Synopsis of the thesis on

Studies for developing shoot cultures of *Leptadenia reticulata* (Retz.) Wight and Arn and *Tylophora indica* (Burm. F.) Merrill with phytochemical analysis

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INTRODUCTION

Leptadenia reticulata (Retz.) Wight and Arn is a multipurpose medicinal plant of family Asclepiadaceae. According to Athara-Veda the plant is a life and strength giver nature (Arya et al., 2003) and therefore commonly called as "Jivanti" (to give life). It is distributed in tropical and subtropical parts of Asia and Africa (Parabia et al., 2007) and in India it is reported in the Sub-Himalayan tract of Punjab, Uttar Pradesh and throughout the Deccan Peninsula. Plant contains many important bioactive compounds such as α-amyrin, β-amyrin, ferulic acid, luteolin, diosmentin, rutin, lupeol, β-sitosterol, stigmasterol, laptadenol, n-triaconate, p-coumaric acid etc. (Subramanian and Lakshman, 1977; Sastry et al., 1985; Prashanth et al., 2003; Rajanna et al., 2009; Bawra et al., 2010; Geetha et al., 2011; Pal et al., 2012).

Tylophora indica (Burm. F.) Merrill commonly known as "Indian ipecac" or "Antamul" is another medicinally important plant of the Asclepiadaceae family and is distributed throughout Southern and Eastern part of India in plains, forests and hilly places in India and normally found in Uttar Pradesh, Bengal, Assam, Orissa, Himalayas and sub Himalayas (Anonymous, 1969; 1978a). The plant is traditionally used for the treatment of respiratory diseases in Ayurvedic medicine, particularly for the treatment of asthma and bronchitis and hence it named as 'asthma plant' (Anonymous, 1978b; Kirtikar and Basu, 1991). This plant also possesses immunomodulatory, hypoglycaemic, anti-allergic and antimicrobial properties. Pharmacological activity is mainly attributed to the presence of alkaloids like tylophorine (Gellert, 1982), tylophorinine (Gopalakrishnan et al., 1979), tylophorinidine (Mulchandani et al., 1971), tyloindicines A-J (Ali et al., 2001), septidine etc. (Gupta et al., 2010; Gurav et al., 2011) and other compounds such as kaempferol, quercetin, and α & β-amyrins (Ali, 2008). The destruction caused by harvesting the roots and leaves as a source of the drug and lack of organized cultivation has led to a rapid decline in the wild population and has resulted in its enlistment as an endangered species India.

The exhaustion of important medicinal plant as natural resources due to over-exploitation has threatened their existence in wild and to conserve these important medicinal plants technique like plant tissue culture have to be adopted for their large-scale propagation. As many wild plants show a poor rate of multiplication, *in vitro* methods of propagation are helpful in regenerating those plants which are in high demand. Different methods like organogenesis and somatic embryogenesis are successful system which offers a rapid propagation of plants and can meet the need of pharmaceutical industries. Somatic

embryogenesis has overriding advantages compared to other propagation methods as it produces a somatic embryo (SE) with a functional meristem, a vascular system and a root/shoot axis in a single step (Bassuner et al., 2007). Regeneration of plantlets through direct and indirect organogenesis are also worthwhile for production of large number of plants and have their own benefits. One of them is that *in vitro* plants can be assessed for important metabolites which are synthesized by *in vivo* plants. But *in vitro* cultures are known to synthesize less metabolites as compared to *in vivo* plants, which can be enhanced by treating the cultures with elicitors (Zhao et al., 2005). Elicitors are classified mainly in two types: biotic or abiotic which trigger a series of morphological, physiological, biochemical and molecular changes in plants (Namdeo, 2007). One of the commonly used biotic and abiotic elicitors are yeast extract (YE) and salicylic acid (SA) respectively, which are commonly used for enhancement of important secondary metabolites (Patil et al., 2013; Maqsood and Abdul, 2017).

