CHAPTER 4

RESULTS AND DISCUSSION

The present chapter is mainly divided into two parts: Development of *in vitro* cultures and phytochemical analysis. The first objective was to establish cultures using different explants of *Leptadenia reticulata* and *Tylophora indica*. The second objective was to analyze the *in vitro* cultures qualitatively and quantitatively for the targeted metabolites. Last objective was to enhance the selected metabolites content in cultures by elicitation.

Leptadenia reticulata (Retz.) Wight and Arn

Establishment of callus and shoot cultures was done using leaf and nodal explants and the results are summarized below.

4.1 Development of *In vitro* Cultures

In vitro cultures when tried to develop using MS medium fortified with different PGRs, the observations revealed that establishing cultures was difficult due to heavy bacterial contamination. Therefore an antibacterial assay was performed at first to nullify the bacterial contamination by selecting suitable antibiotic.

4.1.1 Antibacterial Assay

Initially the explants were treated with $HgCl_2$ alone to avoid contaminants, but it failed to control the bacterial contamination hence a small study of antibacterial assay using different antibiotics was carried out. This primary screening helped in selection of a suitable antibiotic which could be further utilized along with other surface sterilizing agents. This assay was done using disc diffusion method where bacterial contaminant was grown on plates containing antibiotic octodiscs (Hi media) and the zone of inhibition was checked with respect to bacterial growth. The result showed that gentamycin (Fig. 7a), kanamycin and streptomycin (Fig. 7b) gave more zone of inhibition but they had one semitransparent ring around clear zone which made it unclear zone. Maximum clear zone of inhibition was observed in tetracycline (Fig. 7b) and rest of the antibiotics appeared with semitransparent/unclear zone of inhibition (Table 4). Hence initially both streptomycin and tetracycline were used for the treatment of explants. Though tetracycline showed maximum zone of inhibition, it was observed during *in vitro* studies that streptomycin could prevent contamination better as compared to tetracycline. Therefore streptomycine was utilized to treat the explants for *in vitro* culture establishment.

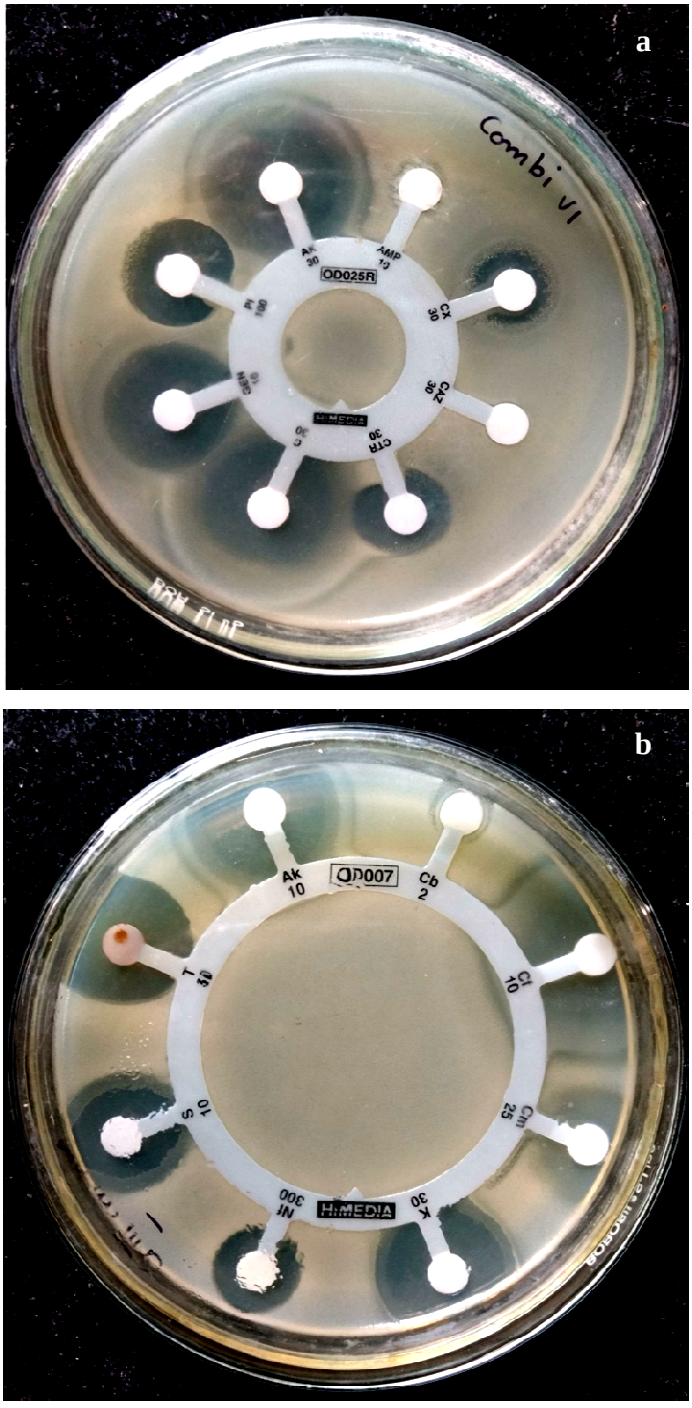

Figure 7. Antibiotic assay using disk diffusion method: (a) Gentamycine (GEN) showed clear zone of inhibition and (b) maximum clear zone of inhibition in streptomycin and tetracycline (T).

Table 4. Effect of different antibiotics on inhibition of bacterial growth.

Sr. No.	Antibiotics	Concentration (µg)	Type of zone	Zone of inhibition
1	Amikacin (AK)	30	Semitransparent ring around clear zone	++
2	Ampicillin (AMP)	10	-	-
3	Cefoxitin (CX)	30	Irregular	+
4	Ceftazidine (CAZ)	30	-	-
5	Ceftriaxone (CTR)	30	Irregular	+
6	Chloramphenicol (C)	30	Semitransparent ring around clear zone	+++
7	Gentamicin (GEN)	10	Semitransparent around clear zone	+++
8	Piperacilin (PI)	100	Clear	++
9	Carbenicillin (Cb)	100	-	-
10	Ciprofloxacin (cf)	10	Semitransperant ring around clear zone	++
11	Co-Trimazine (cm)	25	Unclear zone	+
12	Kanamycin (K)	30	Semitransperant ring around clear zone	+++
13	Nitrofurantoin (Nf)	300	Clear	+
14	Streptomycin (S)	10	Clear	++
15	Tetracyclin (T)	30	Clear	+++

+: less inhibition, ++: moderate inhibition, +++: highest inhibition

4.1.2 Leaf Explant

Entire leaves of *L. reticulata* were collected from plants growing in botanical garden and excised explants were placed in the MS medium supplemented with sucrose (3%) and different PGRs, the morphogenic response was evaluated at the end of six weeks. Medium without PGR served as control and result showed only swelling of explants which failed to survive after four weeks.

4.1.2.1 Effect of PGRs on Callus Growth and Morphology

MS medium was fortified with individual and combinations of cytokinins (5-20 µM) and auxins (0.1-1.0 µM) to assess their effect on leaf explants.

- **Individual cytokinins (BA and Kn)**

Presence of cytokinins in the medium is known to induce morphogenic response and hence media was fortified with different cytokinins i.e. BA/Kn (5-20 µM) individually. Out of all the concentrations tried, BA (15 µM) was the only concentration which was able to induce callus formation in 30% cultures (Table 5). Callus was induced from the cut margins of the explants by end of second week. It slowly proliferated till four weeks and then the explants along with callus, was transferred to fresh medium with the same concentration for further growth. Subculture enhanced the growth of callus which was off-white, friable and it covered the entire lamina of leaf till the six weeks (Fig. 8a). It started to turn brown in further weeks and completely became black by eight weeks. Similarly when effect of Kn was evaluated in the same concentration range (5-20 µM), the response was observed only in terms of swelling of explants in all the concentrations by third week (Fig. 8b) and then after they failed to survive.

- Individual cytokinins results depicted that BA was beneficial for callus differentiation as compared to Kn.

- **Individual auxins (IAA, NAA and 2,4-D)**

Auxins are reported to induce callus from leaf explant, hence the MS medium was fortified with IAA/NAA/2,4-D in concentration range of 0.1-1.0 µM. In IAA, lower concentration (0.1 µM) was able to induce callus in 50% cultures. Greenish-brown friable callus formation was observed from the periphery of explants after two weeks. This callus was slow growing and less proliferative in nature and even subculture could not help in enhancing the growth of callus till six weeks (Fig. 9a). Further increase in concentration (0.5-1.0 µM) resulted in only swelling of leaves.

When another auxin NAA was tried (0.1-1.0 µM), the callus was greenish-white, friable in comparison to IAA, and the response enhanced to 60% at 0.5 µM NAA. The callus induction was observed from cut end of leaf explants after two weeks. Callus growth increased till three weeks and a small cluster was formed within six weeks (Fig. 9b), but later

the growth stopped and it turned black. Further increase in NAA concentration to 1.0 µM decreased the callus growth as well as response.

When NAA was replaced with 2,4-D, the color and texture of callus changed to white and it became more friable in comparison to the earlier auxins (Fig. 9c). Moreover result also showed enlargement of leaves especially at higher concentration of 2,4-D. Maximum callus growth was observed at 1.0 µM and 70% cultures were able to survive till six weeks (Table 5).

- The effect of individual auxin proved to be beneficial for callus growth in leaf explants as compared to individual cytokinins. The morphogenic response in individual PGRs only accounted for formation of callus, which failed to enter in any organogenic pathway. Thus, further experimental work was carried out to evaluate the synergistic effect of cytokinins and cytokinins with auxins on organogenesis.

Table 5. Individual and synergistic effect of different PGRs on callus growth and morphology in leaf explants of *L. reticulata* (6 weeks).

PGRs	Callus growth and morphology	Proliferation	Response (%)
BA (15 µM)	Off-white, friable	++	30
Kn (5-20 µM)	No response	-	-
IAA (0.1 µM)	Greenish-brown, friable	+	50
NAA (0.5 µM)	Greenish-white, friable	++	60
2,4-D (1.0 µM)	White, friable	+	70
Kn (20 µM) + $AdSO_4$ (10 µM)	Brown, friable	+	30
BA (20 µM) + NAA (0.5 µM)	Greenish-white, friable	+++	70
BA (15 µM) + 2,4-D (1.0 µM)	Off-white, semi friable	+++	80
Kn (10 µM) + IAA (1.0 µM)	Greenish, off-white, friable	+	40
Kn (20 µM) + NAA (1.0 µM)	White, semi-friable	+	50
Kn (10 µM) + 2,4-D (1.0 µM)	White, semi-friable	+	50

+: less proliferation, ++: moderate proliferation, +++: highest proliferation

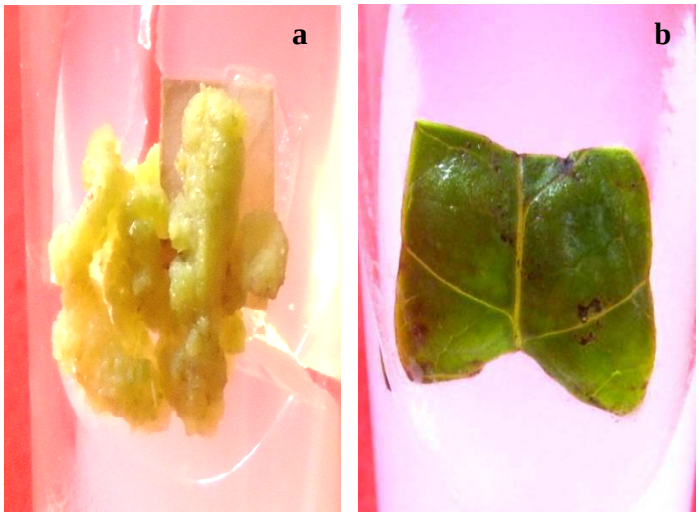

Figure 8. Effect of individual cytokinins on callus differentiation from leaf explants of *L. reticulata*: (a) Off-white, friable callus in the presence of BA (15 µM) after six weeks and (b) swelling of leaf explants in Kn (10 µM) within three weeks.

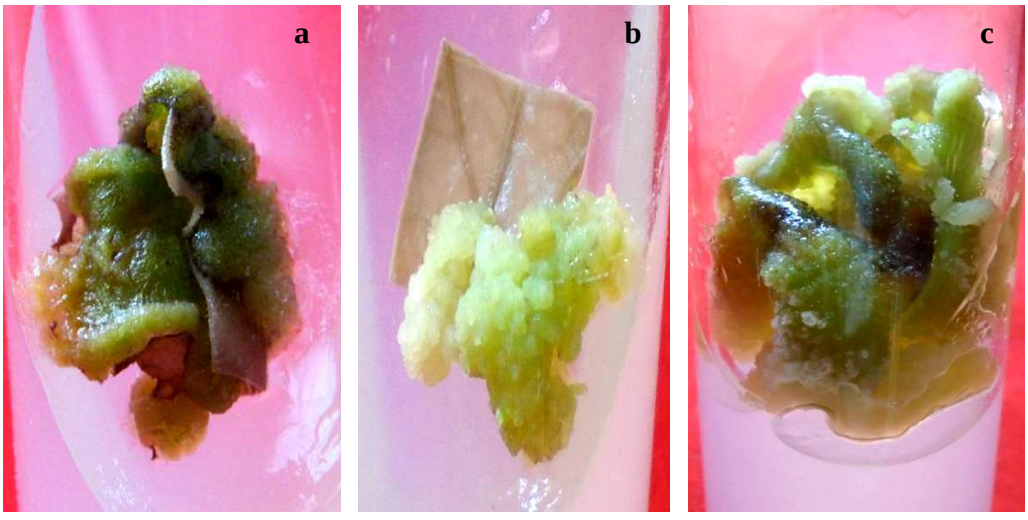

Figure 9. Effect of individual auxins on callus formation from leaf explants of *L. reticulata* at the end of six weeks: (a) Greenish-brown, friable callus on medium supplemented with IAA (0.1 µM), (b) greenish-white, friable callus differentiated in NAA (0.5 µM) and (c) white, friable callus in presence of 2,4-D (1.0 µM).

4.1.2.2 Synergistic Effect of Cytokinins and Auxins on Callus Growth and Morphology

When synergistic combinations of two cytokinins i.e. BA with Kn/$AdSO_4$, Kn with $AdSO_4$ and cytokinins with auxins i.e. BA/Kn with IAA/NAA/2,4-D was tried, observations revealed that only three combinations entered in the somatic embryogenesis pathway which is discussed afterwards and rest all formed only callus which is summarized below.

- **Kn and $AdSO_4$**

As individual Kn was unable to generate morphogenic response, it was combined with $AdSO_4$ (5-10 µM) and their effect was evaluated. Lower concentrations of Kn (5-10 µM) with all concentrations of $AdSO_4$ failed to form callus. Whereas at higher level of Kn (20 µM) with $AdSO_4$ (10 µM) differentiated brownish friable callus from the cut margin of leaf (Fig. 10a). The growth of callus was slow as well as the response obtained was very less (30%).

- **BA and NAA/2,4-D**

As from the above result it could be concluded that individual BA was able to induce a response for callus induction but individual auxins NAA and 2,4-D were better. Thus, a combination of BA with NAA/2,4-D was tried to understand their effect on leaf explants. BA and NAA was able to further enhance the growth of callus. Maximum callus proliferation was recorded at higher concentration of BA (20 µM) with NAA (0.5 µM) in 70% cultures (Table 5), but these cultures could not sustain after six weeks. Greenish-white, friable callus differentiated from the midrib of leaf explants on adaxial surface during second week. Growth enhanced in the third week and it spread towards abaxial surface of explant. After four weeks callus was transferred to the similar medium composition for further growth and it was observed that it became more friable and proliferative (Fig. 10b).

2,4-D is commonly utilized for differentiating callus and hence its synergistic effect with BA was evaluated. Lower level of BA (5 µM) and 2,4-D (0.1 µM) differentiated callus but the rate of proliferation was less which increased at higher concentration of BA (15-20 µM) with 2,4-D (1.0 µM). Maximum proliferation was recorded in medium supplemented with BA (15 µM) and 2,4-D (1.0 µM) within six weeks in 80% cultures. It resulted with induction of off-white, semi friable callus from midrib of explants by second week. Then it proliferated in further weeks and covered the entire lamina of leaf explants till six weeks (Fig. 10c). BA-2,4-D combination proved to be better for callus growth as compared to all the other PGRs combinations (Table 5).

