DISCUSSIONS CHAPTER V

5.1 *Torenia crustacea*

The explant surface sterilization of *T. Crustacea* had most effective explant survival through treatment using 0.1% mercuric chloride for 2 min, leading to 63.33±4.71% of the cultured explant to survived for 21 days after culture. The effectiveness of this treatment can be attributed to mercuric chloride's strong antimicrobial properties, which efficiently eliminate surface contaminants without causing toxicity to the explants. The 60% survival rate is relatively high compared to similar studies (RR), suggesting that *T. crustacea* possesses moderate tolerance to this sterilization protocol. Further optimization could investigate alternative sterilant or combinations to enhance this survival rate further.

While most effective shoot proliferation and multiplication were observed in the MS media supplemented with 1mg/L BAP and 0.2 mg/L NAA and the highest average shoot length was observed. This combination likely optimizes the balance between cytokinin and auxin, promoting cell division and shoot elongation (RR). The observed highest average shoot length underscores the critical role of BAP in enhancing apical dominance and elongation in *T. crustacea*.

For maximum average number of rooting, it was observed that MS media supplemented with 1mg/L IAA was optimum. This aligns with IAA's role as a primary auxin in stimulating root initiation and elongation. The observed rooting efficiency highlights the responsiveness of *T. crustacea* to auxin-induced rhizogenesis. After that the hardening was successfully conducted and all the plantlets survived in green house at 28 ^{0}C ± 5 ^{0}C at 70% relative humidity. This high survival rate indicates the protocol's effectiveness in acclimatizing T. crustacea plantlets to ex vitro conditions. The controlled temperature and humidity likely minimized stress during the transition, allowing the plantlets to establish robust physiological mechanisms.

In the RAPD assay out of the ten RAPD primers used four RAPD primer (OPC-3, OPC-7, OPC-9, and OPC-10) did not formed any DNA bands. Three RAPD primers (OPC-2, OPC-4, and OPC-6) formed similar monomorphic DNA bands in both the wild and micropropagated *T. crustacea* plant and the other three RAPD primers (OPC-1, OPC-5, and OPC-8) formed different polymorphic DNA bands in wild and micropropagated *T. crustacea* plant, hence the somaclonal variation is confirmed in the micropropagated *T. crustacea* plant. This variation could be attributed to stress-induced genetic changes

during in vitro culture, particularly in media supplemented with exogenous hormones. While somaclonal variation can be undesirable for maintaining genetic fidelity, it also presents opportunities for generating novel traits. Future studies could focus on minimizing undesirable variations through reduced hormonal concentrations or alternative propagation techniques such as somatic embryogenesis.

While comparing the antioxidant potential of wild and tissue cultured *T. crustacea*, the tissue cultured extract formed comparatively higher gallic acid, quercetin content (using HPLC), antioxidant capacity and free radical scavenging activities.

Some bioactive compounds identified in the wild methanolic extract of *T. crustacea* namely- Trimethylsilyl dimethylphosphinate serves as an effective electrolyte additive that improves the electrochemical performance in high-voltage lithium-ion batteries (Han et al., 2015), Methyl sterculate is primarily used in organic chemistry research in ring opening oxidation and metabolism studies (Pearson et al., 1972), Oleic acid is a monounsaturated omega-9 fatty acid commonly found in various animal and vegetable fats and oils. Oleic acid is widely used in the food industry for its nutritional benefits, such as lowering bad cholesterol and raising good cholesterol (Chochos & Choulis, 2011), Arsenous acid, tris(trimethylsilyl) ester primarily used as a chemical reagent in organic synthesis it is used to treat rheumatism, skin disease etc. (Manikandan et al., 2019).