The aim of present study was to assess the potential of leaf and nodal explants in both plants for organogenesis and embryogenesis. These *in vitro* cultures were further analysed qualitatively and quantitatively for p-coumaric acid in *L. reticulata* and lupeol in *T. indica*. The cultures were also subjected to elicitor(s) treatment to evaluate their effect on targeted metabolite. Further the molecular technique will be used to assess the effect of elicitor(s) on cultures.

OBJECTIVES

- Establishment of callus and shoot cultures for *L. reticulata* and *T. indica*
- Chemical profiling of *in vivo* and *in vitro* cultures
- Quantification of targeted metabolites in selected species
- Elicitation of targeted metabolites in cultures and gene expression analysis using molecular technique

MATERIALS AND METHODS

The present study has been carried out on two important medicinal plants of Asclepiadaceae family i.e. *Leptadenia reticulata* and *Tylophora indica.* The experimental work is mainly divided into two parts: Development of *in vitro* cultures and their phytochemical analysis.

❖ **Establishment of Cultures**

❑ **Plant Material:** Leaves and nodes

❑ **Methodology:**

- Healthy twigs of *L. reticulata* and *T. indica* were collected from plants growing in Botanical Garden and Arboretum of The M.S. University of Baroda
- Entire leaves and nodes were excised and kept in running tap water for 1 h
- Then they were washed thoroughly using labolene
- The detergent was removed and rinsed with D/W
- Only *L. reticulata* explants received PVP (0.01%) treatment, which was removed by rinsing with distill water
- Then the explants of both the species were surface sterilized using bavistin (0.01%), streptomycin (0.01%) and $HgCl_2$ (0.1%) for 4 min each and rinsed with D/W after each treatment
- Square piece of leaves (1 cm^2) containing midrib and nodes containing opposite axillary buds were placed vertically in Murashige and Skoog's (1962) medium (MS) fortified with sucrose (3%) and different plant growth regulators (PGRs)/additives

❑ **PGRs used for establishing cultures:**

Different cytokinins i.e. 6-benzyladenine (BA), kinetin (Kn), adenine sulphate ($AdSO_4$) and auxins i.e. indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 2,4-dichlophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA) individually and their synergistic combinations as well as gibberellic acid (GA_3) and coconut water were utilized to establish shoot and callus cultures for both selected species.

<u>Leaf explants</u>

✓ **Control:**

- Basal MS medium

✓ **Individual cytokinins:**

- MS + sucrose (3%) + BA/ Kn (5-20 µM)

✓ **Individual auxins:**

- MS + sucrose (3%) + IAA/ NAA/ 2,4-D (0.1-1 µM)

✓ **Combination of cytokinins:**

- MS + sucrose (3%) + BA/ Kn (5-20 µM) + $AdSO_4$ (5-20 µM)

✓ **Combination of cytokinins and auxins:**

- MS + sucrose (3%) + BA/ Kn (5-20 µM) + IAA/ NAA/ 2,4-D (0.1-1 µM)

Nodal explant

✓ **Individual cytokinins:**

- MS + sucrose (3%) + BA/ Kn (5-20 µM)

✓ **Combination of cytokinins:**

- MS + sucrose (3%) + BA/ Kn (5-20 µM) + $AdSO_4$ (5-20 µM)

✓ **Supplementation of natural additive:**

- MS + sucrose (3%) + CW (2-8%)

Development of plantlets from somatic embryos

Somatic embryos were transferred to the following static and liquid media supplemented with different PGR combinations for further development:

- MS + sucrose (3%) + Kn (5 µM) + IAA (0.1 µM)
- MS + sucrose (3%) + Kn (5 µM) + NAA (1 µM)
- MS + sucrose (3%) + BA (10 µM) + $AdSO_4$ (10 µM)
- MS + sucrose (3%) + GA_3 (1 µM)
- MS + sucrose (3%) + CW (4 µM)

