"A study on *in vitro* propagation and comparative analysis of bioactive compounds in some selected wild and tissue-cultured medicinal plants of Bodoland Territorial Region, Assam"

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CONCLUSION CHAPTER-VI

In the present experiment *in vitro,* mass propagation technique of five medicinal plants namely- *Lindernia crustacea, Lindernia pusilla, Phlogacanthus thyrsiformis, Enydra fluctuans,* and *Hygrophila auriculata* has been successfully standardized, along with the antioxidant potentials of wild and *in vitro* propagated plants have been studied and the bioactive compounds in the wild and *in vitro* plants have been studied. From the experiment it was observed that all the explants responded well in all the basal media at any PGR concentrations, but the explants responded differently in the different basal media. The explant response was observed best in the MS medium supplemented with only cytokinin (i.e. BAP). Explant multiplication of all the plants was highest in the higher cytokinin to auxin ratio was used in the MS media. And explant rooting was observed best in the higher auxin ratio in the MS media. To study the genetic stability of the *in vitro* plants produced from different explants of the plants RAPD assay was experimented using 10 different RAPD primers, this experiment concludes that the *in vitro* plants contain variation in the genomes of the *in vitro* propagated plants as compared to the wild plant. From the experiment it also can be concluded that the *in vitro* propagated plants contain higher antioxidant properties, total phenol content, total flavonoid content, total antioxidant capacity, and DPPH free radical scavenging activity as compared to the wild plant methanolic extract. HPLC analysis for the investigation of gallic acid and quercetin content in wild and *in vitro* plants revealed that the *in vitro* plants produced higher gallic acid and quercetin content as compared to the wild plant. The GC MS study revealed some important bioactive components in both wild and *in vitro* plants. It became evident from a comparison of the many bioactive chemicals produced by tissue-cultured and wild plants that there is a variation in their production. It might be the somaclonal variation present in the tissue-cultured plant. A thorough study is required to determine what affects the production of bioactive compounds in plants.

The performed research will help contribute in commercial production and extraction of bioactive compounds from the *Lindernia crustacea, Lindernia pusilla, Phlogacanthus thyrsiformis, Enydra fluctuans, and Hygrophila auriculata*.

Medicinal plants are the primary source of pharmaceuticals and the majority of human population in the world relies on the medicinal plant extracts as primary healthcare to treat many diseases, identification of the active compounds in the plant extracts may lead to discovery of new drug. Since the medicinal plants are being consumed continuously for its medicinal and food resources, the availability of some these resources in nature is reducing day by day, some of these may get extinct in near future. Therefore, conservation and mass production of these medicinal plants are utmost important. Plant tissue culture may be an alternate and ultimate solution to address this issue, in this technique any plant cell, tissue, or organ are used to grow inside a controlled and aseptic environment in the culture media supplemented with plant growth regulators. This technique is very important for mass and rapid propagation of uniform, and disease-free plants; it is also very useful for the preservation of endemic plants and genome transformation. It is also very much effective tool for production of secondary metabolites and bioactive compounds from plant extracts. Plant genome modification, plant improvement, transformation for vaccine production, and production of secondary metabolites are widely performed using this technique.

In the present experiment five medicinal plants were selected from Tamulpur and Kokrakjar district of Assam, i.e.- *Torenia crustacea, Lindernia pusilla, Phlogacanthus thyrsiformis, Enydra fluctuans,* and *Hygrophila auriculata*. This experiment aimed to standardize *in vitro* mass propagation technique, study of genetic stability in micropropagated plants using RAPD assay, comparison of antioxidant capacity using different antioxidant assays, quantitative gallic acid and quercetin content in the wild and *in vitro* plants were detected using HPLC assay, the bioactive constituents in wild and micropropagated plants were identified using GC-MS analysis.

Surface sterilization is one of the most crucial steps in the tissue culture process, for *in vitro* propagation, the nodal explant of the plants was selected for *in vitro* culture. The explant surface sterilization of the collected medicinal plants was conducted using 0.1% mercuric chloride for 1-5 min followed by incubation of the sterilized explants inside the growth chamber under a controlled and aseptic condition in the growth medium supplemented with growth regulators (BAP, IAA, NAA). The *in vitro* plant genome may

undergo somaclonal variation due to the different *in vitro* conditions and growth regulators. Therefore, detection of somaclonal variation utmost important in the *in vitro* plants. Therefore, the genetic stability study was conducted in the *in vitro* grown plants and were tested for genetic stability using RAPD assay. Plant extracts were prepared in the 70:30 methanolic aqueous solution for different *in vitro* assays. The comparative antioxidant capacity of wild and *in vitro* plants was studied through the total phenol content, total flavonoid content, total antioxidant capacity, and DPPH free radical scavenging activity. The quantitative gallic acid and quercetin content in the wild and *in vitro* plants were studied using HPLC analysis, and finally the bioactive compounds in the wild and *in vitro* plants were identified using GC- MS analysis.

From the experiment the following observations were obtained as outcome:

1. Standardization of *in vitro* propagation protocol and study of genetic stability in the *in vitro* plants using RAPD assay:

In the *in vitro* propagation of *T. crustacea,* explant surface sterilization was reported highest in the treatment using 0.1% mercuric chloride for 2 min, leading to 63.33±4.71% of the cultured explant to survived after 21 days of culture. 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. For maximum average number of rooting, it was observed that MS media supplemented with 1mg/L IAA was optimum. 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. 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.