Again, in the *in vitro* methanolic extracts of *T. crustacea* - Octamethylcyclotetrasiloxane is primary function is as a conditioning agent and emollient, It may also be used as a probe liquid for nuclear magnetic resonance cryoporometry (NMRC) to analyze the pore size distribution (PSD) of oil-bearing tight sandstone (Liu et al., 2017), D-mannitol, 1,1'-O-1,16-hexadecanediylbis- is primarily used as a sweetener and stabilizer in the food and beverage industry due to its ability to preserve the texture and quality of food products during freezing and drying processes (O'Neil et al., 2013), the chronic administration of perfluorodecanoic acid may induce weight loss and increases liver weight and lipid content in mice. Perfluorodecanoic acid reduces mRNA expression of the genes encoding IL-1β, IL-18, and cellular inhibitor of apoptosis 2 (cIAP2), as well as caspase-1, -3, and -7 in mouse liver. It has been found in marine life and as a contaminant in surface water. Formulations containing perfluorodecanoic acid have been used commercially as wetting agents and flame retardants (Li et al., 2022).

Lindernia pusilla is used in Sri Lankan traditional medicine, this herb is found in the paddy fields and watery areas, flowering herb, bearing small leaves and creeping stems, the flowers are like Wilanda, therefore this herb is also known as Wilandavenna (Ediriweera, 2015). T. et al., 2016 developed *in vitro* propagation and *in vitro* flowering was of *Lindernia antipoda* L. (Alston), in this study *in vitro* shoot initiation was obtained in the half strength MS medium supplemented with 1m/L BAP, most efficient rapid shoot multiplication and the most efficient *in vitro* flowering was obtained in the MS medium supplemented with 1mg/L BAP and 0.2mg/L NAA (T. et al., 2016).

Rajila & Arunprasath, 2020 developed an efficient *in vitro* propagation of *Lindernia madayiparense,* in this experiment most efficient callus initiation was successfully developed in MS medium supplemented with in 0.5mg/L NAA& BAP, highest shoot multiplication was observed in the MS medium supplemented with 0.5mg BAP + 0.5mg NAA, and most efficient rooting was observed in the MS medium supplemented with 1mg of NAA. 5 compounds are obtained in the GC-MS analysis of *in vitro* callus extract.

In conclusion, the study demonstrates significant advancements in the micropropagation of *T. crustacea* while revealing opportunities for optimization. The findings on genetic stability, antioxidant potential, and unique bioactive compounds highlight the plant's potential for commercial applications in pharmaceuticals and nutraceuticals. Future research could build on these results by exploring genetic markers for variation, optimizing metabolite production, and scaling up propagation techniques for industrial use.

5.2 *Lindernia pusilla*

In the present experiment in the explant surface sterilization of *L. pusilla* most effective explant survival was observed in the explant treatment using 0.1% mercuric chloride for 3 min, 76.67±4.71% of the cultured explant survived after 21 days of culture. These findings align with prior studies emphasizing the efficacy of mercuric chloride as a surface sterilant for medicinal plants.

Most effective shoot proliferation and multiplication were observed in the MS media supplemented with 1mg/L BAP and 0.2 mg/L NAA and the highest average shoot length

was observed. The results suggest a synergistic effect of BAP and NAA in promoting shoot organogenesis in the species.

Maximum average number of rooting were observed in the MS media supplemented with 0.5mg/L IAA. After that the hardening was successfully conducted and all the plantlets survived in green house at $28^0C \pm 5^0C$ at 70% relative humidity.

In the RAPD assay out of the 14 RAPD primers used four RAPD primer 7 RAPD primers (OPC-1, OPC-7, OPC-8, OPA-1, OPA-4, OPA-12, and OPA-13) formed similar monomorphic DNA bands in both the wild and micropropagated *L. pusilla* plant and the other 7 RAPD primers (OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-9, and OPA-2) formed different polymorphic DNA bands in wild and micropropagated *L. pusilla* plant, hence the somaclonal variation is confirmed in the micropropagated *L. pusilla* plant. This variation, likely arising during tissue culture processes, provides a basis for potential selection of genetically diverse lines for further research or applications. Such genetic variation has been similarly documented in other micropropagated plants, offering opportunities for breeding and conservation.

In the comparative study of the antioxidant potential of wild and tissue cultured *L. pusilla*, the tissue cultured extract formed comparatively higher gallic acid, quercetin content (using HPLC), antioxidant capacity and free radical scavenging activities. This enhancement in secondary metabolite production might be attributed to stress conditions in vitro, which are known to induce metabolic pathways responsible for antioxidant biosynthesis.