❖ **Rooting**

❑ **Methodology:**

- Healthy elongated shoots were harvested from eight week old cultures and leaves from last 2-3 nodes were removed
- These shoots were initially placed in sterile D/W and then in bavistin solution (0.01%) for 1 min each
- After this, the shoots were placed in MS liquid rooting medium of different strength with the last two to three nodes being immersed in medium
- The base of the culture tube was covered with aluminum foil till the root induction started

❑ **Media used for rooting**

- ½MS / ¼MS + sucrose (1%) - Control
- ½MS/ ¼MS + sucrose (1%) + IBA (2-8 µM)

- ½MS/ ¼MS + sucrose (1%) + NAA (2-8 µM)

❖ **Hardening and Acclimatization**

- Plantlets which developed from somatic embryos, leaf and nodal explants were initially transferred to thermocol cups filled with cocopeat:sand (1:1) and covered with plastic bags to maintain humidity and kept in culture room at 25 ± 2 °C for four weeks
- After this these plantlets were transferred to the pots filled with same substrates to greenhouse for gradual acclimatization
- Full grown plants were then placed in soil under full sunlight in Botanical garden

❖ **PHYTOCHEMICAL ANALYSIS**

In vivo and different *in vitro* cultures were analyzed qualitatively and quantitatively for their biosynthetic potential. The chemical profiling was done using HPTLC for both plants, and in *L. reticulata* p-coumaric acid whereas for *T. indica* lupeol was quantified.

Chemical profiling

❑ **Methodology:**

- Healthy *in vivo* shoots of *L. reticulata* and *T. indica* collected from the Botanical Garden and *in vitro* callus and shoot cultures were analyzed for chemical profiling
- The samples were dried in oven at 40 °C and powdered using mortar-pestle
- 1 gm dried powder of *in vivo* and *in vitro* samples were extracted using 10 ml hexane, it was refluxed in water bath for 10 min at 50 °C
- The extracts were then filtered and collected in evaporation dish, and the procedure was repeated twice using same solvent
- Same procedure was repeated three times using ethyl acetate and methanol
- All the extracts were kept overnight at room temperature for evaporation and next day the marc was reconstituted to 1 ml using the same solvent and stored in freezer (0 °C)

❑ **Mobile phases and TLC plate development:**

All the extracts were spotted as a band (8 mm length and 12 mm distance between tracks) on a precoated Silica gel G60 F_{254} (20 x 20 cm, 0.2 mm thick, E. Merck Ltd., Mumbai, India) with the help of Linomat V automatic sample applicator.

Following mobile phases were used for different extracts-

(1) *L. reticulata*-

- Hexane extract- toluene:ethyl acetate:formic acid (7.2:2.5:0.3 v/v)
- Ethyl acetate extract- toluene:ethyl acetate:methanol (6.4:2.7:0.9 v/v)
- Methanol extract- toluene:ethyl acetate:formic acid:methanol (6.0:2.7:0.6:0.7 v/v)

(2) *T. indica*-

- Hexane extract- toluene:methanol (9:1 v/v)
- Ethyl acetate extract- toluene:ethyl acetate:methanol (6:3:1 v/v)
- Methanol extract- toluene:ethyl acetate:formic acid (6.5:3.0:0.5 v/v)

Freshly prepared mobile phases were poured in CAMAG twin through glass chamber and chamber was saturated for 45 min. The plates loaded with samples were allowed to develop in respective mobile phase and after development, they were air dried. Scanning was done at different UV wavelengths e.g. 254 nm, 366 nm and 525 nm (after derivatization using anisaldehyde-sulfuric acid reagent) in CAMAG TLC scanner 3 linked to winCATS software.

Quantification of Metabolites

10 µl of standard and samples were applied as a band on TLC plates. The chambers were saturated with respective mobile phases for 45 min. In *L. reticulata*, p-coumaric acid was quantified in methanol extract by developing the plates in toluene:ethyl acetate:methanol (7.2:2.5:0.7 v/v), air dried and scanned at 254 nm. Whereas in *T. indica* hexane extract was used for lupeol quantification and the plates were developed in toluene:methanol (9:1 v/v), air dried and scanned at 525 nm. The respective peak areas were recorded for the compounds in all the samples.