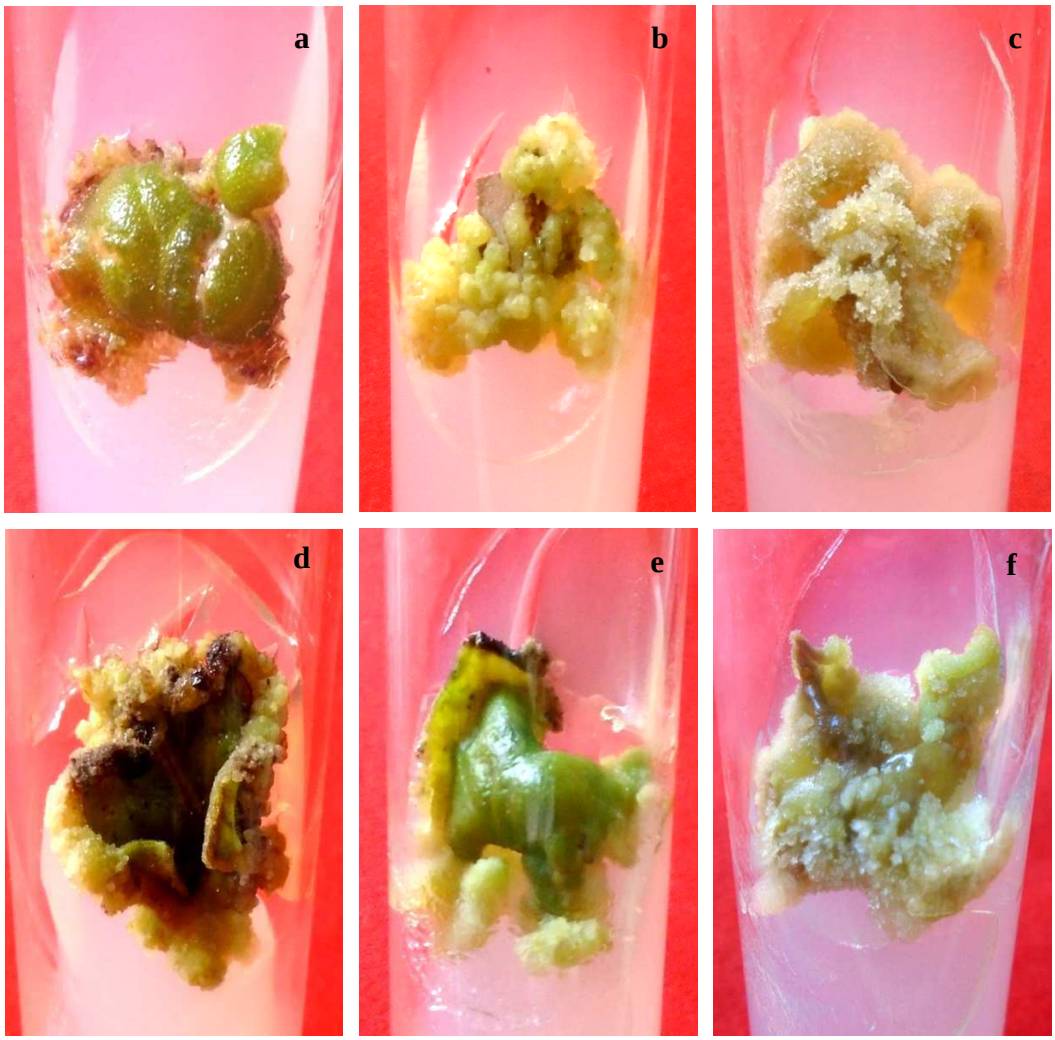

Figure 10. Synergistic effect of PGRs on callus growth and morphology from leaf explants of *L. reticulata* at the end of six weeks: (a) Brown, friable callus in Kn (20 µM) + $AdSO_4$ (10 µM), (b) greenish-white, proliferative friable callus in BA (20 µM) + NAA (0.5 µM), (c) off-white, semi friable callus in BA (15 µM) + 2,4-D (1.0 µM), (d) greenish, off-white, friable callus in Kn (10 µM) + IAA (1.0 µM), (e) white, semi-friable callus in Kn (20 µM) + NAA (1.0 µM) and (f) white, semi-friable callus in Kn (10 µM) + 2,4-D (1.0 µM).

- **Kn and IAA/NAA/2,4-D**

As it was earlier noted that presence of individual Kn failed to give any response, it was combined with individual auxins to induce a response in leaf explants. The synergism of Kn with IAA formed callus in 40% cultures at moderate level of Kn (10 µM) with IAA (1.0 µM) (Table 5). Callus induction was observed after third week and it was greenish, off-white and friable in nature. It ceased to grow after sixth week (Fig. 10d), and later it gradually turned brown and finally became black along with the explant.

Combining Kn and NAA in the media, improved callus growth and response slightly. It was observed that lower concentrations of Kn (5-10 µM) with NAA (0.1-1.0 µM) evoked less response and the highest response (50%) was recorded in Kn (20 µM) and NAA (1.0 µM) (Table 5). In this the callus differentiation was initiated in two weeks and it was white and semi-friable. Callus was induced from margins of leaf explants and grew towards the lower lamina of leaves by the end of six weeks (Fig. 10e).

When Kn and 2,4-D were combined, it failed to improve the response, however the callus growth and morphology remained similar i.e. white and semi-friable. Only few combinations differentiated callus with very less response and in presence of Kn (10 µM) + 2,4-D (1.0 µM) in 50% cultures formed callus. The callus induction was observed after two weeks which further proliferated slightly by four weeks, then it slowed down (Fig. 10f) and after six weeks turned brown.

- It was observed that all combinations of Kn with cytokinins/auxins induced poor growth and response for callus in comparison with BA combinations. Thus BA-NAA and BA - 2,4-D proved to be beneficial as they showed maximum callus growth within six weeks and were further utilized for phytochemical analysis.

Leaves of *L. reticulata* were excised and square piece of lamina containing midrib was inoculated as cut region would help for efficient absorption of nutrients and growth regulators (Sarwar and Skirvin, 1997). MS medium fortified with individual cytokinins induced varied response as BA induced callusing with meagre response whereas Kn failed to induce any morphogenic response. These results are in line with previous reports on leaf explants of *Gerbera jamesonii* (Kumar et al., 2004), *Metabriggsia ovalifolia* (Ma et al., 2011), *Viola canescens* (Khajuria et al., 2017) and *Scaevola sericea* (Liang et al., 2020). Whereas in *Azadirachta indica* (Akula et al., 2003), BA failed to induce any callus from leaf explant. In

L. reticulata, the callus formed in presence of few concentrations of BA and it was friable in nature and similar findings are reported for leaf explant of *Hemidesmus indicus* (Shanmugapriya and Shivakumar, 2011). Similarly, leaves of *Stevia rebaudiana* (Janarthanam et al., 2009), *Biophytum sensitivum* (Kala et al., 2014) and *Anisochilus carnosus* (Reshi et al., 2017) induced only callogenesis and no caulogenesis in medium containing BA. Generally $AdSO_4$ is used to enhance the organogenesis, but in the present study when it was combined with Kn, it only induced callus, and the same is also documented in *Lilium longiflorum* (Zăpârţan, et al., 1999-2000). Whereas Garro-Monge et al. (2008) reported callus formation in *Aloe barbadensis* when placed in BA+$AdSO_4$+2,4-D. The variation in morphogenic responses is due to the antagonistic effect of cytokinins which inhibits the synthesis of IAA oxidase isoenzyme and in turn stops the production of endogenous IAA which in turn adversely affects the morphogenic responses (Lee, 1971).

When MS medium was fortified with different concentrations of IAA, greenish-brown friable callus was developed within six weeks. Similar results are documented for *Tecomella undulata* (Patel and Patel, 2013) and *H. indicus* (Pathak and Joshi, 2017) where callus was formed with similar morphology in presence of IAA. Whereas in leaf explant of *Gmelina arborea*, both compact and friable callus was formed depending on the concentrations of IAA (Ujjwala et al., 2013). Individual IAA induced only callus from leaf explant of *Plumbago rosea* and *P. zeylanica* (Das and Rout, 2002). Changing IAA with NAA in the medium improved the response in terms of callus proliferation, and the texture also changed to greenish-white and friable. This type of change has been reported by Shanmugapriya and Shivakumar (2011) in *H. indicus* where the callus changed to dark green and compact in medium containing NAA. Individually NAA can induce callus from leaf explants as observed in *Justicia gendarussa* (Agastian et al., 2006), *Ophiorrhiza japonica* (Kai et al., 2008) and *P. zeylanica* (Lubaina and Murugan, 2012). The difference in callus morphology is due to the variation in physiological activity of the auxins within the plant tissue (Anjusha and Gangaprasad, 2017).

When MS medium was supplemented with different concentrations of 2,4-D, it induced white friable callus and the response was better as compared to IAA and NAA. Whereas in *H. indicus* (Purohit et al., 2015) and *Ocimum sanctum* (Mishra, 2015), compact callus was formed in medium having 2,4-D. But the superiority of this auxin for callus formation is also documented in *Tylophora indica* (Faisal and Anis, 2003), *Tinospora cordifolia* (Bhalerao et al., 2013), *Anthurium andreanum* (Thokchom and Maitra, 2017), *Oldenlandia umbellate*

(Saranya et al., 2019) and *Polyalthia bullata* (Kamarul Zaman et al., 2020). It was concluded that when cytokinins and Auxins were used individually, they failed to differentiate shoots from leaf explants, which is in line with reports on *Spilanthes acmella* (Singh and Chaturvedi, 2012).

As leaf explants of *L. reticulata* were unable to differentiate shoots in presence of individual cytokinins or auxins, thus their combinations were tried as the ratio of these PGRs is reported to influence organogenesis (Skoog and Miller, 1957; Bhojwani and Razdan, 1996). Earlier studies have stated that the main function of cytokinin is to stimulate the cell division and form adventitious bud. Whereas auxins are known to influence the initiation of cell division and organize meristems which differentiate organs along with promotion of vascular differentiation (Gaspar et al., 2003). However, when cytokinins and auxins were combined in the present study, most of the combinations induced only callus. This might be due to the fact that auxins play important role in callus induction and when combined with cytokinins induces a different morphogenic response (Yang et al., 2010) which is also reported in several studies (Baskaran et al., 2011; Aghaali et al., 2019).

The observations revealed that when BA and NAA were used individually it showed little response for callus formation, whereas the % response as well as callus proliferation increased when they were added together, which is in corroboration with results in *Poncirus trifoliata* (Tzatzani et al., 2018). In the present study greenish-white friable callus was observed in presence of BA-NAA, and this combination in the leaf explant of *Abelmoschus esculentus* formed callus which was white and green, compact and friable (Kabir et al., 2008). Whereas other report on *Celosia argentea* showed greenish-reddish-white compact and friable callus (Bakar et al., 2014). Many previous studies *Abutilon indicum* (Seth et al., 2017) and *Allophylus edulis* (Messias et al., 2019) have documented that the callus feature and colour changes in different concentrations of BA-NAA. The callus failed to regenerate shoots which might be due to the quiescent cells of the explants and with respect to PGRs involvement (Phillips, 2004). Similarly the callus formed from leaf explant of *Adhatoda vasica* (Mandal and Laxminarayana, 2014) and *Corallocarpus epigaeus* (Narayan, 2016) in presence of BA-NAA also failed to induce shoots.

The combination of BA-2,4-D induced more profuse callus as compared to individual BA and 2,4-D which is in line with report on *L. reticulata* (Patel et al., 2014), *Piper permucronatum* (Santos et al., 2016) and *Limonium sinense* (Dam et al., 2017). The synergistic effect of BA-2,4-D on profuse callus formation is in accordance with earlier

reports on *A. indicum* (Rout et al., 2009; Seth and Panigrahi, 2019), *S. acmella* (Nabi et al., 2018) and *Eriobotrya japonica* (Deng et al., 2020). Whereas Mehaboob et al. (2019) depicted that MS media fortified with BA (0.5 mg/l) + 2, 4-D (2.0 mg/l) + NAA (0.5 mg/l) formed maximum callus in *Zingiber officinale*. One of the reason is the presence of 2,4-D in the medium which is known to be efficient in stimulating the cell division in plant tissue (Gaspar et al. 1996). Previous study on *L. reticulata* (Martin, 2004; Bharat et al., 2011) stated that media is supplemented with NAA/2,4-D individually or in combination with BA it formed callus which is similar with present investigation. Whereas results documented by Hariharan et al. (2002) and Patel et al. (2014) reported that optimum somatic embryos and shoot differentiation respectively in presence of BA and NAA from leaves of *L. reticulata*. Many combinations induced callus from leaf explant of *L. reticulata* but maximum callus proliferation was observed in presence of BA-NAA and BA-2,4-D, but the later proved slight better as compared to former and similar results were reported in *Cerasus humilis* (Wang et al., 2016) and *Brucea mollis* (Das et al., 2018). The effectiveness of BA with NAA/2,4-D was observed to be better as compared to Kn which is also reported for *Hemidesmus indicus* (Pathak and Joshi, 2017) and *Gynura aurantiaca* (Cui et al., 2019).

Combination of Kn with IAA induced greenish, off-white and friable callus which was different from other combinations. Previously Pathak and Joshi (2017) reported callus with varying morphology i.e. greenish-white, compact and brown, friable-nodular callus in different combinations of PGRs. Whereas when Kn-NAA was used the callus again became white-semi friable. This might be due to the endogenous auxin concentrations (Lane, 1978). Similarly leaf explant of many species only induced callus when placed in Kn-IAA/NAA like *Stevia rebaudiana* (Sivaram and Mukundan, 2003), *Euphorbia nivulia* (Sunandakumari et al., 2005), *Populus ciliate* (Thakur and Srivastava, 2006), *Withania coagulans* (Jain et al., 2011) and *Ophiorrhiza pectinata* (Midhu et al., 2019). The combination of Kn-2,4-D also evoked callogenesis, but response was less as compared to combination of BA-2,4-D which is in accordance with results in *Limonium sinense* (Dam et al., 2017). Generally BA/Kn-2,4-D was responsible for the formation of embryogenic callus (Dam et al., 2017; Sangra et al., 2019), whereas in *L. reticulata* both these combinations failed to induce somatic embryos which is in accordance with the report on *Macrotyloma uniflorum* (Mohamed et al., 2004) and *O. pectinata* (Midhu et al., 2019). Generally individual auxins (Yang et al., 2009; Mousavi et al., 2012) and combinations of cytokinins and auxins (Dhar and Joshi, 2005; Lim et al., 2009) are considered to be responsible for callus induction. BA-NAA, BA-2,4-D, Kn-NAA and Kn-2,4-

D combinations induced only callus in leaf explant in various plants like *Solanum tuberosum* (Kumar et al., 2014), *Lantana camara* (Veraplakorn, 2016), *A. indicum* (Seth and Panigrahi, 2019) and *Papaver rhoeas* (Aghaali et al., 2019).

In the present study, it was noted that the callus which differentiated from leaf explants when excised and subcultured turned brown within a week. Even it was transferred to a fresh medium along with the explants resulted in browning and similar results are documented for the same plant (Bharat et al., 2011) and other plants like *Pinus sylvestris* (Anderson and Levinsh, 2002), *Bougainvillea* spp. (Anand et al., 2017) and *Isodon amethystoides* (Duan et al., 2019). Thus further proliferation of callus was arrested which is in line with other studies (Ling et al., 2007; Chugh et al., 2009; Mondal et al., 2014). The first reason is programmed cell death caused by environmental stress, the other possible reason is that there is formation of quinones from phenolic compounds in the plant cell (Gao, 1999). Another reason may be oxidation adversely affecting the regenerative capacity of the plant tissue (Quisen et al., 2009).

4.1.2.3 Synergistic Effect of PGRs on Somatic Embryogenesis

It was interesting to note that combination of cytokinins or cytokinin (5-20 µM) and auxin (0.1-1.0 µM) induced a varied morphogenic response. The type of PGRs played a vital role in either differentiating callus or callus which formed somatic embryos. In the combinations which were responsible for somatic embryogenesis, only cotyledonary stage of SEs were taken into consideration for counting after six weeks and detailed results are described below.