1,2,4,5-Cyclohexanetetrol (key precursor in the biosynthesis of terpenes and steroids) (Rosatella & Afonso, 2022), 4-Aminohex-5-Enoic Acid can be used in treatment of neurological conditions, neuroscience research related to GABAergic pathways (Jung et al., 1977), Trisiloxane, 1,1,3,3,5,5-Hexamethyl- which are Wetting agent, emulsifier and foam builder used in cosmetics, shampoo, lotions etc. (Rayasam et al., 2022), Panaxydol, Tms is an important bioactive compound that can be used as potential therapeutic for delaying liver inflammation (H. S. Kim et al., 2016), Trimethylsilyl-Di (Timethylsiloxy)-Silane is primarily used as a chemical reagent in organic synthesis, particularly for the protection of hydroxyl groups and activation of carboxylic acids (Arya et al., 1990).

The *in vitro* propagated methanolic extract of *L. pusilla* plant contains Methylthiane S ,S-Dioxide is primarily used as intermediate chemical to synthesis of biologically active compound and pharmaceuticals, synthesis of pyrrole derivatives (Karthik et al., 2016), anthracene, 9-Ethyl-9,10-Dihydro-10-Trimethylsilyl- is used in preparation of nanostructured thin films and inhibitor in human protein kinase CK2 (López-Rojas et al., 2023), Panaxydol, Tms is an important bioactive compound that can be used as potential therapeutic for delaying liver inflammation (M.-Y. Kim et al., 2024).

Another experiment for *in vitro* rapid shoot multiplication of *Lindernia antipoda* (L.) Alston was successfully conducted on the half strength MS medium supplemented with 1mg/L BAP and 0.2mg/L NAA (T. et al., 2016). (Prihantini et al., 2018) developed *in vitro* propagation of *Artemisia annua* and studied production of antioxidant capacity of tissue cultured extractrs.

The callus produced in the MS media supplemented with 0.5mg/L NAA and 0.5mg/L BAP showed highest total phenol and DPPH radical scavenging activity. Also, the GC MS analysis revealed highest content of caffeic acid in the callus produced in this media. This experiment concluded that the tissue cultured *A. annua* bears higher pharmaceutical properties compared to that of wild plant where GC-MS profiling provides a comprehensive framework for understanding the biochemical and genetic dynamics of tissue culture systems.

In an experiment of evaluation of phenolic compounds in wild and tissue cultured *Ageratina pichichensis*. Though in some cases the tissue cultured extract showed lower antioxidant activity compared to the wild plant for DPPH, ABTS, and TBARS. There are some compounds found in the *in vitro* plants were not found in the wild plant such as caffeic acid (CfA), epicatechin (EPI), and p-coumaric acid (pCA). Concluding that the wild and *in vitro* plants of *A. pichichensis* produce antioxidant activity and phenolic compounds especially CC and CSC which may be a potent biotechnological alternative for extraction of bioactive compounds (Motolinia-Alcántara et al., 2023).

5.3 *Phlogacanthus thyrsiformis*

The explant surface sterilization of *P. thyrsiformis* most effective explant survival was observed in the explant treatment using 0.1% mercuric chloride for 2 min and 3 min,

76.67±4.71% of the cultured explant survived after 21 days of culture. In the present experiment the highest shoot multiplication and highest shoot length was obtained in the BM2 media where MS media was supplemented with 1mg/L BAP and 0.2mg/L NAA, and rooting was best in the MS medium supplemented with 1mg/L IAA. This finding aligns with the established role of BAP as a cytokinin that promotes cell division and shoot proliferation, particularly when combined with low concentrations of auxins like NAA, which support shoot elongation and tissue differentiation. Rooting was most effective in the MS medium supplemented with 1 mg/L IAA, emphasizing the auxin's pivotal role in root induction and elongation by enhancing cell elongation and division in root primordia.

In an experiment of *in vitro* propagation of *Bacopa monneiri* the highest shoot initiation of *Bacopa monneiri* explants were observed best in the MS medium supplemented with 3mg/l BAP and rooting of the *B. monneiri* explants was best in the MS medium supplemented with 2mg/L BAP and 3mg/L IAA (Gurnani et al., 2012). This comparison indicates that specific hormonal balances are required for different plant species and suggests the need to fine-tune protocols for optimal micropropagation efficiency.