In the *in vitro* propagation of *L. pusilla,* explant surface sterilization was reported highest in the treatment using 0.1% mercuric chloride for 2 min, leading to 76.67±4.71% of the

cultured explant to survived after 21 days of culture. In case of *L. pusilla* most effective shoot proliferation and multiplication and highest average shoot length was observed in the MS media supplemented with 1mg/L BAP and 0.2 mg/L NAA. Highest average number of rooting were observed in the MS media supplemented with 0.5mg/L IAA. In the RAPD assay out of the 14 RAPD primers 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 while 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.

Explant surface sterilization of *P. thyrsiformis* was reported highest in the treatment using 0.1% mercuric chloride for 2 min and 3 min, leading to 76.67±4.71% of the cultured explant to survived after 21 days of culture. In case of *P. thyrsiformis,* 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. In the RAPD assay out of the total ten RAPD primers four primers (OPC 2, OPC-8, OPC-9, OPC-10) did not bind any targets, out of the six RAPD primers five primers (OPC-1, OPC-3, OPC-4, OPC-5 and OPC-7) formed polymorphic DNA bands in wild and tissue cultured *P. thyrsiformis*, only one RAPD primer (OPC-6) formed monomorphic bands.

In the explant surface sterilization of *E. fluctuans* most efficient explant survival (76.67±4.71%) was obtained in 3 min and 4 min treatment with 0.1% mercuric chloride after 28 days of explant culture in basal medium. Most efficient shoot multiplication of *E. fluctuans* explants were observed in the MS medium with 2 mg/L BAP (BM6) and highest root formation was obtained in the MS medium supplemented with 1mg/L IAA. 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 (OPC-03, OPC-07, OPC-09, OPC-10) did not bind any target DNA templates and out of the rest six RAPD primers the five RAPD primers (OPC-10, OPC-02, OPC-04, OPC-05, OPC-06) formed polymorphic DNA bands in wild and tissue cultured *E. fluctuans.*

And 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 and highest average shoot length was observed in the MS media supplemented with 1mg/L BAP and 0.5 mg/L NAA. Maximum average number of rooting were observed in the MS media supplemented with 1mg/L IAA. 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.

2. Comparative antioxidant capacity tests of wild and *in vitro* 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 (Table: 8.1).

Table 8.1: Comparative antioxidant capacity of wild and *in vitro* plant, result represents mean ±SE value of triplicate experiment, $p<0.05$

Sl no	Plant	Compound	Wild plant (µg/mg)	*In vitro* plant (µg/mg)
1	*T. crustacea*	Total phenolic content (GAE)	64.7+5.3	69+3.4
		Total flavonoid content (QE)	44±3.7	48±3
		Total antioxidant capacity (AAE)	143.7±4.2	126±4.2
		Radical scavenging activity IC_{50} (DPPH)	28.11±1.25	28.677±1.13
2	*L. pusilla*	Total phenolic content (GAE)	54.7 ±5.3	61±3.4
		Total flavonoid content (QE)	20±7	24±7.7
		Total antioxidant capacity (AAE)	94±7.2	114.7±7.2
		Radical scavenging activity IC_{50} (DPPH)	40.70±0.50	45.19±0.84.

3	*P. thyrsiformis*	Total phenolic content (GAE)	206.6667±4.92	237.66±3.4
		Total flavonoid content (QE)	106±4.54	125.66±1.69
		Total antioxidant capacity (AAE)	182.33±4.1	228.33±3.39
		Radical scavenging activity IC_{50} (DPPH)	49.33±1.24	56.33±1.24
4	*E. fluctuans*	Total phenolic content (GAE)	54.7 ±5.3	61 ±3.4
		Total flavonoid content (QE)	20±7	24±7.7
		Total antioxidant capacity (AAE)	94±7.2	114.7±7.2
		Radical scavenging activity IC_{50} (DPPH)	37.70±2.33	45.19±2.84.
5	*H. auriculata*	Total phenolic content (GAE)	154.7 ±5.3	184±3.4
		Total flavonoid content (QE)	112±3	124±2.6
		Total antioxidant capacity (AAE)	94±7.2	114.7±7.2
		Radical scavenging activity IC_{50} (DPPH)	67.70±0.50	74.19±0.84

3. Comparative gallic acid content and quercetin content in wild and *in vitro* plants using HPLC assay: HPLC was used for quantification of gallic acid and quercetin content in the wild and *in vitro* propagated plants. from the HPLC assay for quantitative detection of gallic acid and quercetin content in the wild and *in vitro* plant, the *in vitro* plant produced higher content of gallic acid and quercetin content as compared to the wild plant (Table 8.2).

Table: 8.2: quantitative detection of gallic acid and quercetin content in wild and *in vitro* plant, result represents mean ±SE value of triplicate experiment, $p<0.05$.

Sl no	Plant	Compound	Wild	*In vitro* plant
1	*T. crustacea*	Gallic acid	91.66±1.24	99.33±1.24
		Quercetin	82.73±1.42	86.33±0.53
2	*L. pusilla*	Gallic acid	10.302± 0.064	42.94± 0.23
		Quercetin	2.13± 0.035	2.26± 0.032
3	*P. thyrsiformis*	Gallic acid	117.31±2.06	129.24±4.06
		Quercetin	85.67±1.81	85.66±1.82
4	*E. fluctuans*	Gallic acid	8.05±0.65	10.19±1.18
		Quercetin	2.01±1.09	5.12±1.08
5	*H. auriculata*	Gallic acid	43.1±1.33	56.27± 0.78

	Quercetin	41.57±0.96	42.7±1.28

4. Comparative analysis of active compounds in the wild and *in vitro* plants: The active compounds in the wild and *in vitro* plants were identified using GC-MS analysis based on the peaks observed on the chromatograms. Diverse group of active compounds were identified with important characteristics including antimicrobial, antioxidant and antiviral activities were exhibited in the *in vitro* and wild plants.