- **BA and Kn**

A combination of both cytokinins i.e. BA and Kn was tried in concentration range of 5-20 µM. Both the cytokinins at lower concentration (5 µM) induced less SEs (3.70 ± 0.95), and this number increased to 15.30 ± 2.15 in 90% cultures when BA was kept constant at 5 µM and Kn level was increased to 10 µM. Further raising the Kn concentration to 15 and 20 µM, it reduced the number of SEs. Addition of 10 µM BA with Kn (5-20 µM) gradually decreased the no. of SEs with increased levels of Kn. The frequency of differentiating SEs enhanced when BA level reached to 15 µM with Kn (5 µM) i.e. 20.90 ± 2.86 SEs in 90% cultures (Table 6). This was one such combination which simultaneously differentiated indirect as well as direct embryos from the leaf explants. In indirect differentiation of SEs, initially off-white,

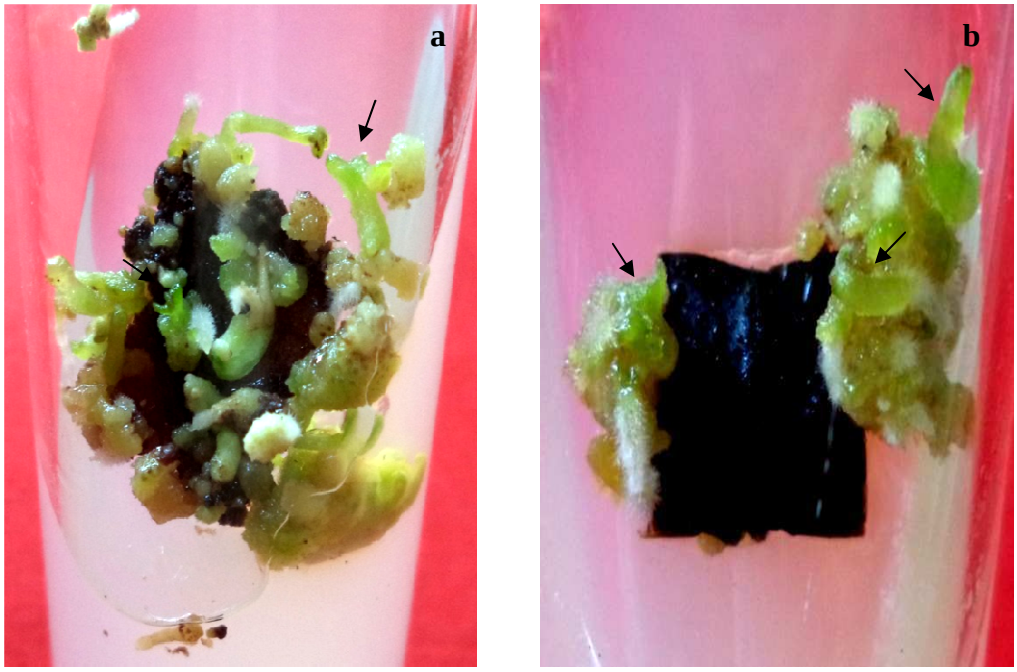

Figure 11. Induction of somatic embryos (SEs) from leaf explants of *L. reticulata* in presence of BA (15 µM) + Kn (5 µM) at the end of six weeks: (a) Direct SEs with heart and torpedo stages and (b) indirect differentiation of embryos with globular and elongated globular stages.

friable callus was formed by the end of first week. This callus was friable and it proliferated fast in next week, and simultaneously started to differentiate somatic embryos by third week. These embryos passed through globular, heart and torpedo stages till six weeks, but by end of eight weeks the callus started to turn brown and hindered the further development of SEs. The response for direct formation of SEs was very less (30%), however they were able to develope into heart and torpedo stages within six weeks (Fig. 11a). An asynchronous development was observed in both direct and indirect pathways, therefore all SEs were not able to reach torpedo/cotyledonary stages and few remained in globular or elongated globular stage (Fig. 11b). At higher concentration of both PGRs (20 µM) rapidly decreased the frequency of SEs to 2.70 ± 0.81 and the percent response also dropped.

- **BA and $AdSO_4$**

Kn was replaced by $AdSO_4$ in the medium and the effect of its combination with BA was evaluated for leaf explants, it depicted variation in terms of number and response of SEs. The differentiation of SEs was less (3.00 ± 0.69) at lower concentration (5 µM) of BA and $AdSO_4$, the number gradually increased to 10.00 ± 1.61 as the $AdSO_4$ level was enhanced to 20 µM. Increasing BA to 10 µM slightly improved the number of SEs in presence of $AdSO_4$ (5 and 10 µM), but gradual decrease was observed at 15-20 µM. When BA reached to 15 µM, it induced few SEs at lower levels of $AdSO_4$ (5 and 10 µM). However suddenly the frequency of SEs increased at higher concentration of $AdSO_4$ (15 µM) and a maximum 21.70 ± 2.06 SEs were obtained in 100% cultures. In this combination, callus differentiation was initiated from lower end of the cut margin of leaf explants within a week and proliferated by second week. Between three to four weeks, globular embryos were differentiated from which few elongated by four weeks (Fig. 12a). The callus of four weeks was harvested and observed, from which early stages of SEs i.e. globular and elongated globular could be clearly identified (Fig. 12b). The transfer of explants along with callus to fresh medium with similar composition helped in converting embryos to heart and torpedo stage, but few were restricted in elongated globular stage at the end of six weeks (Fig. 12c, d). Different stages of SEs could be identified after six weeks as the growth was asynchronous (Fig. 12e). After that germination ceased and callus along with SEs turned brown to black within eight weeks. Further increasing concentration of both BA and $AdSO_4$ to 20 µM, slightly decreased the number of SEs (19.30 ± 2.09) in 100% cultures (Table 6). Additionally, in this combination callus proliferation remained high

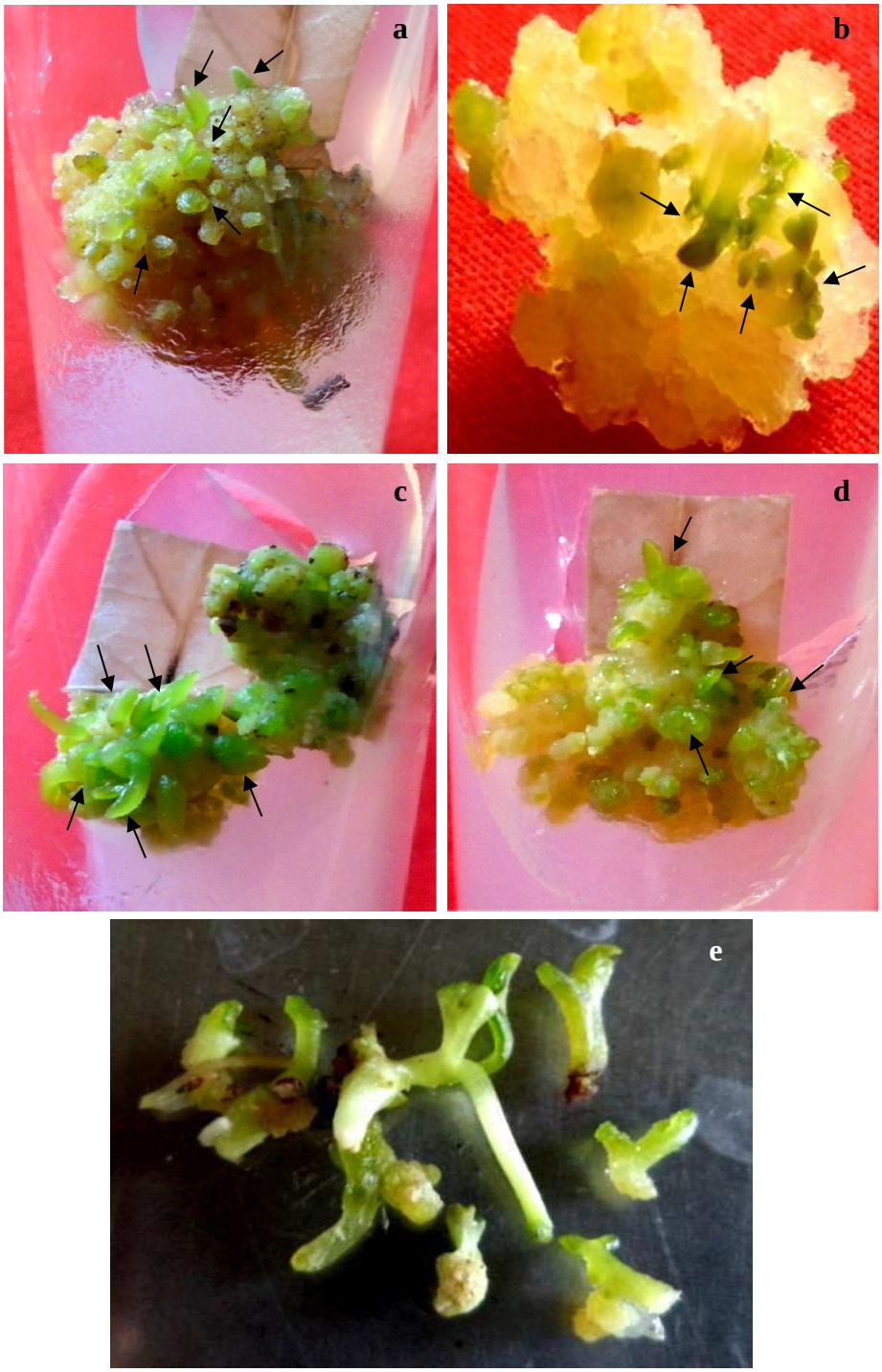

Figure 12. Differentiation of somatic embryos (SEs) in an optimized medium with BA (15 µM) + $AdSO_4$ (15 µM) from leaf explants of *L. reticulata*: (a, b) Induction of globular embryos by four weeks, (c, d) heart and torpedo stage embryos within six weeks and (e) different stages of embryos separated from callus after six weeks.

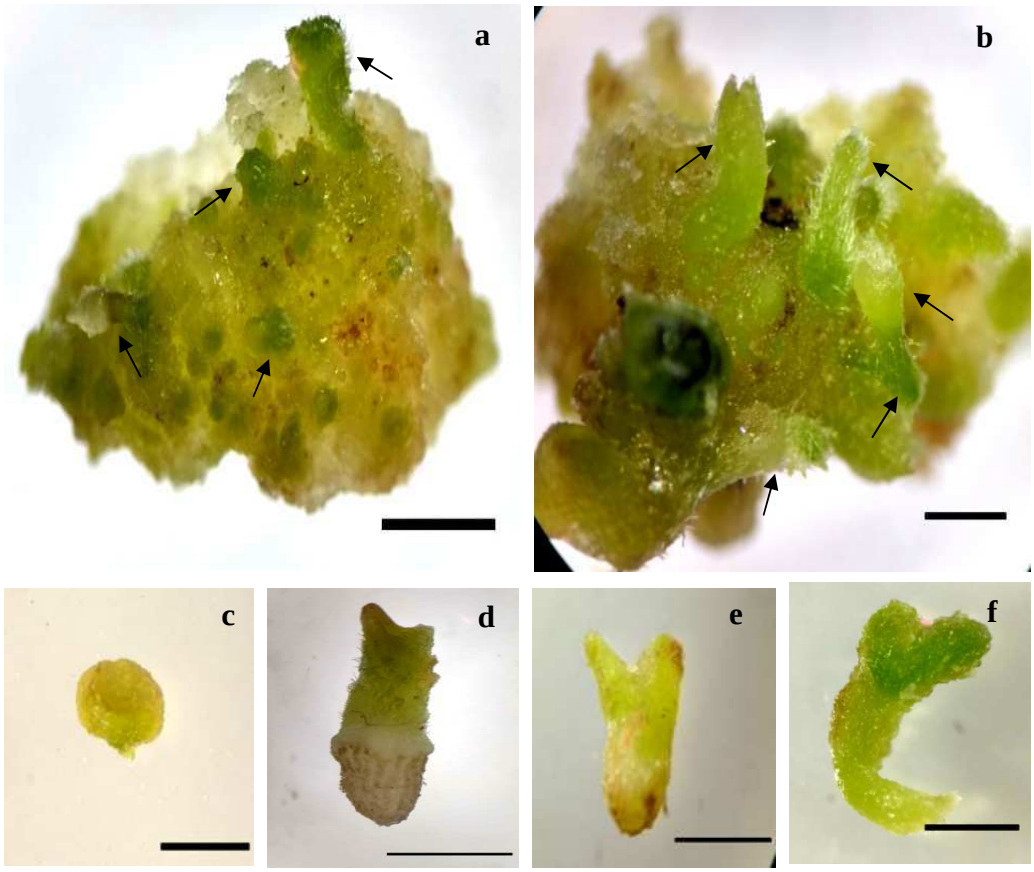

Figure 13. Microscopic observation of somatic embryos (SEs) differentiated on optimized medium BA (15 µM) + $AdSO_4$ (15 µM): (a) Globular and elongated globular embryos at four weeks, (b) different stages of embryos by end of six weeks and (c-f) individual stages of SEs - (c) globular, (d) early heart stage, (e) heart and (f) torpedo. (Bar = 0.5mm)

compared to BA-Kn, but the number of SEs obtained were more or less similar with above combination.

Embryogenic calli which formed in above combination was further observed under microscope. This was done for identifying the different stages of embryos. Four weeks embryogenic calli showed globular embryos on the surface of callus (Fig. 13a) which converted into different stages (Fig. 13b). Embryos with distinct stages like globular, early heart, heart and torpedo were easily separated and observed (Fig. 13c-f).

Table 6. Effect of different PGRs on induction of somatic embryos from leaf explants of *L. reticulate* (6 weeks).

PGRs (µM)				No. of somatic embryos/explant*	Response (%)
BA	Kn	$AdSO_4$	IAA		
0	0	0	0	0 i	0
5	5	0	0	3.70 ± 0.95 gh	80
5	10	0	0	15.30 ± 2.15 c	90
5	15	0	0	11.10 ± 1.55 cd	90
5	20	0	0	8.70 ± 1.36 de	90
10	5	0	0	6.40 ± 0.92 ef	90
10	10	0	0	7.50 ± 1.50 ef	90
10	15	0	0	9.60 ± 1.50 de	90
10	20	0	0	10.60 ± 1.48 cd	90
15	5	0	0	20.90 ± 2.86 a	90
15	10	0	0	14.00 ± 1.99 c	90
15	15	0	0	9.70 ± 1.73 de	90
15	20	0	0	9.20 ± 1.43 de	90
20	5	0	0	8.90 ± 1.25 de	90
20	10	0	0	7.60 ± 0.95 ef	90
20	15	0	0	4.80 ± 0.97 fg	80
20	20	0	0	2.70 ± 0.81 gh	70
5	0	5	0	3.00 ± 0.69 gh	90
5	0	10	0	3.80 ± 0.75 gh	90
5	0	15	0	8.10 ± 1.39 ef	80

5	0	20	0	10.00 ± 1.61 cde	90
10	0	5	0	12.10 ± 1.66 c	90
10	0	10	0	14.00 ± 2.04 c	90
10	0	15	0	10.40 ± 1.82 cd	80
10	0	20	0	7.50 ± 1.73 ef	80
15	0	5	0	3.30 ± 1.09 gh	70
15	0	10	0	4.50 ± 1.17 fg	80
15	0	15	0	21.70 ± 2.06 a	100
15	0	20	0	14.30 ± 1.39 c	100
20	0	5	0	9.70 ± 1.64 de	90
20	0	10	0	11.50 ± 1.01 cd	100
20	0	15	0	18.60 ± 1.39 ab	100
20	0	20	0	19.30 ± 2.09 ab	100
5	0	0	0.1	3.80 ± 0.52 gh	50
5	0	0	0.5	3.83 ± 0.55 gh	60
5	0	0	1.0	6.86 ± 0.94 ef	70
10	0	0	0.1	9.00 ± 1.21 de	70
10	0	0	0.5	12.25 ± 1.20 c	80
10	0	0	1.0	7.71 ± 1.65 ef	70
15	0	0	0.1	5.43 ± 1.21 fg	70
15	0	0	0.5	4.20 ± 0.72 fg	50
15	0	0	1.0	2.75 ± 0.41 gh	40
20	0	0	0.1	2.33 ± 0.27 gh	30
20	0	0	0.5	1.50 ± 0.35 gh	20
20	0	0	1.0	0 i	0

*Values represent mean ± SE. Means (n = 10) followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test.

- **BA and IAA**

Auxins when combined with cytokinins are known to induce embryogenic callus from leaf explant. Thus, BA was combined with IAA in concentration ranges of 0.1-1.0 µM and their results were evaluated. Low level of both BA (5 µM) and IAA (0.1 µM) resulted with less embryos (3.80 ± 0.52) in 50% cultures. Slight increase in the concentration of both PGRs facilitated formation of high number of embryos as BA (10 µM) with IAA (0.5 µM)

differentiated 12.25 ± 1.20 embryos with 80% response. The number of SEs were comparatively less than the optimum number obtained in above combination. BA-IAA formed greenish-white, friable callus within three weeks which proliferated in further weeks and became embryogenic. Induction of globular shaped SEs was observed within four weeks (Fig. 14a), after that the callus was transferred on the fresh medium having same PGRs concentrations. Subculture assisted significant proliferation of callus compared to BA-Kn as well as in germination of SEs, as they converted into heart and torpedo stages by end of six weeks (Fig. 14b). The number of embryos and the response declined when both PGRs level reached to 20 µM (Table 6).

- It was noted from somatic embryogenesis study that a combination of cytokinins proved to be better as compared to cytokinin with auxin combination.

Indirect somatic embryogenesis is a multi-step process in which the somatic cells dedifferentiates and form callus, in which embryogenic clumps originate and develop into SEs (Williams and Maheswaran, 1986; Von Arnold et al., 2002). Cytokinins are known to play important role in this transition as well as in stimulating divisions in pro-embryogenic cells (Feher et al., 2003). Although there are reports on the use of individual cytokinins for initiation of somatic embryogenesis in various species (Sagare et al., 2000; Cheruvathur et al., 2013b; Li et al., 2014), generally combinations of cytokinins and cytokinins with auxins are reported to have inductive effect on SEs.