In the *in vitro* propagation of potato, shoot initiation for sprouts were best in the MS medium supplemented with 2mg/L BAP and 3mg/L KIN, and for nodal explant 2mg/L BAP+ 1mg/L NAA, and 4mg/L KIN and 1mg/L IAA.

GC-MS analysis showed wild methanolic extract of *P. thyrsiformis* contains some important bioactive compounds i.e. methanol, oxo-, benzoate which is used in the naturally occurring oils of clove, tuberose, used in perfumes, also as flavouring agents, disinfectant additives, dye carrier, and solvents (resin, cellulose ether and esters, rubber) (Lewis, 2016), trisiloxane 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]- bears antioxidant properties (Momin & Thomas, 2020); 2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane; Asarone, is an important bioactive compound which possesses anti-anxiety, anti-depressant, anti-Parkinson's, anti-Alzheimer's, anti-cancer, anti-epileptic, anti-thrombic, anti-hyperlipidemic, anti-cholestatic, and radioprotective activities (Sandhir et al., 2021); Isoelemicin is commonly used as pharmaceutical adjuvant, nutritional, and cosmetics etc. (Ezealisiji & Awucha, 2020).

On the other end in the tissue cultured methanolic extract of *P. thyrsiformis* extract cyclotetrasiloxane, octamethyl- contains antimicrobial properties (Olaoye et al., 2024); Formic acid is used as antibacterial agent, preservative and in manufacturing (Okoye-Chine et al., 2022); Silandrone is used as anabolic androgenic steroid (Elks, 2014); and Arsenous acid, tris(trimethylsilyl) has an important role in nematostatic effect, and bears anticancer and antioxidant properties (Wiraswati et al., 2023).

For explant surface sterilization of *U. kirkiana* stock plants mercuric chloride was proved to be more effective for explant decontamination over sodium hypochloride, and calcium hypochlorite (Mng'omba et al., 2012). In the current experiment 0.1% mercuric chloride was found sufficient for surface sterilization of *P. thyrsiformis* nodal explants. The selected nodal explants of *P. thyrsiformis* was successfully established in the 3 min treatment with 0.1% mercuric chloride, where 80% of the explants survived after 21 days of explant culture. This treatment achieved a balance between effective microbial decontamination and minimal tissue damage. Mercuric chloride treatment for longer duration period resulted tissue damage of the explants causing explant mortality, e.g. 0.1% mercuric chloride treatment for 5 min showed 0% explant contamination rate, with 30% explant survival rate and 70% of the tissue damaged due to the exposure of the tissue in the 0.1% mercuric chloride for longer period. This indicates the cytotoxic effects of prolonged exposure to mercuric chloride.

In an experiment by Boruah, 2020, 0.1% mercuric chloride treatment for 3 min showed highest explant survival rate (68%) and lowers the bacterial contamination (18%). Treatment for longer duration (5min) decreased the contamination rate (8%) but also decrease the explant survival rate (35%). The fungal contaminations did not show any significant differences at different duration of explant treatment (Boruah, 2020). In the surface sterilization of banana cv. by Shukla, S., *et al.,* 2019 the maximum explant survival rate (73.25%) was observed while the explants were treated with 0.1% mercuric chloride for 2 min. while explant treatment using 0.1% mercuric chloride for 8 min showed minimum rate of explant survival of banana cv. after 25 days of explant culture.

G Hashmi et al., 1997 demonstrated the use of RAPD markers for identification of somaclonal variation in peach. In this experiment out of 35 RAPD primers, ten primers revealed polymorphism in the regenerants of peach through callus culture.

In this experiment, the antioxidant properties like phenolics, flavonoid, radical scavenging activities were observed comparatively higher in the tissue cultured extracts of *P. thyrsiformis* than in the wild plant. In an experiment of comparison of phenolics in wild and *in vitro Thalictrum foliolosum* plants using HPLC, *in vitro* plants possessed higher content of phenols (1489.80±9.15 µg GAE/g) and flavonoids (783.58±8.23 µg RE/g) than those of the wild plant extracts (Mishra et al., 2021).

