venkatesh et al., 2014; Rukmini & Suvarnalatha Devi, 2014). Three compounds were detected in leaf extracts of *Morus alba* were detected using gas chromatography and the separated products were identified using mass spectroscopy, viz- 9,12,15 octadecatriodenic acid ethyl ester, gibberellic acid, and linolenic acid (Emniyet et al., 2015). 13 distinct compounds were detected and identified in the extract of *Ficus religiosa* using GC- MS analysis that bears important antimicrobial and antioxidant activities; ethyl isoallocholate, 4-methoxy phenol, phenol, and octadecanoic acid (Saravanan et al., 2014). GC MS analysis of *Vitis sitosa* revealed presence of 9,12,15 octadecatriodenic, which possesses many biological activities like hepatoprotective, anti-cancer, anti-inflammatory etc. (Gobalakrishnan et al., 2014).

Another investigation of GC-MS for identification bioactive compounds in the leaf extract of *Phyllodium pulchellum* revealed presence of 10 compounds bearing therapeutic activities, some important compounds like-9-Cyclohexylnonadecane (15.93), 9-Octadecenamide, (Z) (18.89), 2,2,2-trifluoroethyl 2- methyltetrahydro-5-oxo-3-furancarboxylate (10.47) etc. were identified in the extracts of *Phyllodium pulchellum.* Concluding that the GC-MS is an effective technique and high sensitivity which helps for separation, characterization and identification of bioactive compounds (Velmurugan & Anand 2017)

El-Naggar et al., 2023 established a successful efficient *in vitro* micropropagation and callus formation for *Urginea maritima* to study important bioactive compounds in the *in vitro* callus extracts using GC-MS analysis. Twenty-five and twenty-nine compounds were identified in the methanolic callus and bulbs of *U. maritima* respectively. Some important bioactive compounds were identified in the callus culture extract bearing anti-inflammatory, anticancer, hepatoprotective, and antiviral properties also these identified bioactive compounds can be used as antioxidant, antibiotic, anti-cancer, and anti-inflammatory for the drug formulations (El-Naggar et al., 2023).

Another experiment for analysis of GC-MS in the extracts of *in vitro* and wild *Pluchea lanceolata* plant revealed presence of diverse phytochemicals and active compounds. Medicinally important triterpenoids i.e.- lupeol and alpha amyrin found in *Pluchea lanceolata* extracts were quantified using high-performance thin-layer chromatography, in the *in vitro* leaf extracts 1.232 µg/g dry weight of lupeol and 2.129 µg/g dry weight were recorded (Mamgain et al., 2023).