In the present study, it was observed that type and concentration of PGRs significantly affected somatic embryogenesis and among all the combinations tried, BA-Kn, BA-AdSO_4 and BA-IAA induced SEs from leaf explant. This justifies the key role of BA in inducing SEs (Chang, 1991) and these observations are comparable with previous report on same plant (Martin, 2004). Similarly, the profound effect of BA on induction of somatic embryos from the leaf explant has been well documented in many other studies (Girija et al., 2000; Chitra Devi and Narmathabai, 2011; Sane et al., 2012; Salma et al., 2019; Sedaghati et al., 2019; Liang et al., 2020). Recently, Yumbla-Orbes et al. (2020) documented that SEs of *Eustoma grandiflorum* matured better when placed in media containing BA, and it was also observed from previous report of cassava that cytokinins are effective for germination of SEs (Taylor et al., 2001; Mongomake et al., 2015).

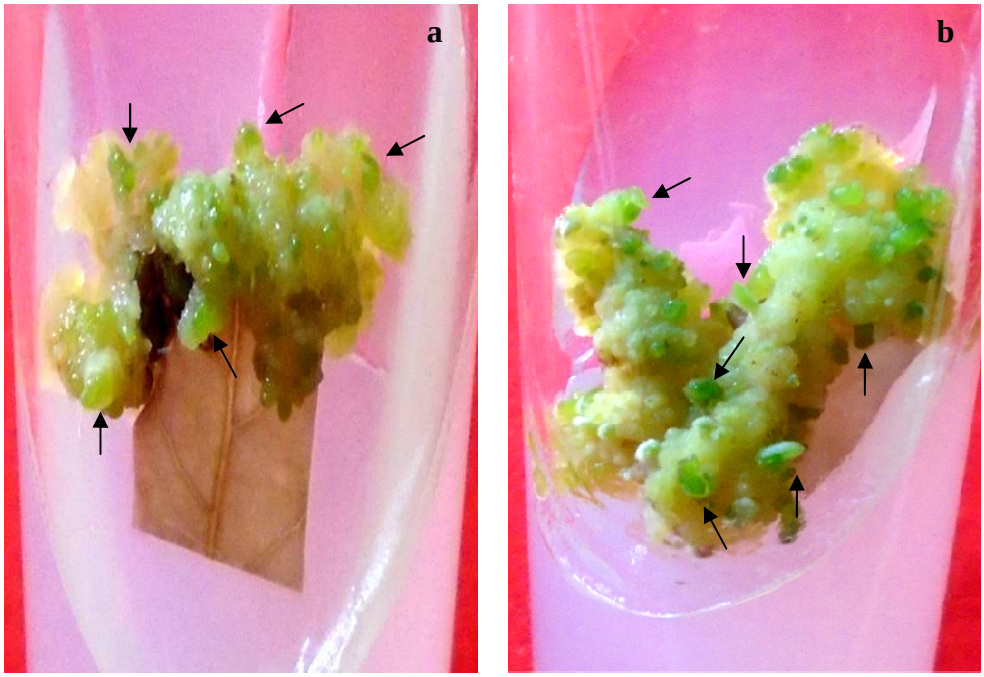

Figure 14. Differentiation of somatic embryos (SEs) in the presence of BA (10 µM) + IAA (0.5 µM) from leaf explants of *L. reticulata*: (a) Induction of globular embryos from callus within four weeks and (b) different stages at the end of six weeks.

In the present investigation, BA alone induced callusing but when combined with Kn or $AdSO_4$, it differentiated SEs. A combination of BA and Kn is rarely reported for somatic embryogenesis e.g. in *Solanum trilobatum* (Dhavala et al., 2009) and *Moringa oleifera* (Devendra et al., 2012). On the other hand some researchers have suggested the use of other PGRs along with BA and Kn for e.g. Li et al. (2008) obtained optimum SEs when leaf explant of *Picea koraiensis* were placed on media containing BA (1.1 μM) + Kn (1.16 μM) + NAA (8.06 μM). Whereas SE formation in *Jatropha curcas* has been documented in presence of multiple PGRs like BA+Kn+TDZ+IAA (Mweu et al., 2016).

Another cytokinin $AdSO_4$ has stimulatory effect on somatic embryogenesis when combined with other PGRs especially cytokinins. In the present study optimum SEs were obtained in BA-$AdSO_4$ which is in accordance with the report in *Phaseolus vulgaris* (Cabrera-Ponce et al., 2015). Whereas combination of BA (13.3 μM), $AdSO_4$ (271.5 μM) and NAA (2.7 μM) has been reported for somatic embryo formation in *Anacardium occidentale* (Martin, 2003). Taha et al. (2007) reported that SE formation in *Phoenix dactylifera* was optimum in presence of BAP+2iP+$AdSO_4$. Similarly in *Acacia senegal* leaf explants, $AdSO_4$ has beneficiary effect on SE induction as well as germination when added together with other PGRs (Rathore et al., 2012). Whereas Jha et al. (2007) reported that SEs of *J.curcas* was developed in presence of $AdSO_4$ alone. Additive effect of $AdSO_4$ when combined with other cytokinins is also documented in many plants viz. *Acacia farnesiana* and *A. schaffneri* (Ortiz et al., 2000), *Coffea arabica* and *C. canephora* (Samson et al., 2006), *Carthamus tinctorius* (Walia et al., 2007a), *Bixa orellana* (Parimalan et al., 2011), *A. senegal* (Rathore et al., 2012), *C. tinctorius* (Walia et al., 2007b) and *Carica papaya* (Al-Shara et al., 2020). It is due to the fact that $AdSO_4$ retards the degradation of cytokinins in the medium by feed-back inhibition or by competing for the enzyme systems involved in cytokinin metabolism which in turn facilitates somatic embryogenesis (Van Staden et al., 2008).

Auxins has very important role in somatic embryogenesis process because they provide the signal for both cell polarization and the asymmetric division for somatic cells (Jimenez, 2005; Pagnussat et al., 2009). Especially when auxins are combined with cytokinins they regulate cell division and differentiation and, therefore, are beneficial in inducing SEs (Pinto et al., 2011). It was noted from the experiment that the combination of BA and IAA also induced SEs in *L. reticulata* which is also reported in *J. curcas* (Sardana et al., 2000), *Hybanthus enneaspermus* (Shekhawat and Manokari, 2016) and *Vitis vinifera* (Das et al., 2002; Alavijeh et al., 2016). Midhu et al. (2019) reported that embryogenic callus in

Ophiorrhiza pectinata developed in presence of IAA (3 mg/l) which further transferred to medium having combination of BA (0.2 mg/l) and IAA (0.1 mg/l) for induction of SEs. The observations revealed that when BA was combined with IAA it induced embryogenic callus but combination of BA+NAA when used it formed non-embryogenic callus, this is in corroboration with report on *Desmodium motorium* (Chitra Devi and Narmathabai, 2011). Similarly beneficial effect of IAA and inhibitory effect of NAA on embryogenic callus formation was documented for *Eucalyptus grandis* (Nakhooda and Mandiri, 2016). In the present study, SEs differentiated and germinated in similar media and PGR combination, whereas in earlier study for the same plant SEs were formed in presence of BA (2 mg/l) and NAA (0.5 mg/l), and were further transferred to Kn (1 mg/l) for germination (Hariharan et al., 2002). Similarly, combination of BAP (4.40 µM) and NAA (2.68 µM) was used for optimum differentiation of SEs by Sathyanarayana et al. (2008). In other studies on *L. reticulata*, SEs were induced in presence of BA (8.87 µM) and IBA (2.46 µM) (Martin, 2004). Whereas in the study of Rathore et al. (2013), the differentiated SEs failed to develop into plantlets. Furthermore the number of embryos developed in the present study is higher as compared to earlier reports. It was also noted that auxin 2,4-D is commonly used for induction of SEs (Horstman et al., 2017). But in the present study 2,4-D had inhibitory effect on individually or in combination with cytokinins for differentiation of SEs, and similar observations were made by Martin (2004) for the same plant.

4.1.2.4 Development of Somatic Embryos

Mature SEs which were in torpedo or cotyledonary stages (Fig. 15a) were transferred to static and liquid media fortified with different PGRs i.e. BA (15 µM) + $AdSO_4$ (15 µM), GA_3 (0.5-2.0 µM) and CW (4%) for their germination into plantlets. When BA (15 µM) + $AdSO_4$ (15 µM) was tried, SEs germinated into healthy plantlets in static medium but the response was less, whereas in liquid medium embryos turned brown along with medium within two weeks. Presence of GA_3 in static medium proved to be beneficial for the development of SEs, and 1 µM was suitable in converting SEs into healthy plantlets within two weeks (Fig. 15b) as compared to the other two concentrations. In liquid medium embryos failed to survive and turned black within a week. Whereas the CW failed to improve response for development of plantlets because conversion frequency was very less in static medium and embryos failed to survive in liquid medium.

Figure 15. Development of SEs in GA_3 (1.0 µM) derived through leaf explants of *L. reticulata*: (a) Torpedo stage, (b) germination of an embryo into a plantlet after two weeks, (c) differentiation of secondary SEs (SSEs) and embryogenic callus from hypocotyl region by four weeks, (d) further induction of direct SSEs at six weeks and (e) development of SSEs into plantlets, differentiation of indirect embryos from callus at the end of eight weeks.

An interesting result for differentiation of direct secondary SEs (SSEs) occurred in GA_3 medium from the hypocotyls of plantlets by fourth week and simultaneously embryogenic callus was also differentiated (Fig. 15c). Subculture was done on the medium with same PGR concentration which induced repeatitive SSEs within six weeks (Fig. 15d). SSEs elongated and converted into plantlets and simultaneously callus differentiated SSEs which were in globular stage at the end of eight weeks (Fig. 15e).

- All combinations showed development of SEs but only in static medium as liquid medium turned black and therefore static medium was beneficial for the development of SEs in *L. reticulata.* Developing medium containing GA_3 proved to be advantageous as repetitive somatic embryos were formed in the same medium which could help in obtaining large number of plantlets from single explant.

The conversion of mature SEs into plantlets is an essential phase, as mature SEs are known to accumulate sufficient storage proteins and develop into normal plants (Rai et al., 2008). Many studies suggested that this stage requires different PGRs as compared to induction medium (Pasternak et al., 2002; Mohamed et al., 2004). In this study, when developmental media was fortified with GA_3, optimum growth of SEs was observed which is in accordance with earlier study on the same plant in which presence of GA_3 along with BA facilitated maximum conversion of SEs into plantlets (Martin, 2004). It has been generally reported that for maturation of SEs, removal of cytokinin and auxin from medium proved to be beneficial (Hazubska-Przybyl et al., 2020), and the same was observed in the present study. The beneficial effect of GA_3 for conversion of SEs into plantlets has been documented previously in *Gossypium hirsutum* (Ganesan and Jayabalan, 2004), *Carthamus tinctorius* (Kumar et al., 2008), *Jatropha curcas* (Cai et al., 2011), *Hemidesmus indicus* (Cheruvathur et al., 2013b), *Curcuma amada* (Raju et al., 2014), *Abutilon indicum* (Seth et al., 2017), *Haworthia retusa* (Kim et al., 2019) and *Valeriana officinalis* (Abdi et al., 2019). Whereas Daniel et al. (2018) reported that in *Abelmoschus esculentus* differentiated SEs were developed in presence of BAP and GA_3 along with glutamine and casein hydrolysate. The efficiency of GA_3 could be due to gene activation for the development of SEs (Sharmin et al., 2014).

The observations from the present results has also revealed that during development of SEs, both direct as well as indirect secondary somatic embryos differentiated from

hypocotyles of the plantlets in the same medium. This could be due to the fact that embryogenic cells can act independently from neighbouring cells and they either undergo further stages of germination or they can continue to differentiate into secondary embryos (Raemakers et al., 1995). Similarly secondary somatic embryogenesis is also documented in *Anacardium occidentale* (Martin, 2003), *Piper nigrum* (Ramakrishnan Nair and Dutta Gupta, 2006), *Carthamus tinctorius* (Kumar et al., 2008), *Desmodium motorium* (Chitra Devi and Narmathabai, 2011), *Camellia assamica* (Bajpai and Chaturvedi, 2021) and other species (Karami et al., 2008; Bao et al., 2012; Cheruvathur et al., 2013). If the right protocol is developed then the process of generating direct and indirect secondary somatic embryos is easy and quick method and can be kept for extended period of time (Arrillaga et al., 1994; Pavlovic et al., 2012). It also shows high multiplication rate and increased level of genetic uniformity (Bao et al., 2012).

4.1.3 Nodal Explants

To develop shoot cultures, nodal explants were utilized and they were placed in MS medium fortified with different cytokinins (5-20 µM) and their effect was monitored till eight weeks.

4.1.3.1 Effect of Individual Cytokinins on Shoot Regeneration

MS medium with 3% sucrose without PGRs served as a control which failed to form shoots. Cytokinins are known to stimulate the axillary quiescent meristem to develop into shoot and hence they are commonly used for the same. Thus, to achieve better response, basal medium was fortified with different cytokinins individually for multiple shoot regeneration.

- **Individual cytokinins (BA and Kn)**

Nodes of *L. reticulata* were placed in media fortified with individual cytokinins like BA and Kn in concentration range from 5-20 µM. BA at lower concentration (5 µM) formed shoots (2.20 ± 0.28) and their length of 2.95 ± 0.25 cm was recorded in 100% cultures by end of eight weeks (Table 7). Gradually when BA level was increased to 20 µM, shoot regeneration frequency remained same. In presence of BA, axillary bud break was observed within a week and immediately the axillary bud of the opposite node proliferated by second week. Both the buds elongated into shoots in further weeks and after four weeks, they were subcultured on the fresh medium. This induced more shoot buds to emerge from both sides of

the node in the sixth week and simultaneously the earlier shoots elongated by eight weeks (Fig. 16a).

Replacing BA with another cytokinin (Kn) formed more than one shoot in all the concentrations tried. Eight weeks result reveled that from lower to higher concentration (5-20 µM), slight increase in number of shoots as well as response was recorded but it was less in comparison to BA. Whereas shoot length continually increased till 20 µM and it was more in Kn than the former cytokinin. Only 2.33 ± 0.19 shoots with a length of 3.43 ± 0.36 cm were recorded for Kn at 20 µM in 60% cultures (Table 7). Kn containing medium assisted better growth of *L. reticulata* shoots as they grew tall and healthy with broadened leaf (Fig. 16b).

- It can be concluded from the study for individual cytokinins that BA gave better response in terms of number of shoots and percent response.

4.1.3.2 Synergistic Effect of Cytokinins on Shoot Regeneration

Individual cytokinins failed to form multiple shoots, therefore synergistic combinations of cytokinins in same concentration range was used (5-20 µM).

- **BA and Kn**

It is documented that synergistic combination of cytokinins in the medium promotes the growth of axillary buds and multiple shoot formation from nodes. BA and Kn individually formed slightly more than two shoots therefore their combined effect was evaluated. Initially at lower concentration (5 µM) of both PGRs, it only formed 2.17 ± 0.15 shoots and length was also less (2.41 ± 0.46 cm). Further increasing the level failed to enhance the number as well as response. A total 2.40 ± 0.60 shoots were recorded in 50% cultures when media was supplemented with BA (10 µM) and Kn (10 µM) among all the concentrations (Table 7). The axillary buds proliferated within a week from both nodes which formed small shoots at the end of two weeks. Within four weeks another bud emerged from one side and then nodal explant was transferred to fresh medium, which helped in branching of shoots within six weeks. Shoot elongation was monitored and data was recorded till eight weeks (Fig. 17a). The synergistic combination of BA with Kn remained similar in terms of shoot number to that of individual cytokinins.

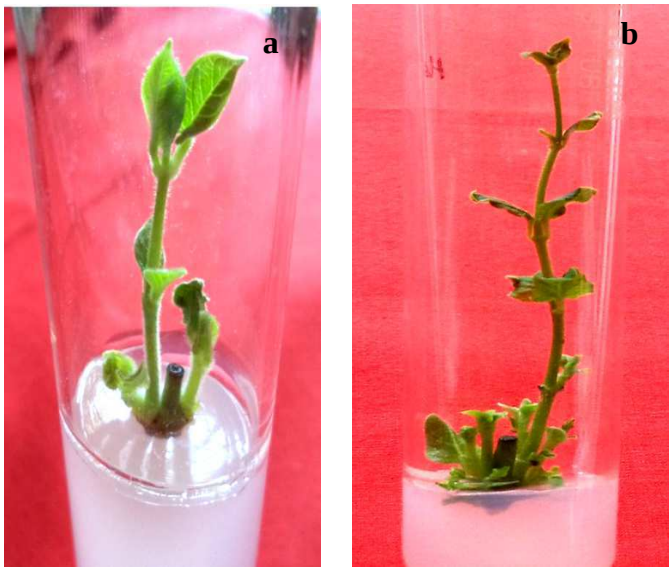

Figure 16. Effect of individual cytokinins on shoot regeneration from nodal explant of *L. reticulata* at the end of eight weeks: (a) Shoot formation in presence of BA (5 µM) and (b) elongated shoot in medium containing Kn (20 µM).