Elicitation of cultures

Callus cultures developed from leaf explants in *L. reticulata* and shoot cultures developed through nodal explant in *T. indica* were used for elicitation studies.

L. reticulata

Leaf callus which differentiated on optimized MS + sucrose (3%) + BA (20 µM) + NAA (0.5 µM) medium served as a control. This medium was fortified with different concentrations of yeast extract (YE) (25, 50, 100 and 200 mg/l) / salicylic acid (SA) (25, 50, 100 and 200 µM). Leaf explants were placed on the media and calli were harvested after fourth and fifth week. The samples were dried and will be quantified for targeted metabolites.

T. indica

Shoots developed from nodal explant on optimized medium MS + sucrose (3%) + Kn (10 µM) + $AdSO_4$ (15 µM) served as a control and analysed for lupeol synthesis. These shoots will be treated with different concentrations of YE (25, 50, 100 and 200 mg/l) / SA (25, 50, 100 and 200 µM). Control and treated shoots will be harvested at weekly intervals, dried and quantified for lupeol.

RESULTS

❖ **Establishment of cultures**

L. reticulata

Cultures were established using leaf and nodal explants in Murashige and Skoog's (MS) medium supplemented with sucrose (3%) and different concentrations of cytokinins and auxins. The morphogenic response in leaf explants varied according to the PGR's present in the medium. In some combinations differentiation of direct and indirect shoot bud formation was observed but they failed to form shoots. Simultaneously in these combination callus differentiated somatic embryos and the optimum 21.70 ± 2.06 was recorded with 100% induction in a synergistic combination of BA (15 µM) and $AdSO_4$ (15 µM).

In nodal explants, media fortified with individual and synergistic combinations of cytokinins regenerated shoots and optimum 5.10 ± 0.33 shoots with 100% response was obtained in BA (10 µM) and $AdSO_4$ (10 µM).

T. indica

Shoot cultures were developed using leaf and nodal explants in MS medium fortified with sucrose (3%) and different concentrations of PGRs. Both the explants had the potency to regenerate shoots and somatic embryos directly or indirectly. In leaf explants callus differentiated an optimum 22.50 ± 0.81 shoots in 80% cultures in presence of BA (10 µM)

and Kn (15 µM). Many synergistic combinations of cytokinins and cytokinins with auxins induced embryogenic calli and highest frequency of embryos (26.00 ± 1.13) was recorded in presence of Kn (5 µM) and IAA (1 µM) with 80% induction. These somatic embryos were transferred to liquid medium augmented with GA_3 (1 µM) which facilitated the further developments of plantlets which reached to a length of 9.38 ± 0.35 cm with 90% germination within four weeks.

Nodal explants were also able to form multiple shoots in presence of cytokinins. An optimum 4.10 ± 0.22 shoots with 100% response was noted at eight weeks in BA (10 µM) and Kn (5 µM). Medium fortified with BA and $AdSO_4$ induced axillary buds to divide and form shoots and simultaneously at base of the explants morphogenic callus proliferated which differentiated shoot buds at the end of eight weeks. Transfer of this callus cluster to same medium resulted in growth of shoot buds to shoots. Similarly when Kn-$AdSO_4$ combination was tried similar morphogenic response was observed but the basal callus differentiated somatic embryos which developed into plantlets after transfer it to earlier optimized medium for germination of somatic embryos.

❖ **Rooting**

In *T. indica* shoots developed through leaf/nodal explants were utilized for rooting and optimum 9.75 ± 0.74 roots/shoot with 80% response was obtained in ¼MS medium supplemented with 4 µM of IBA. When another auxin NAA (2-8 µM) tried, roots were developed but maximum 6.33 ± 0.51 roots were formed in ½MS medium with NAA (4 µM) which was less as compared to IBA. In the rooting study, somatic embryo induction was also observed on the stem portion by fourth week in NAA fortified medium. These embryos can be further developed in media optimized for development of somatic embryos.