5.4 *Enydra fluctuans*

This study is the first report on the *in vitro* propagation and comparative study of antioxidant, and bioactive compounds in wild and micropropagated medicinal plants. In this experiment comparative total phenol content, total flavonoid content, total antioxidant capacity, and free radical scavenging activity using DPPH assay were conducted, also HPLC assay were conducted for the quantitative comparison of gallic acid and quercetin content in the wild and micropropagated plants. Finally, the bioactive compounds were screened and identified using GC-MS analysis in wild and micropropagated plants.

In the present experiment the explant surface sterilization of *E. fluctuans* most efficient explant survival (76.67±4.71%) was obtained after 28 days of explant culture in basal medium while 3 min and 4 min treatment with 0.1% mercuric chloride. Most efficient shoot multiplication of *E. fluctuans* explants were observed in the MS medium with 2 mg/L BAP (BM6), highest root formation was obtained in the MS medium supplemented with 1mg/L IAA. These results highlight the delicate balance required in sterilization protocols to maximize survival while effectively eliminating microbial contaminants.

In the genetic stability study of micropropagated plants using RAPD assay using 10 RAPD primers, it was observed that out of the ten RAPD primers four primers bind any target DNA templates and out of the rest six RAPD primers the five RAPD primers formed polymorphic DNA bands in wild and tissue cultured *E. fluctuans.* These results suggest that micropropagation techniques may induce somaclonal variation. Such variations could serve as a double-edged sword, providing a potential source of genetic diversity but necessitating careful monitoring to maintain genetic fidelity for medicinal applications.

Again, in the antioxidant assay of wild and tissue cultured *E. fluctuans* methanolic extract, the tissue cultured plant extract formed higher content of gallic acid and quercetin in the HPLC analysis, and higher antioxidant content of radical scavenging activities, and antioxidant activities in the tissue cultured extract compared to the wild extract.

GC-MS analysis identified a range of bioactive compounds, methanolic extract in wild *E. fluctuans* Norvaline has a role of bacterial metabolite, a hypoglycemic agent and a neuro protective agent (De et al., 2016), Metoprolol is used to treat high blood pressure, chest pain due to poor blood flow to the heart, and a number of conditions involving an abnormally fast heart rate (Koch-Weser, 1979), Ethyl Nipecotate is an enantiopure cholinergic drug that has been shown to have antinociceptive and anti-inflammatory properties (Rustum, 1995), Ritalinic Acid is the drug commonly prescribed to children for treatment of attention-deficit hyperactivity disorder is the ester dl-methylphenidate (Ritalin) (Lockridge et al., 2018), L-Glutamic acid help treat epilepsy and muscular dystrophy (Mok et al., 2006), treat low blood sugar (hypoglycemia) in people with diabetes adenine, 9-(2,3-dideoxy-beta. -D-glycero-pent-2-enofuranosyl) can be used anti-HIV activity (Jafari-Vayghan et al., 2020).
The *in vitro* propagated methanolic extract of *E. fluctuans* contains Hentriacontane bears anti-microbial, anti-tumoral, and anti-inflammatory activities (Ouellette & Rawn 2015). Z,z-6,28-heptatriactontadien-2-one is used as vasodilator, larvicidal effects on A. aegypti and A. stephensi (Mallikadevi et al., 2012), 2-Azetidinecarboxylic acid is used as teratogenic agent, causes the production of abnormal proteins with impaired biological activity (Rubenstein et al., 2006), L-Norvaline has a role of bacterial metabolite, a hypoglycemic agent and a neuro protective agent (De et al., 2016), Ritalinic acid is the drug commonly prescribed to children for treatment of attention-deficit hyperactivity.

A study on antioxidant activities of micropropagated berry crops in genus *Vacciniun* L. by Debnath SC, Goyali JC*.,* 2020, reported that the *in vitro* propagated plants performed higher antioxidant activity compared to the plants grown *ex vivo.* Another study by Dakah et al., 2014 reported that the *in vitro* grown treated with 1.5 and 1 mg/L of kin plus 0.1 mg/L of NAA methanolic extract of *Ziziphora tenuior* L. showed more antioxidant properties compared to the wild grown plant.