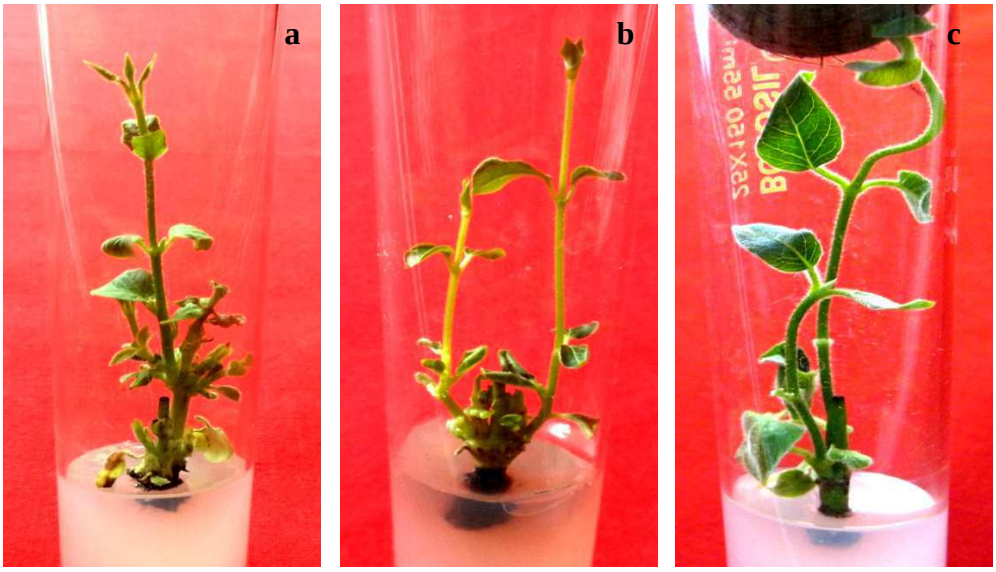

Figure 17. Effect of synergistic cytokinins on shoot regeneration from nodal explant of *L. reticulata* at the end of eight weeks: (a) Shoot formation in combination of BA (10 µM) + Kn (10 µM), (b) development of shoot in the presence of Kn (10 µM) + $AdSO_4$ (10 µM) and (c) maximum shoot length in medium supplemented with Kn (10 µM) + $AdSO_4$ (5 µM).

- **BA and $AdSO_4$**

$AdSO_4$ is a cytokinin which is known to facilitate multiple shoot regeneration when added with other cytokinins. Thus, it was added together either with BA or Kn and the response it induced in these combinations were evaluated. When BA was kept constant to 5 µM and fortified with different levels $AdSO_4$ (5-20 µM), the shoot number could only reach to 3.90 ± 0.22 shoots with 2.49 ± 0.33 cm length. The optimum number of shoots i.e. 5.10 ± 0.33 was achieved in 100% cultures having 3.74 ± 0.60 cm length in presence of 10 µM of BA and $AdSO_4$. In this combination axillary bud emerged as shoot by second week from both nodes (Fig. 18a), and this elongated in further weeks. New buds were formed from both sides within four weeks (Fig. 18b). Then the nodes were transferred to fresh medium which helped in multiplication and elongation of shoots within six weeks (Fig. 18c). At the end of eight weeks, healthy shoots with significantly increased length and formed a number of branches (Fig. 18d). Further raising BA concentration to 20 µM, failed to induce multiples and shoots became stunted with hyperhydricity, moreover response for shoot regeneration also gradually declined (Table 7).

- **Kn and $AdSO_4$**

BA was replaced with Kn and combined with $AdSO_4$, showed a profound effect on length of shoots. Lower concentration of Kn (5 µM) with $AdSO_4$ (5-20 µM) formed few shoots as 20 µM induced 2.20 ± 0.28 shoots with 2.99 ± 0.38 cm length (Fig. 17b). Elevating the concentration of Kn to 10 µM with 5 µM $AdSO_4$ resulted in elongating shoots to a maximum length of 4.12 ± 0.68 cm, but it formed very less number of shoots (Fig. 17c). Increasing the concentration of $AdSO_4$ to 10 µM enhanced number of shoots to 3.10 ± 0.33 in 100% cultures. Higher concentrations (15-20 µM) of Kn with all $AdSO_4$ levels decreased the shoot number, its length and response (Table 7).

- From the above experiment it was concluded that the combination of BA with $AdSO_4$ was beneficial for multiple shoot regeneration from nodal explants. Additionally Kn-$AdSO_4$ showed significant result with respect to improved shoot length. However it was also noted that axillary shoot regeneration was strongly influenced by different seasons.

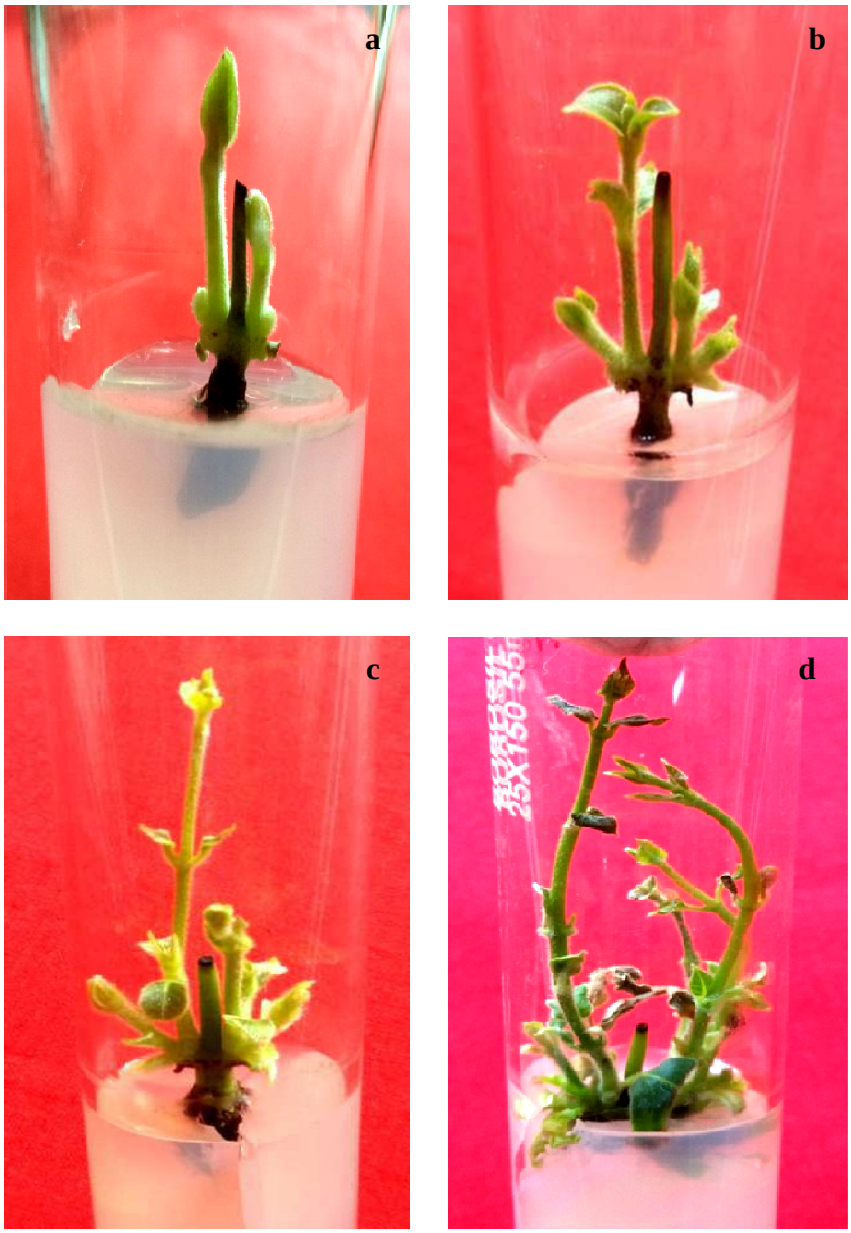

Figure 18. Axillary shoot regeneration in optimized medium fortified with BA (10 µM) + $AdSO_4$ (10 µM) from nodal explant of *L. reticulata*: (a) Emergence of opposite axillary buds into shoots by two weeks, (b) induction of new buds after four weeks, (c) induction of multiple shoots at six weeks and (d) healthy elongated shoots at the end of eight weeks.

Table 7. Effect of PGRs on shoot regeneration from nodal explants of *L. reticulata* (8 weeks).

PGRs (µM)			No. of shoots/explant*	Shoot length (cm)*	Response (%)
BA	Kn	$AdSO_4$			
5	0	0	2.20 ± 0.28 bc	2.95 ± 0.25 bc	100
10	0	0	1.78 ± 0.28 de	2.09 ± 0.21 cd	90
15	0	0	2.11 ± 0.29 bcd	2.84 ± 0.20 bc	90
20	0	0	2.00 ± 0.18 cde	2.02 ± 0.08 cd	90
0	5	0	1.67 ± 0.27 ef	1.88 ± 0.19 d	30
0	10	0	1.20 ± 0.18 ef	2.13 ± 0.19 cd	50
0	15	0	1.80 ± 0.33 de	3.13 ± 0.28 ab	50
0	20	0	2.33 ± 0.19 bc	3.43 ± 0.36 ab	60
5	5	0	2.17 ± 0.15 bcd	2.41 ± 0.46 bc	60
5	10	0	1.33 ± 0.27 ef	1.80 ± 0.07 d	30
5	15	0	1.80 ± 0.18 de	1.70 ± 0.16 d	50
5	20	0	1.83 ± 0.18 de	1.89 ± 0.15 d	60
10	5	0	1.33 ± 0.27 ef	2.45 ± 0.18 bc	30
10	10	0	2.40 ± 0.60 bc	2.57 ± 0.3 bc	50
10	15	0	1.40 ± 0.22 ef	1.37 ± 0.09 d	50
10	20	0	1.25 ± 0.22 ef	1.42 ± 0.15 d	40
15	5	0	1.00 ± 0.00 f	1.60 ± 0.00 d	10
15	10	0	1.40 ± 0.22 ef	1.76 ± 0.14 d	50
15	15	0	2.00 ± 0.24 cde	2.42 ± 0.29 bc	60
15	20	0	1.25 ± 0.22 ef	1.34 ± 0.10 d	40
20	5	0	1.33 ± 0.27 ef	1.60 ± 0.29 d	30
20	10	0	1.00 ± 0.00 f	1.50 ± 0.14 d	30
20	15	0	1.00 ± 0.00 f	1.30 ± 0.07 d	20
20	20	0	1.00 ± 0.00 f	0.90 ± 0.07 d	20
5	0	5	2.40 ± 0.25 bc	2.15 ± 0.24 cd	100
5	0	10	3.60 ± 0.57 b	2.80 ± 0.26 bc	100
5	0	15	3.90 ± 0.33 b	2.76 ± 0.27 bc	100
5	0	20	3.90 ± 0.22 b	2.49 ± 0.33 bc	100

10	0	5	3.90 ± 0.26 b	2.59 ± 0.35 bc	100
10	0	10	5.10 ± 0.33 a	3.74 ± 0.60 ab	100
10	0	15	3.50 ± 0.45 b	3.05 ± 0.33 bc	100
10	0	20	2.30 ± 0.25 bc	2.13 ± 0.23 cd	100
15	0	5	2.10 ± 0.22 bcd	2.83 ± 0.37 bc	100
15	0	10	2.10 ± 0.26 bcd	2.16 ± 0.21 bc	100
15	0	15	3.00 ± 0.33 bc	2.14 ± 0.29 cd	60
15	0	20	2.13 ± 0.37 bcd	2.23 ± 0.35 bc	80
20	0	5	1.80 ± 0.18 de	2.16 ± 0.51 cd	50
20	0	10	2.00 ± 0.35 cde	1.91 ± 0.23 d	40
20	0	15	2.67 ± 0.54 bc	1.74 ± 0.23 d	30
20	0	20	1.75 ± 0.41 de	1.69 ± 0.21 d	40
0	5	5	1.00 ± 0.20 f	1.76 ± 0.31 d	100
0	5	10	1.50 ± 0.21 ef	2.67 ± 0.28 bc	100
0	5	15	2.00 ± 0.28 cde	2.80 ± 0.36 bc	100
0	5	20	2.20 ± 0.28 bc	2.99 ± 0.38 bc	100
0	10	5	1.90 ± 0.17 de	4.12 ± 0.68 a	100
0	10	10	3.10 ± 0.33 bc	2.48 ± 0.15 bc	100
0	10	15	2.70 ± 0.28 bc	2.74 ± 0.28 bc	100
0	10	20	1.90 ± 0.28 de	3.82 ± 0.38 ab	100
0	15	5	1.80 ± 0.28 de	2.59 ± 0.29 bc	100
0	15	10	2.70 ± 0.25 bc	2.52 ± 0.23 bc	100
0	15	15	2.40 ± 0.25 bc	2.16 ± 0.22 cd	100
0	15	20	2.60 ± 0.21 bc	2.50 ± 0.26 bc	100
0	20	5	2.00 ± 0.25 cde	2.32 ± 0.18 bc	80
0	20	10	1.29 ± 0.17 ef	1.88 ± 0.14 d	70
0	20	15	1.20 ± 0.18 ef	2.00 ± 0.22 cd	60
0	20	20	1.20 ± 0.18 ef	1.43 ± 0.17 d	60

*Values represent mean ± SE. Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

4.1.3.3 Elongation of Shoots

One common observation which was noted during the above study was that the nodes when placed in different PGRs combinations, regenerated multiple shoots, but shoots remained stunted. Therefore these shoots were transferred to the medium fortified with different concentrations (2-8%) of CW for another four weeks. At lower concentration (2%) it failed to elongate all the shoots simultaneously, and only few shoots elongated to a length of 4.49 ± 0.29 cm (Fig. 19a). Increasing the concentration to 4% proved advantageous because it was able to elongate all the shoots and highest length of 6.72 ± 0.38 cm could be achieved (Fig. 19b). Further raising the level of CW to 6-8%, similar result was observed like 2% and only few shoots elongated whereas rest of shoots remained stunted. Average length was 5.34 ± 0.44 cm and 5.36 ± 0.41 cm for respective concentrations was recorded (Fig. 19c, d; Fig. 20).

- On the basis of above experiment it was concluded that 4% CW favored formation of healthy elongated shoots.

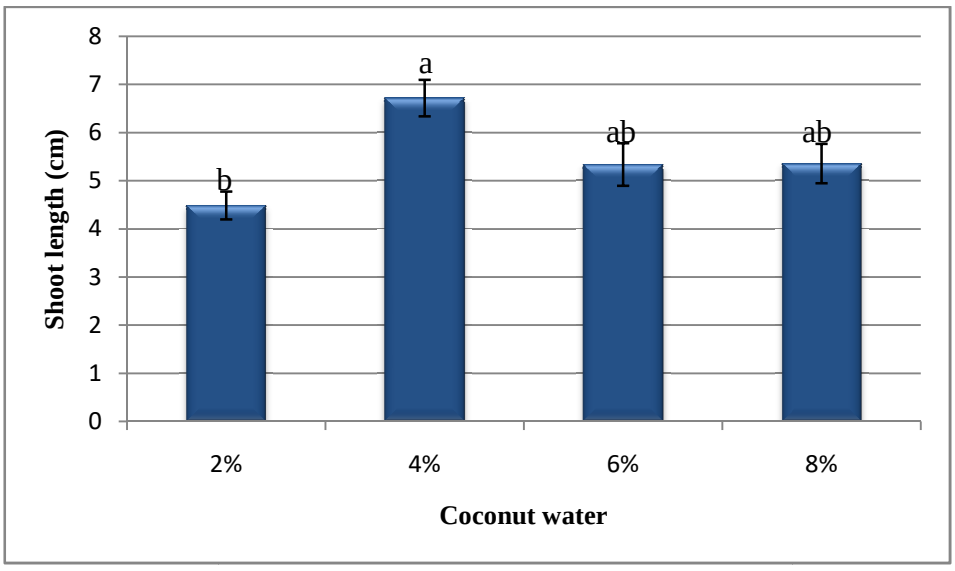

Figure 20. Elongation of axillary shoots in presence of CW at the end of four weeks.