❖ **Hardening and Acclimatization**

In *T. indica*, young and healthy plantlets obtained from somatic embryos and leaf/ nodal explants were removed from the culture medium, washed gently with distilled water and carefully transferred to cups filled with mixture of cocopeat:sand (1:1). They were watered every alternate day till four weeks, then plants were shifted to greenhouse for another four weeks. Plants were removed gently from pots without hampering their roots and potted in the garden soil. They grew healthy in the garden with 100% survival after three month.

❖ **Phytochemical analysis**

❑ **Chemical profiling**

Different *in vivo/in vitro* shoots and callus samples of *L. reticulata* were utilized for the extraction using three solvents i.e. hexane, ethyl acetate and methanol for each sample. Hexane extract of all the samples were subjected to chromatographic separation on TLC plates in optimized mobile phase and when observed under UV_{254} variation was observed in chemical profiling as confirmed through changes peak areas and by observing chromatograms. Further observing plates at 366 nm also confirmed change in metabolite synthesis as banding pattern changed amongst the sample and the same can be observed after densitometry scanning of plate which reveals variation in chromatograms. When plate was derivatized, variation in number of bands was recorded and further confirmed through peak areas and chromatograms. Similar results were also recorded for ethyl acetate and methanol extracts of the samples when separated on TLC plates.

Similarly for *T. indica* the *in vivo* and *in vitro* samples were harvested and extraction was done using above mentioned three solvents. Variation in peak number and their areas was observed between all the samples under different UV ranges and these showed the comparison of banding pattern between all the samples.

❑ **Quantitative analysis**

In *L. reticulata, in vivo* shoots and *in vitro* samples detected the presence of p-coumaric acid in methanol extract after scanning at 254 nm. But the quantity of p-coumaric acid was higher in *in vitro* callus cultures as compared to *in vivo* and *in vitro* shoots.

Whereas for *T. indica* samples, derivatization of plates confirmed the presence of lupeol in hexane extract of all the samples and further densitometric analysis revealed that the area under the curve (AUC) obtained after 525 nm scanning was different for all the samples. Lupeol quantity was recorded higher in nodal derived *in vitro* shoots in comparison with *in vivo* and leaf derived *in vitro* shoots.

❖ **Elicitation of cultures**

In *L. reticulata* elicitation of callus cultures was carried out using YE and SA. Elicitated cultures were harvested at four and five weeks, dried at 40 °C and stored 0 °C. These elicitated samples will be quantified for the targeted metabolite.

In *T. indica,* shoot cultures of optimised media will be treated with elicitors, harvested at weekly intervals, dried and analysed for the content of the targeted metabolite.

Further investigation on gene expression analysis will be carried out for elicitated cultures using molecular technique.

DISCUSSION

Regeneration studies in *L. reticulata* revealed that leaf explants in presence of BA and IAA induced somatic embryos. Synergistic combinations of cytokinins and auxins have proved to be beneficial for somatic embryogenesis as they regulate cell cycle, division and differentiation (Pinto et al., 2011). When nodal explants of *L. reticulata* were placed in medium fortified with BA and $AdSO_4$, it formed optimum number of shoots. Singh et al. (2017) also reported that presence of BAP in combination with $AdSO_4$ in culture media have the potency for multiple shoot induction in potato. *In vitro* callus and shoot cultures were evaluated for its biosynthetic potential using chemical profiling and the result depicted that variation in banding pattern as well as peak areas between them which might be due to presence of PGRs in the medium (Lee et al., 2011). HPTLC fingerprint has been used to assess the efficiency of *in vitro* cultures in plants like *Bacopa monnieri* (Patni et al., 2010) and *Withania somnifera* (Shetty and Chandra, 2012). Further quantifying p-coumaric acid in cultures revealed that it varied in content between *in vivo* and *in vitro* samples. Amongst all the cultures the highest content was recorded in leaf callus formed in media supplemented with BA and NAA, whereas the lowest content was in callus which formed in 2,4-D fortified media, Nikam and Savant (2009) have documented similar observations in callus culture of *Ceropegia juncea*. *In vitro* culture as compared to *in vivo* plants was able to synthesise a higher content of p-coumaric acid and similarly withanolide content in *in vitro* cultures of *Withania coagulans* was high as compared to wild plants (Jain et al., 2011).