In an experiment of comparative analysis of bioactive compounds in the micropropagated *Saraca asoca* leaf explants in MS medium supplemented with 0.3mg/L BAP and 0.6mg/L 2,4-D, the methanolic extracts of *in vitro* callus showed a significant antibacterial activity against *Salmonella typhi* (ZOI 14 mm) and *Enterococcus faecium* (17mm).

From the result it was observed that the callus extract showed higher antioxidant and antimicrobial activity compared to the wild plant. Also, the LC-MS analysis of the methanolic callus extract revealed eight compounds were identified mainly quercetin, flavonoids, epiafzelechin, and naringenin which can be used as therapeutic drugs (Vignesh et al., 2022).

5.5 *Hygrophila auriculata*

In the present experiment in the explant surface sterilization of *H. auriculata* most effective explant survival was observed in the explant treatment using 0.1% mercuric chloride for 4 min, 56.67±4.71% of the cultured explant was survived after 21 days of culture. Most effective shoot proliferation and multiplication were observed in the MS media supplemented with 1mg/L BAP and 0.5 mg/L NAA and the highest average shoot length was observed. Maximum average number of rooting were observed in the MS media supplemented with 1mg/L IAA highlighting the auxin's role in stimulating root development by promoting cell elongation and differentiation in root tissues. These results underscore the importance of fine-tuning plant growth regulators for optimal plant regeneration in tissue culture studies.

After that the hardening was successfully conducted and all the plantlets survived in green house at 28^0C±5^0C at 70% relative humidity. This success indicates that the acclimatization protocol effectively mitigated the stress of transition from in vitro to ex vitro conditions, ensuring the establishment of healthy, viable plants.

In the RAPD assay out of the ten RAPD primers four primers (OPC-3, OPC7, OPC-9, OPC-10) did not bind any target sequence on the template forming no DNA bands in the gel electrophoresis. Two RAPD primers (OPC-6, and OPC-8) formed similar monomorphic DNA bands in both the wild and micropropagated *H. auriculata* plant and the other three RAPD primers (OPC-1, OPC-2, OPC-4, and OPC-6) formed different

polymorphic DNA bands in wild and micropropagated *H. auriculata* plant, hence the somaclonal variation is confirmed in the micropropagated *H. auriculata* plant.

In the comparative study of the antioxidant potential of wild and tissue cultured *H. auriculata*, the tissue cultured extract showed comparatively higher antioxidant activities in total phenol, total flavonoid, total antioxidant capacity, and free radical scavenging activity in DPPH assay. Also, the tissue cultured plant extract showed higher gallic acid, and quercetin content (using HPLC), as compared to the wild plant.

Another study of *in vitro* plant regeneration of *Hygrophila Schulli* (buch. ham) M. R. & S.M. Almeida was conducted by Aasim et al. (2019). In this experiment the cotyledonary node and cotyledonary leaf explants of *Hygrophila schulli* were highest in the MS media supplemented with 1mg/l BAP.

In another experiment of *in vitro* propagation, and evaluation of antioxidant activities and phytochemical profiling of wild and micropropagated Curcuma larsenii Maknoi & Jenjitikul plant, highest shoot multiplication were observed in the MS medium supplemented with 2 mg/L BA and 0.5 mg/L NAA, and highest roots were observed in the MS medium supplemented with 4 mg/L BA and 0.5 mg/L IAA.

The *in vitro* plant leaf extracts performed higher antioxidant activity than the wild plant and other organs of *in vitro* plants. both the wild and *in vitro C. larsenii* exhibited antioxidant activity concluding that the *in vitro C. larsenii* plants can be an important alternative as biotechnological tool for extraction of bioactive substances (Saensouk et al., 2024).

GC-MS analysis identified a range of compounds, methanolic extract of *H. auriculata*-1,1,1,3,5,5,7,7,7-nonamethyl-3-(trimethylsiloxy) tetrasiloxane that can be used in the biological activity research compound and phytochemical analysis (Gideon & Ladan, 2023), 3-methylsalicylic acid, 2tms derivative used in analytical chemistry for compound analysis using GC-MS specifically in metabolomic studies (Abadie et al., 2022).