Each bar shows the mean values (n = 10) and error bar as standard error. Bars having same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Nodes of *L. reticulata* when cultured on BA supplemented medium, failed to form multiple shoots and similarly nodal explants of *Vitex negundo* (Chandramu et al., 2003),

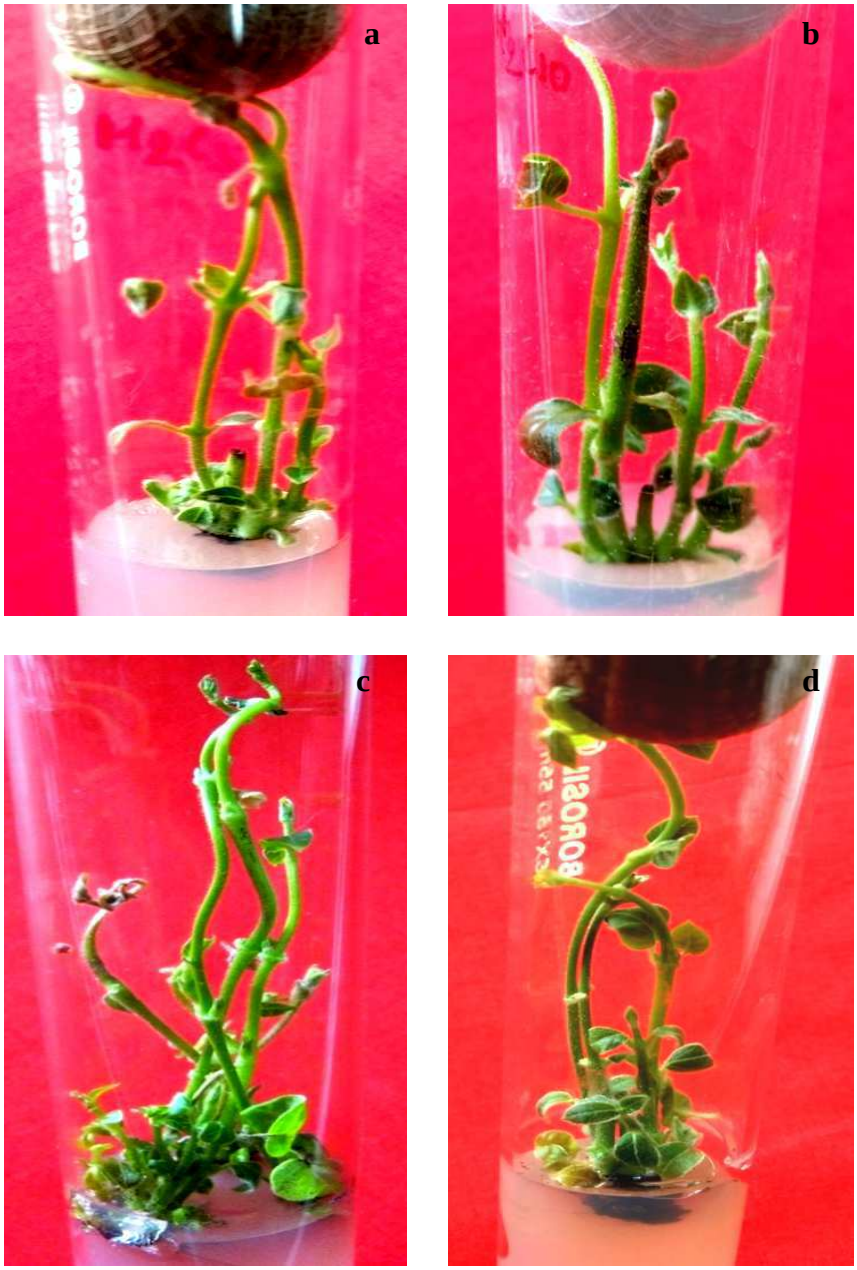

Figure 19. Effect of CW on axillary shoot elongation in *L. reticulata* by end of four weeks: (a) 2%, (b) 4%, (c) 6% and (d) 8%.

Tinospora cordifolia (Gururaj et al., 2007) and *Dendrobium longicornuare* (Dohling et al., 2012) are reported to be less responsive in BA fortified medium. Likewise, when Kn was added as individual PGR, it also induced meagre response which is in corroboration with results of other Asclepiadaceae members like *Hemidesmus indicus* (Siddique and Bari, 2006; Shekhawat and Manokari, 2016), *Caralluma lasiantha* (Aruna et al., 2012) and *Decalepis salicifolia* (Ahmad et al., 2018). In line with present study, many studies also stated poor response for shoot regeneration in presence of individual BA and Kn such as in *Clematis viticella* and *C. integrifolia* (Parzymies and Dabski, 2012), *Sophora tonkinensis* (Jana et al., 2013), *Cucumis sativus* (Alam et al., 2015), *H. indicus* (Pathak et al., 2017), *Clitoria ternatea* (Mishra et al., 2019) and *Hedyotis biflora* (Revathi et al., 2019). Further, augmentation of MS medium with combination of BA and Kn also failed to induce multiple shoots. This results are in agreement with previous results for nodal cultures of *Holarrhena antidysenterica* (Ahmed et al., 2001), *Terminalia arjuna* (Pandey et al., 2006), *Cucumis anguria* (Margaret et al., 2014) and *Dendrobium chrysotoxum* (Kaur, 2017). Recently antagonistic effect of two cytokinins on shoot regeneration from nodal explant is reported in *Glycyrrhiza glabra* (Shaheen et al., 2020).

It was noted that nodes of *L. reticulata* failed to induce multiple shoots in presence of BA with Kn, but replacing Kn with another cytokinin $AdSO_4$ facilitated maximum shoot regeneration, which is previously observed for nodal explant of *Cocculus hirsutus* (Meena et al., 2012). In line with present study, beneficial effect of $AdSO_4$ on shoot regeneration from nodal explant of *L. reticulata* is documented by Rathore et al. (2013). Earlier Arya et al. (2003) depicted formation of 4.93 ± 0.77 shoots in presence of BA (18 μM) and IAA (0.6 μM) in *L. reticulata,* but in the present study total 5.10 ± 0.33 shoots with 100% response was recorded in combination of BA (10 μM) and $AdSO_4$ (10 μM). Similarly, the shoot number as well as % response was also higher as compared to earlier report on same plant by Parabia et al. (2007), however they have used Kn (10 mg/l) with IBA (1 mg/l) as PGRs. Similarly Bharat et al. (2011) also suggested combination of Kn with IBA for nodal explant of *L. reticulata*. The synergistic effect of cytokinins i.e. BA with $AdSO_4$ proved to be beneficial which was in accordance with previous studies on same plant where other cytokinins (BA and Kn) are beneficial (Shekhawat et al., 2006; Sudipta et al., 2011). Whereas combination of BA and NAA has been documented in other studies (Satyanarayan et al., 2008; Dhawan and Damor, 2013), but the reported shoot number and response is less as compared to present protocol.

The combination of cytokinins are required for cell expansion, its division, regulation of shoot formation and axillary bud multiplication, they also regulate protein synthesis which is required for formation and functioning of mitotic spindles (Mok and Mok, 2001). In *L. reticulata*, optimum shoot regeneration was observed at same concentration (10 μM) of BA and $AdSO_4$ whereas in *Dendrocalamus strictus* lower BAP (4 mg/l) with higher $AdSO_4$ (15 mg/l) evoked maximum shoot formation (Pandey and Singh, 2012). The beneficial effect of BA and $AdSO_4$ is also documented in nodal explant of *Phaseolus vulgaris* (Gatica-Arias et al., 2010). Murashige (1974) has mentioned that addition of adenine in the form of $AdSO_4$ act as a catalyst on the organization of enhanced shoot bud proliferation. This is because $AdSO_4$ is considered as a rich source of nitrogen and thus it improves the shoot multiplication (Ahmad et al., 2018). While some studies suggested inclusion of auxin along with BA-$AdSO_4$ for better shoot proliferation. PGR combination of BA+$AdSO_4$+NAA has been documented for optimum shoot proliferation from axillary buds of Asclepiadaceae plant species *H. indicus* (Misra et al., 2003) and *Decalepis salicifolia* (Ahmad et al., 2018) as well as in *Sida cordifolia* (Sivanesan and Jeong, 2007). Whereas multiple shoot regeneration in *Bacopa monnieri* is reported in presence of BA+$AdSO_4$+IAA (Ramesh et al., 2006) and in *Hedyotis biflora* medium is supplemented with BAP, Kn, $AdSO_4$ and IAA along with additives like ascorbic acid, arginine and citric acid (Revathi et al., 2019). Favourable effect of $AdSO_4$ on axillary shoot regeneration has been well documented in *H. antidysenterica* (Raha and Roy, 2001), *Melia azedarach* (Husain and Anis, 2004), *Curcuma angustifolia* (Shukla et al., 2007), *Petrocarpus marsupium* (Husain et al., 2008), *Ficus religiosa* (Siwach and Gill, 2011), *Solanum tuberosum* (Singh et al., 2017) and *Decalepis arayalpathra* (Ahmad et al., 2018).

The observations also revealed that for efficient regeneration, combination of BA and $AdSO_4$ was better as compared to Kn and $AdSO_4$ which is in line with previous study on nodal explant of *Nyctanthes arbor-tristis* (Rout et al., 2008). It may be due to the fact that BA has more permeability across plasma membrane and high cell uptake which facilitates bud break, efficient proliferation and multiple shoot formation (Malik et al., 2005). However, the shoots regenerated from nodal cultures coulden't elongate properly and earlier reports in *L. reticulata* states the same (Arya et al., 2003; Parabia et al., 2007). This is one of the adverse effects of high concentration of cytokinins (Hu and Wang, 1983; Gonbad et al., 2014) and BAP has often been reported to have inhibitory activity on shoot elongation (Figueiredo et al., 2001; Purkayastha et al., 2008). But when these shoots were transfered to medium containing coconut water it facilitated their elongation and similar observations on nodal explants have

been reported in *Eclipta alba* (Baskaran and Jayabalan, 2005) and *Capsicum annuum* (Mythili et al., 2017).

When nodes of *L. reticulata* were utilized for establishment of nodal cultures it was observed that the shoot regeneration frequency was affected by season. Maximum shoot regeneration was recorded from nodes in the period of summer (Feb-June). In monsoon the frequency was not affected but higher contamination was recorded (July-September), whereas the shoot forming capacity of nodal explant was least during winter (October-January). The season of explant collection is a crucial factor for establishment of *in vitro* cultures (Hu and Wang, 1983). Previously Benmahioul (2009) also reported the effect of the season on response of *Pistacia vera* explants and frequency of contamination which is in accordance with present study. The explants collected during autumn (dormant axillary bud) from adult trees were contaminated after a few days. However, the best results were recorded with nodal segments taken in full growth (spring). In line with present study Kumar et al. (2010) also documented better growth in summer and successive decrease in monsoon and winter in *Gmelina arborea*. Similarly, nodal explants of *Dendrocalamus asper* (Singh et al., 2011c) and *D. hamiltonii* (Singh et al., 2012) have maximum regeneration frequency during summer whereas winter was unsuitable for *in vitro* shoot establishment. Similarly nodal explants showed significant variation in morphogenic response during different months of the year in *H. antidysenterica* (Kumar et al., 2005), *Vitex negundo* (Steephen et al., 2010), *Stevia rebaudiana* (Verma et al., 2011), *Dalbergia sissoo* (Vibha et al., 2014) and *Terminalia bellerica* (Dangi et al., 2014)

4.1.4 Rooting of shoots

Elongated eight weeks old shoots regenerated from nodal explants were utilized for the rooting experiment (Fig. 21a). They were first transferred in the basal medium of different strengths like full and half of MS media with 1% sucrose without any PGR. Basal MS medium of both the strengths failed to form roots, and shoots died after two weeks. Then shoots were placed in the IBA supplemented medium of both strength at 2 and 4 µM concentrations, and response was evaluated within four weeks. Full strength MS medium with 2 µM IBA, formed only 1.5 ± 0.35 tiny roots of few cm length (1.15 ± 0.04 cm). The response was also very less (20%) but rate of shoot survival improved to 90%. When the level was increased to 4 µM, number slightly enhanced but it was maximum number of roots 1.67 ±

0.27 as well as length (1.9 ± 0.05 cm) in 30% cultures and the shoots survival (90%) remained similar within four weeks (Fig. 21b).

Rooting was also tried in half strength MS medium with IBA in same concentrations. In this strength when medium was augmented with 2 µM of IBA, 1.5 ± 0.35 roots were formed which was similar with full strength but length was slightly increased to 1.53 ± 0.19 cm. Roots were formed only in 20% cultures but 100% shoots survived in this concentration up to four weeks. Further raising the level of IBA (4 µM) in half strength medium adversely affected the root formation and shoot survival too as it failed to form roots and only 20% shoots were survived (Fig. 22).

- It was concluded that MS medium supplemented with IBA encouraged rooting in shoots.

Rooting of nodal derived shoots depicted that basal MS medium of different strengths (full and half) failed to induce roots which is also reported in *Ajuga bracteosa* (Kaul et al., 2013), *Rubia cordifolia* (Khadke et al., 2013) and *Hemidesmus indicus* (Pathak and Joshi, 2017). When different strengths of MS medium was augmented with IBA, it induced rooting of shoots which is in corroboration with previous results documented for rooting of *L. reticulata* shoots (Martin, 2004; Parabia et al., 2007; Satyanarayana et al., 2008; Bharat et al., 2011; Dhawan and Damor, 2013). Whereas other studies on same plant suggested that addition of activated charcoal along with IBA facilitates rooting (Sudipta et al., 2011; Patel et al., 2014). On the contrary Arya et al. (2003) have shown that BA and IAA combination could induce rooting in *L. reticulata*. They have also suggested that full strength medium is better which is also in accordance with present results. Similarly, full MS medium augmented with IBA has proved to be helpful for *in vitro* rooting in shoots of *Brunfelsia calycina* (Liberman et al., 2010), *Solanum nigrum* (Bhat et al., 2010), *Gynura procumbens* (Banu et al., 2017), *Genipa amecana* (de Souza et al., 2019), *Zinjiber officinale* (Mehaboob et al., 2019) and *Bacopa monnieri* (Tata, 2020). There are many other reports on *B. monnieri* (Joshi et al., 2010), *Munronia pinnata* (Gunathilake et al., 2008), *Camellia sinensis* (Bidarigh and Azarpuor, 2013), *Allamanda cathartica* (Khanam and Anis, 2018) and *Portulaca quadrifida* (Pathak et al., 2019) where rooting was induced in presence of IBA.

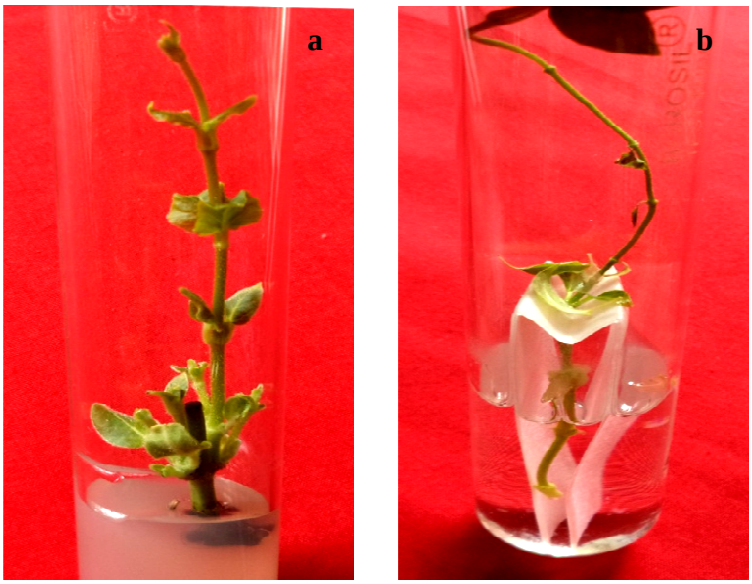

Figure 21. Rooting of *L. reticulata* micro shoots: (a) Elongated eight weeks old shoot and (b) induction of tiny roots in full strength MS medium containing 4 µM of IBA at the end of four weeks.