In *T. indica* leaf callus which was formed in presence of different PGR's varied in its morphogenic response as it differentiated both shoots and somatic embryos. Optimum shoots regenerated in a synergistic combination of BA with Kn in our present study and the same cytokinins together proved to be beneficial in plants like *Stevia rebaudiana* (Sreedhar et al., 2008) and *Withania coagulans* (Jain et al., 2011). A large number of somatic embryos were germinated in combination of Kn with IAA, which is higher as compared to earlier reports (Jayanthi and Mandal, 2001; Chaudhuri et al., 2004; Chandrasekhar et al., 2006; Sahai et al., 2010). Then after plantlets from SE were transferred for further development in which different PGRs tried, GA_3 has been proved to be better for development of SEs and it is in

accordance with earlier report on SE development in *Quercus rubra* (Vengadesan and Pijut, 2009) and *Hemidesmus indicus* (Cheruvathur et al., 2013). The study also confirmed that liquid cultures proved to be beneficial for SE development and it is in line with earlier reports on suspension cultures of *Phoenix dactylifera* which was proved to be better as compared to agar solidified media for SE formation (Fki et al., 2003; Zouine et al., 2005). When nodes were utilized as an explant for shoot culture establishment the synergistic effect of BA (10 µM) and Kn (5 µM) proved to be optimum for regeneration. In *Artemisia vulgaris* a combination of BA with Kn evoked optimum regeneration, whereas individual cytokinins differentiated less number of shoots (Sujatha and Kumari, 2007) which can be compared to the results of the present study. *In vitro* shoots were then utilized for rooting studies and supplementing the medium with auxins viz. IBA and NAA promotes root development (George and Sherrington, 1984). In the present study optimum rooting was observed in ¼MS medium fortified with IBA (4 µM). Similarly in another Asclepiadaceae member *H. indicus*, ¼MS medium fortified with IBA was reported to be optimum for rooting (Pathak and Joshi, 2017). HPTLC fingerprint of the different samples showed a variation in chemical profiling and it may be due to presence of PGRs in the medium and results of Pathak et al. (2017) for *Hemidesmus indicus* also states that shoots that regenerated in media fortified with various PGR differed in their fingerprint. Quantitative analysis of samples revealed that amount of lupeol in the shoots also varied between the samples and it is in line with earlier reports in *H. indicus* in which PGRs of the media affected lupeol biosynthesis (Misra et al., 2005; Pathak et al., 2017).

CONCLUSIONS

In the present study an efficient regeneration was achieved for important plants like *L. reticulata* and *T. indica* using both leaf and nodal explants. Shoot cultures could be successfully established through organogenesis and somatic embryogenesis. Plantlets regenerated through somatic embryos are considered to be true-to-type in nature which can be utilized for mass propagation of species. Chemical profiling of *in vivo* and *in vitro* samples for both the plants showed a similarity in fingerprint, which confirmed that the *in vitro* cultures are capable of synthesizing metabolites that are present in *in vivo* plants. Quantitative analysis recorded high content of targeted metabolites i.e. p-coumaric acid in *L. reticulata* and lupeol in *T. indica* in cultures as compared to *in vivo* shoots. Therefore *in vitro* cultures of both species were treated with elicitors to enhance the targeted metabolites, which can then

become an alternative for large scale production of important metabolites. This in turn will aid in conserving the wild species.

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