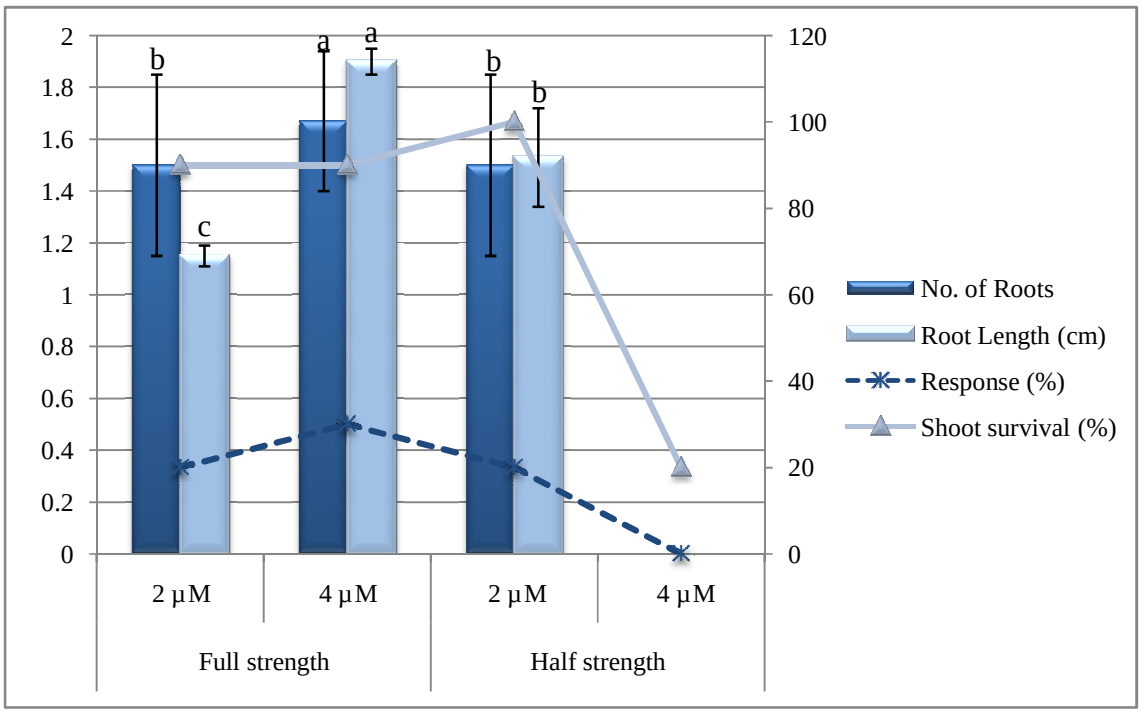

Figure 22. Rooting of *L. reticulata* shoots in different MS medium strengths fortified with IBA.

Each bar shows the mean values (n = 5), error bar as standard error and the line represents % germination. Bars having same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

4.2 Phytochemical Analysis

In this study, *in vitro* shoots and callus regenerated from nodal and leaf explants respectively in the medium fortified with different PGRs were harvested and analyzed for their potency to synthesize metabolites by developing HPTLC fingerprint. Whereas quantification was performed for marker compound *p*-coumaric acid in above samples. Further the culture having highest quantity of *p*-coumaric acid was selected for elicitation using yeast extract and salicylic acid at different concentrations and time interval.

4.2.1 HPTLC Fingerprint

In vivo shoots (L1), *in vitro* shoot and callus samples (L2-L6) were harvested from different media (mentioned in the chapter 3) and extracted using solvents having different polarities i.e. hexane, ethyl acetate and methanol. All the three extracts from the samples (L1-L6) were subjected to chromatographic separation on TLC plates in respective mobile phases followed by derivatization using anisaldehyde-sulphuric acid reagent. Then the plates were scanned at 254, 366 and 525 nm for densitometry analysis and the results are summarized below.

4.2.1.1 Hexane Extract

Hexane extract of all the samples were spotted on TLC plates and subjected to chromatographic separation using optimized mobile phase i.e. toluene:ethylacetate:formic acid (7.2:2.5:0.3 v/v). Then plates were scanned at 254, 366 and 525 nm confirmed the change in banding pattern, peak numbers, and their areas.

- **254 nm**

Scanning at 254 nm showed almost similar profiling of L1 with L2, L3, L4 but it differed for callus samples i.e. L5 and L6 (Fig. 23a). In L1 sample a total 14 peaks were present and maximum area 5973.6 was recorded for peak at Rf (0.90) (Fig. 24a). L2, L3 and L5 samples had almost similar peaks (11, 10, 11), and the highest area obtained for similar peak (Rf 0.78), but their areas varied viz. 10590.4, 8724.5 and 5851.8 respectively (Fig. 24b, c, e). Similarly, L4 sample showed total 14 peaks and maximum area was 11651.8 at Rf 0.88 (Fig. 24d) and L6 sample appeared with 9 peaks among which highest area was 3762.0 at 0.02 Rf (Fig. 24f).

Table 8. Peak areas of different samples at 254 nm for hexane extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	-	-	-	-	-	-	5983.8	17.32	2011.0	15.93	3762.0	30.48
0.03	5182.1	17.18	4570.4	16.21	3537.0	16.83	-	-	-	-	-	-
0.09	-	-	-	-	-	-	-	-	343.0	2.72	-	-
0.16	206.1	0.68	-	-	-	-	-	-	-	-	-	-
0.18	355.5	1.18	205.1	0.73	-	-	442.9	1.28	244.9	1.94	271.3	2.20
0.22	-	-	501.0	1.78	397.7	1.89	834.0	2.41	313.9	2.49	404.2	3.28
0.23	540.6	1.79	-	-	-	-	-	-	-	-	-	-
0.26	-	-	347.4	1.23	-	-	509.6	1.48	-	-	-	-
0.27	590.9	1.96	-	-	349.3	1.66	-	-	-	-	-	-
0.30	-	-	-	-	-	-	-	-	-	-	-	-
0.33	-	-	397.2	1.41	346.2	1.65	343.0	0.99	-	-	-	-
0.34	1030.5	3.42	-	-	-	-	-	-	-	-	-	-
0.37	-	-	323.4	1.15	-	-	-	-	809.0	6.41	-	-
0.38	-	-	-	-	-	-	-	-	-	-	995.6	8.07
0.41	-	-	1821.1	6.46	901.9	4.29	2422.4	7.01	-	-	-	-
0.42	-	-	-	-	-	-	-	-	752.3	5.96	683.1	5.54
0.44	3400.1	11.27	-	-	-	-	-	-	-	-	-	-
0.46	-	-	-	-	-	-	310.1	0.90	-	-	-	-
0.48	2171.0	7.20	-	-	-	-	-	-	-	-	-	-
0.54	-	-	-	-	-	-	-	-	1262.4	10.00	-	-
0.55	838.7	2.78	-	-	-	-	-	-	-	-	1676.1	13.58
0.62	-	-	-	-	-	-	426.2	1.23	-	-	-	-
0.63	-	-	-	-	-	-	608.6	1.76	-	-	-	-
0.64	-	-	-	-	496.3	2.36	-	-	-	-	-	-
0.65	-	-	762.0	2.70	-	-	-	-	509.9	4.04	-	-
0.66	480.4	1.59	-	-	-	-	-	-	-	-	568.3	4.61

0.71	-	-	-	-	1064.8	5.07	-	-	-	-	-	-
0.74	-	-	-	-	-	-	-	-	-	-	-	-
0.75	-	-	-	-	-	-	3830.2	11.09	-	-	-	-
0.77	3262.6	10.82	10590.4	37.56	-	-	2626.1	7.60	-	-	2155.1	17.46
0.78	-	-	-	-	8724.5	41.50	-	-	5851.8	46.35	-	-
0.79	5576.8	18.49	-	-	-	-	-	-	-	-	-	-
0.84	-	-	-	-	-	-	-	-	308.8	2.45	-	-
0.85	-	-	-	-	-	-	-	-	-	-	1825.0	14.79
0.88	-	-	-	-	-	-	11651.8	33.73	-	-	-	-
0.89	-	-	5002.9	17.74	2961.6	14.09	-	-	-	-	-	-
0.90	5973.6	19.81	-	-	-	-	-	-	-	-	-	-
0.94	-	-	-	-	-	-	3854.7	11.16	-	-	-	-
0.95	-	-	3673.2	13.03	2241.8	10.66	-	-	217.8	1.73	-	-
0.96	550.7	1.83	-	-	-	-	-	-	-	-	-	-
0.99	-	-	-	-	-	-	679.9	2.02	-	-	-	-

Chromatograms also showed variation in peaks and comparison was accomplished with respect to all samples (L1-L6) which resulted with unique peak present at particular Rf. The peak at Rf (0.16, 0.23 and 0.34) was unique as it was only present in *in vivo* shoots (L1) and absent in all the *in vitro* samples. Likewise, the peak was common in all the *in vitro* shoot samples (L2-L4) at Rf (0.33 and 0.41) but it was absent in *in vivo* sample (L1) and in vitro callus samples (L5 and L6). Whereas, peak was present at 0.42 Rf in samples L5 and L6, and absent in rest of samples therefore this peak was unique. Some unique peaks were present only in callus sample and *in vivo* sample for e.g. peak at Rf 0.55 and 0.66 was seen in L1 and L6 and L6 which was absent in shoot cultures. Similarly at Rf (0.84 and 0.85) the peak appeared only in L5 and L6 samples which were callus samples and was absent in *in vivo/in vitro* shoot samples (Table 8).

- **366 nm**

Profiling of plate under 366 nm showed the variation in number of bands between the samples (Fig. 23b). Further scanning at same wavelength also confirmed differences in chromatograms among the samples. Total 14 peaks were recorded for L1 sample and maximum area (5973.6) was observed for peak at Rf (0.91) (Fig. 24g). L2-L5 samples had less number of peaks i.e. 6, 6, 10 and 4 respectively, Rf 0.88 exhibited highest peak areas (14346.2, 12966.7, 27755.0 and 440.4) for all above samples which differed greatly among them (Fig. 24h-k). Whereas L6 sample showed maximum area at very low Rf 0.02 (1323.9) (Fig. 24l). The observations revealed that peaks varied in all the samples and comparison resulted in presence of unique peaks. Distinct peaks detected at Rf (0.36, 0.44, 0.51, 0.70, 0.91) were unique because it was only present in L1 sample which was of *in vivo* shoots and absent in all the *in vitro* samples. Rf (0.33, 0.41, 0.74 and 0.88) were present in samples L2-L6 and absent in L1 therefore these peaks were unique in *in vitro* samples. Peak at Rf (0.62) was present in only L2-L4 which was of *in vitro* shoots samples (Table 9).

- **525 nm**

Derivatization of the plates increased the number of bands in all the samples when observed in white light and the band pattern was almost similar in all samples (Fig. 23c). Scanning of plate at 525 nm showed change in peak numbers and area in the chromatograms. Chromatogram showed a total of 15 peaks in sample L1 and maximum area was 25633.3 at Rf (0.76) (Fig. 24m).

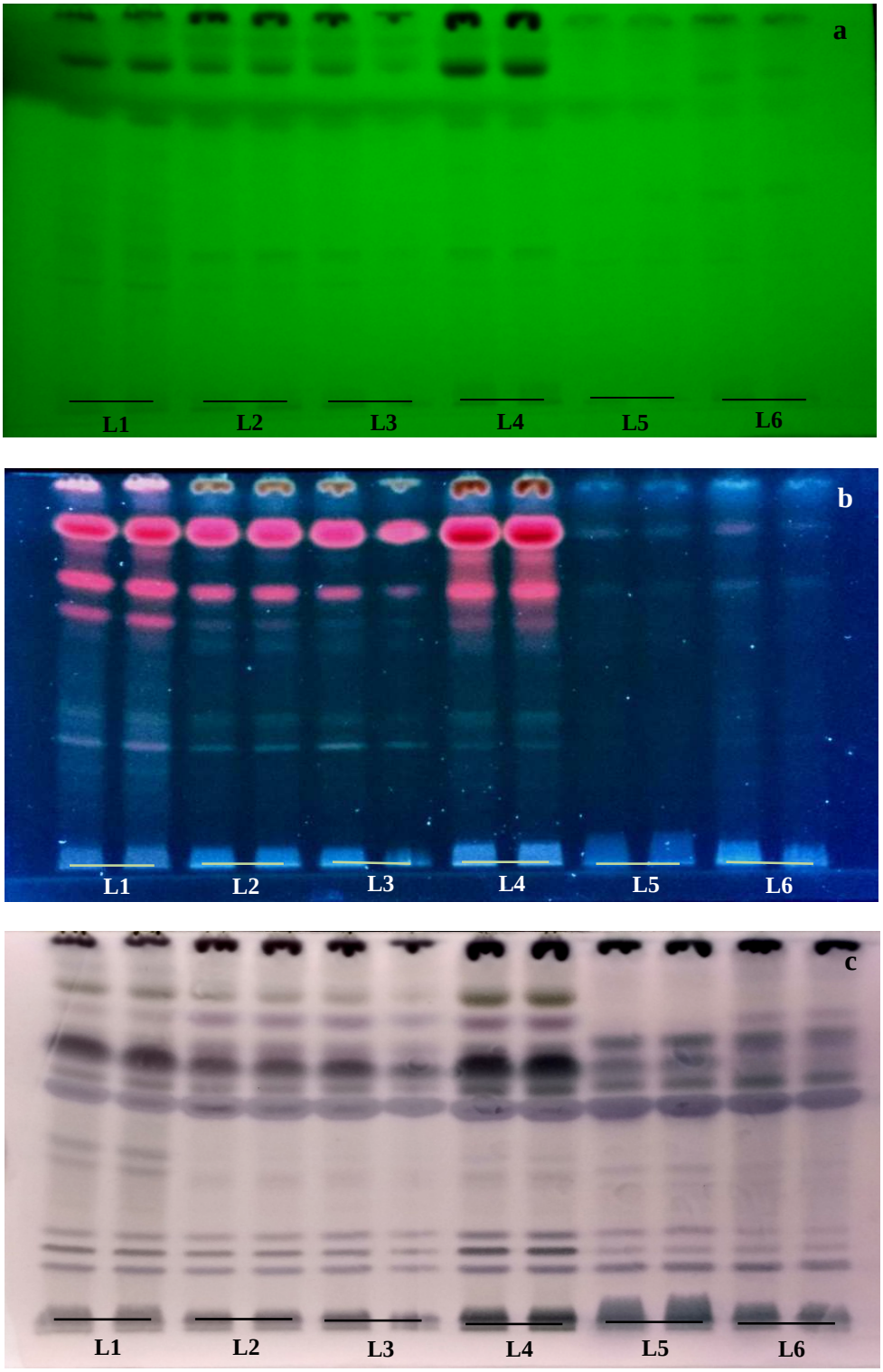

Figure 23: HPTLC fingerprints of hexane extract for different samples of *L. reticulata*: (a) UV 254 nm, (b) UV 366 nm and (c) white light after derivatization.

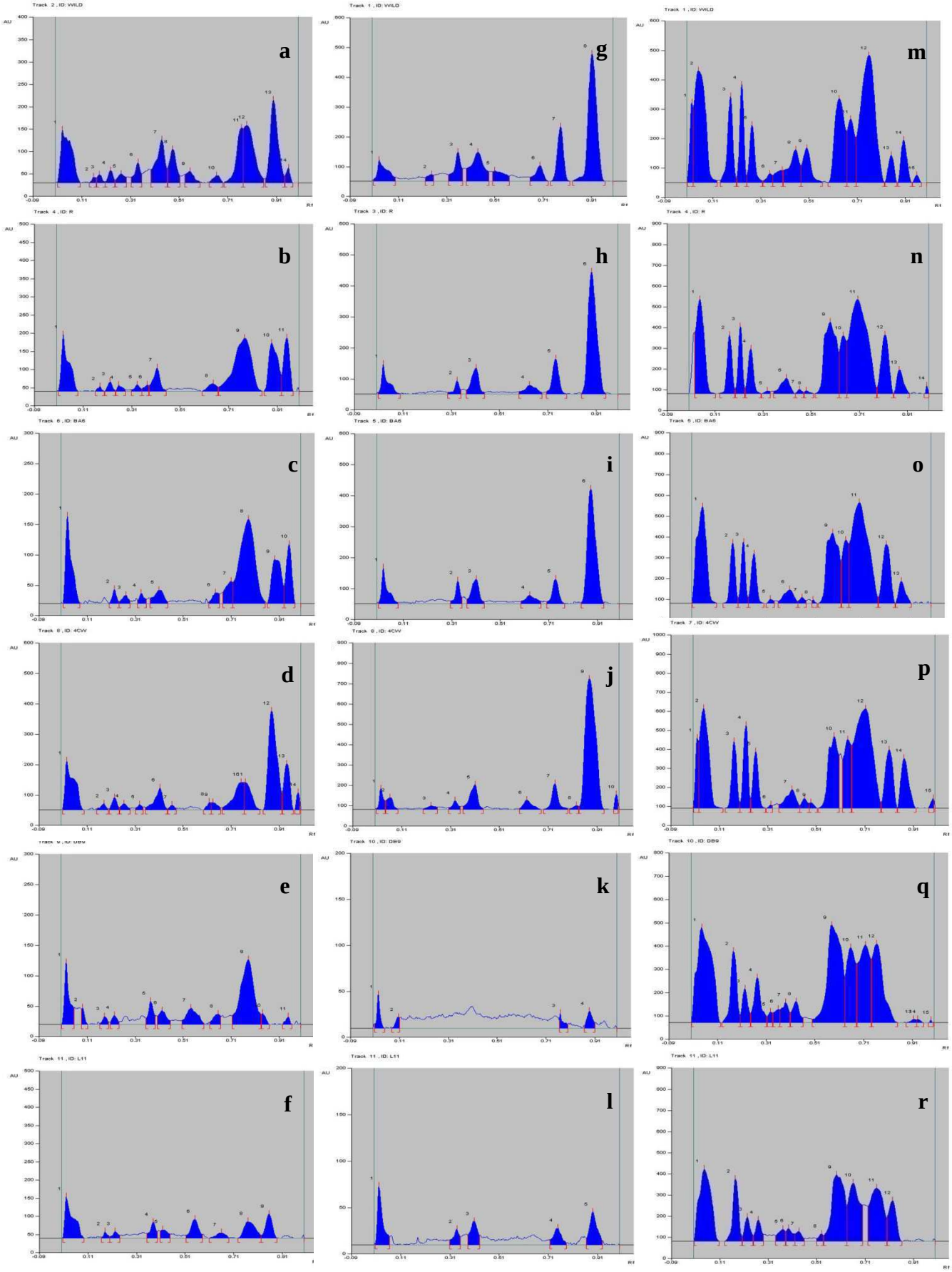

Figure 24. Chromatograms of different samples for hexane extract in *L. reticulata*: (a-f) 254 nm, (g-l) 366 nm and (m-r) 525 nm.

Table 9. Peak areas of different samples at 366 nm for hexane extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	-	-	-	-	-	-	1653.3	3.87	440.4	33.37	1323.9	31.74
0.03	2741.6	8.02	2236.8	8.94	2646.2	11.57	-	-	-	-	-	-
0.06	-	-	-	-	-	-	1616.7	3.78	-	-	-	-
0.10	-	-	-	-	-	-	-	-	191.9	14.54	-	-
0.23	-	-	-	-	-	-	608.1	1.42	-	-	-	-
0.25	579.3	1.70	-	-	-	-	-	-	-	-	-	-
0.33	-	-	904.0	3.61	1423.9	6.23	995.7	2.33	-	-	-	-
0.34	-	-	-	-	-	-	-	-	-	-	400.5	9.60
0.36	2692.4	7.88	-	-	-	-	-	-	-	-	-	-
0.41	-	-	3108.1	12.43	2526.8	11.05	3949.6	9.24	-	-	762.3	18.28
0.44	5078.6	14.86	-	-	-	-	-	-	-	-	-	-
0.51	1663.3	4.87	-	-	-	-	-	-	-	-	-	-
0.62	-	-	-	-	-	-	1626.0	3.81	-	-	-	-
0.63	-	-	1312.5	5.25	1200.7	5.25	-	-	-	-	-	-
0.70	1721.0	5.04	-	-	-	-	-	-	-	-	-	-
0.74	-	-	3104.3	12.41	2105.6	9.21	3271.3	7.66	-	-	-	-
0.75	-	-	-	-	-	-	-	-	-	-	590.5	14.16
0.77	-	-	-	-	-	-	-	-	246.7	18.70	-	-
0.78	4605.7	13.48	-	-	-	-	-	-	-	-	-	-
0.82	-	-	-	-	-	-	426.9	1.00	-	-	-	-
0.88	-	-	14346.2	57.36	12966.7	56.70	27755.0	64.96	-	-	-	-
0.89	-	-	-	-	-	-	-	-	440.6	33.39	1093.9	26.23
0.91	15087.8	44.16	-	-	-	-	-	-	-	-	-	-
0.99	-	-	-	-	-	-	822.6	1.93	-	-	-	-

Table 10. Peak areas of different samples at 525 nm for hexane extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	3381.1	3.44	-	-	-	-	4701.3	3.50	-	-	-	-
0.04	-	-	15321.7	14.52	18529.2	16.88	18698.8	13.90	25060.7	21.49	16901.6	20.33
0.05	17435.1	17.76	-	-	-	-	-	-	-	-	-	-
0.17	-	-	5802.4	5.50	6113.9	5.57	7431.7	5.53	7919.3	6.79	6976.9	8.39
0.18	6544.6	6.67	-	-	-	-	-	-	-	-	-	-
0.21	-	-	5535.1	5.25	-	-	-	-	-	-	-	-
0.22	-	-	-	-	5281.7	4.81	8745.1	6.50	3301.9	2.83	2641.9	3.18
0.23	5842.5	5.95	-	-	-	-	-	-	-	-	-	-
0.26	-	-	4796.6	4.55	5335.2	4.86	6534.1	4.86	-	-	-	-
0.27	4307.0	4.39	-	-	-	-	-	-	5557.4	4.77	2372.0	2.85
0.32	-	-	-	-	-	-	246.5	0.18	-	-	-	-
0.33	-	-	274.9	0.26	363.3	0.33	-	-	789.5	0.68	-	-
0.35	713.8	0.73	-	-	-	-	-	-	-	-	-	-
0.36	-	-	-	-	-	-	-	-	1324.8	1.14	-	-
0.37	-	-	-	-	-	-	-	-	-	-	1648.7	1.98
0.39	-	-	-	-	-	-	-	-	2453.3	2.10	1535.5	1.85
0.40	1328.7	1.35	-	-	-	-	-	-	-	-	-	-
0.41	-	-	2878.9	2.73	2740.1	2.50	4063.4	3.02	-	-	-	-
0.43	-	-	-	-	-	-	-	-	2616.8	2.24	-	-
0.44	-	-	-	-	-	-	-	-	-	-	1156.7	1.39
0.45	4555.4	4.64	-	-	-	-	-	-	-	-	-	-
0.46	-	-	373.2	0.35	601.8	0.55	1195.6	0.89	-	-	-	-
0.49	-	-	266.4	0.25	-	-	505.7	0.38	-	-	-	-
0.50	3896.1	3.97	-	-	-	-	-	-	-	-	-	-
0.51	-	-	-	-	169.1	0.15	-	-	-	-	-	-
0.53	-	-	-	-	-	-	-	-	-	-	652.9	0.79

0.58	-	-	-	-	-	-	15109.5	11.23	24615.8	21.11	-	-
0.59	-	-	17543.1	16.63	17224.3	15.70	-	-	-	-	16608.9	19.98
0.63	11936.2	12.16	-	-	-	-	-	-	-	-	-	-
0.64	-	-	7385.5	7.00	7685.3	7.00	10680.0	7.94	-	-	-	-
0.66	-	-	-	-	-	-	-	-	11946.0	10.24	12450.0	14.97
0.68	6406.9	6.53	-	-	-	-	-	-	-	-	-	-
0.70	-	-	32842.5	31.13	33453.5	30.48	-	-	-	-	-	-
0.71	-	-	-	-	-	-	37778.7	28.09	-	-	-	-
0.72	-	-	-	-	-	-	-	-	15376.5	13.19	-	-
0.76	25633.3	26.11	-	-	-	-	-	-	15079.4	12.93	14054.0	16.90
0.77	-	-	-	-	-	-	-	-	-	-	-	-
0.81	-	-	-	-	9335.9	8.51	9881.6	7.35	-	-	-	-
0.82	-	-	9068.7	8.60	-	-	-	-	-	-	6145.1	7.39
0.85	2167.1	2.21	-	-	-	-	-	-	-	-	-	-
0.87					2905.4	2.65	8322.4	6.19				
0.88	-	-	3139.5	2.98	-	-	-	-	-	-	-	-
0.90	3588.3	3.66	-	-	-	-	-	-	-	-	-	-
0.91	-	-	-	-	-	-	-	-	303.4	0.26	-	-
0.93	-	-	-	-	-	-	-	-	197.4	0.17	-	-
0.96	433.9	0.44	-	-	-	-	-	-	-	-	-	-
0.99	-	-	281.5	0.27	-	-	609.5	0.45	70.5	0.06	-	-

The shoots samples i.e. L2-L4 recorded total peaks (14, 13, 15) and highest area were slight differed (32842.5, 33453.5 and 37778.7) but it was for same peak at Rf 0.70 (Fig. 24n-p). Similarly, total 15 and 12 peaks were showed for callus samples (L5 and L6) and maximum area were obtained for peak at 0.04 Rf but the size of peak was differed as the areas were 25060.7 and 16901.6 respectively (Fig. 24q, r). Although difference was observed in chromatograms resulted with different banding pattern at different Rf and comparison was done to identify unique peaks. Peak appeared at Rf (0.58) was unique for *in vitro* shoot as well as callus samples (L2-L6) and absent in *in vivo* sample (L1). Whereas peak at Rf 0.35 was present in samples L1, L5 and L6, it was absent in *in vitro* shoot samples. In sample L6, the unique peak was obtained at Rf 0.53 (Table 10).

4.2.1.2 Ethyl acetate Extract

Ethyl acetate extract of all samples were separated in toluene:ethyl acetate:methanol (6.4:2.7:0.9 v/v) and observed under different UV ranges which showed variation in profile. This was also confirmed when the plate was scanned at same wavelength. Then it was derivatized using anisaldehyde-sulfuric acid reagent and scanned at 525 nm which also showed change in banding pattern, peak numbers and, their areas.

- **254 nm**

In vivo and *in vitro* shoot samples i.e. L1-L4 showed identical profile when observed under UV 254 nm, while callus samples (L5 and L6) resulted with slight different profiles (Fig. 25a). L1, L2 and L3 sample extracts recorded almost same number of peaks (15), but the maximum areas 14786.8, 7408.5 and 6874.7 were recorded for peaks at Rf (0.91, 0.03, 0.03 respectively) (Fig. 26a-c). L4 sample generated total 18 peaks out of which the maximum area (11372.6) was for peak at Rf 0.89 (Fig. 26d). Both the callus samples (L5 and L6) appeared with same number of peaks (10) and the common peak at Rf 0.14 had maximum areas, but the values were distinct viz. 6228.8 and 11284.6 (Fig. 26e, f). Although the chromatograms showed variation between all samples i.e. *in vivo/in vitro* shoots and callus cultures, presence of some unique peaks was noted. Peaks at Rf 0.15, 0.06, 0.02 and 0.03 were common in all the samples while peak at 0.07 Rf was unique because it only present in callus sample extracts (L5 and L6). In *in vivo* shoot extract, many peaks were unique (Rf 0.24, 0.45, 0.55, 0.66 and 0.86) whereas peak at 0.33 Rf was missing in L1 sample but present in all the *in vitro* sample (L2-L6). Similarly, many peaks (Rf 0.53, 0.63, 0.89 and 0.93) were only present in shoot samples (L2-L4) (Table 11).

Table 11. Peak areas of different samples at 254 nm for ethyl acetate extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	-	-	-	-	-	-	2810.6	4.02	-	-	-	-
0.02	9215.5	11.39	-	-	-	-	3925.8	5.61	4553.1	18.58	4207.3	7.23
0.03	-	-	7408.5	18.60	6874.7	16.86	-	-	-	-	-	-
0.06	2590.7	3.20	1967.1	4.94	2503.5	6.14	223.9	0.32	3989.4	16.28	7431.8	12.77
0.07	-	-	-	-	-	-	-	-	2919.0	11.91	5188.2	8.91
0.09	925.6	1.14	-	-	-	-	-	-	-	-	-	-
0.10	-	-	-	-	-	-	-	-	-	-	1310.9	2.25
0.12	-	-	587.8	1.48	964.5	2.37	-	-	-	-	-	-
0.14	-	-	1214.9	3.05	1276.0	3.13	1502.4	2.15	6288.8	25.66	-	-
0.15	5554.8	6.87	-	-	-	-	-	-	-	-	11284.6	19.38
0.16	-	-	1971.0	4.95	2254.2	5.53	-	-	-	-	-	-
0.18	-	-	-	-	-	-	820.9	1.17	-	-	-	-
0.19	2036.0	2.52	-	-	-	-	-	-	-	-	-	-
0.21	1421.3	1.76	-	-	-	-	764.9	1.09	-	-	7294.1	12.53
0.22	-	-	907.3	2.28	1362.9	3.34	-	-	-	-	-	-
0.23	-	-	-	-	-	-	-	-	844.4	3.44	-	-
0.24	5041.0	6.23	-	-	-	-	-	-	-	-	-	-
0.26	-	-	-	-	-	-	432.0	0.62	-	-	-	-
0.27	-	-	-	-	1385.9	3.40	-	-	-	-	-	-
0.28	-	-	774.3	1.94	-	-	-	-	1058.4	4.32	1997.8	3.43
0.31	6923.8	8.56	-	-	-	-	-	-	-	-	-	-
0.31	-	-	-	-	-	-	-	-	830.3	3.39	-	-
0.33	-	-	-	-	-	-	-	-	-	-	3641.8	6.26
0.34	-	-	2907.3	7.30	2911.0	7.14	4509.9	6.44	2796.1	11.41	-	-
0.36	6645.1	8.21	-	-	-	-	-	-	-	-	-	-
0.37	-	-	-	-	-	-	-	-	-	-	10845.2	18.63

0.38	-	-	-	-	-	-	2941.2	4.20	953.6	3.89	-	-
0.39	-	-	-	-	1980.6	4.86	-	-	-	-	-	-
0.41	-	-	1788.6	4.49	-	-	-	-	-	-	5011.6	8.61
0.42	-	-	-	-	-	-	2193.4	3.13	-	-	-	-
0.45	5627.7	6.96	-	-	-	-	-	-	-	-	-	-
0.49	-	-	-	-	626.6	1.54	-	-	277.9	1.13	-	-
0.50	6110.3	7.55	-	-	-	-	7540.2	10.77	-	-	-	-
0.53	-	-	3729.2	9.36	999.6	2.45	5685.2	8.12	-	-	-	-
0.55	4555.1	5.63	-	-	-	-	-	-	-	-	-	-
0.63	-	-	-	-	-	-	9828.1	14.04	-	-	-	-
0.64	-	-	6964.0	17.49	6788.6	16.65	-	-	-	-	-	-
0.66	6521.6	8.06	-	-	-	-	-	-	-	-	-	-
0.80	-	-	-	-	-	-	370.7	0.53	-	-	-	-
0.83	-	-	-	-	-	-	3900.7	5.57	-	-	-	-
0.84	-	-	2111.9	5.30	1999.0	4.90	-	-	-	-	-	-
0.86	2954.9	3.65	-	-	-	-	-	-	-	-	-	-
0.89	-	-	4741.5	11.91	4697.0	11.52	11372.6	16.25	-	-	-	-
0.91	14786.8	18.28	-	-	-	-	-	-	-	-	-	-
0.92	-	-	-	-	-	-	10620.8	15.18	-	-	-	-
0.93	-	-	2085.6	5.24	1324.8	3.25	-	-	-	-	-	-
0.97	-	-	-	-	2831.4	6.94	-	-	-	-	-	-
0.98	-	-	664.4	1.67	-	-	-	-	-	-	-	-
0.99	-	-	-	-	-	-	540.1	0.77	-	-	-	-

- **366 nm**

Developed fingerprint was observed under UV 366 which also showed different profile from UV 254. Banding pattern visibly looked identical in L1-L4 but it changed in L5 and L6 (Fig. 25b). This is because of variation in band numbers and intensities and it was also confirmed after scanning as it resulted in number of peaks with different peak areas. L1 sample reported total 13 peaks and maximum area 25105.5 found from the chromatogram for peak at Rf (0.55) (Fig. 26g). L2 and L3 samples resulted with 15 and 13 peaks respectively, and peak at same Rf 0.64 had highest areas i.e. 15651.7 and 16032.2 (Fig. 26h, i). Whereas maximum area (22604.2) for peak in L4 sample was at Rf 0.50 (Fig. 26j). Callus samples (L5 and L6) recorded less number of peaks which compared to others viz. 6 and 8, having the maximum areas of 2528.8 and 5503.4 at Rf 0.02 and 0.28 respectively (Fig. 26k, l). There are some common peaks at Rf (0.02) seen in all the samples, whereas peaks at Rf (0.24, 0.66, 0.77) were observed only in L1 sample. Although callus samples generated less number of peaks, there was unique peak at 0.07 Rf which was only present in L5 and L6 samples and sample L6 also had one distinct peak at Rf 0.46 (Table 12).

- **525 nm**

Derivatization of the plate showed that fingerprint was almost similar for L1-L4 and different for L5 and L6 (Fig. 25c). Scanning under 525 nm showed change in the chromatogram in terms of peak numbers and area. L1 sample resulted in 14 peaks and maximum area of 23203.2 at Rf (0.50) (Fig. 26m). Number of peaks was similar for L2 and L3 samples i.e. 19 and 18 peaks and highest area were 15173.4 and 16841.2 for peak at same Rf 0.64 (Fig. 26n, o). Total 15 peaks were noted in L4 sample and at Rf (0.89) maximum area of peak (19821.5) was observed (Fig. 26p). Whereas L5 and L6 sample showed total 13 and 16 peaks, and area 16421.9 and 14828.9 were for peak at Rf 0.33 (Fig. 26q, r). Chromatograms appeared with presence of common peaks between them which were at Rf (0.02, 0.08, 0.10 and 0.17) whereas peaks appeared at Rf (0.33, 0.63, 0.81 and 0.97) were only present in *in vitro* samples (L2-L6). One unique peak was noted in sample L5 at Rf (0.45) and *in vitro* shoots (L2-L4) also showed distinct peaks at Rf (0.06 and 0.53) (Table 13).

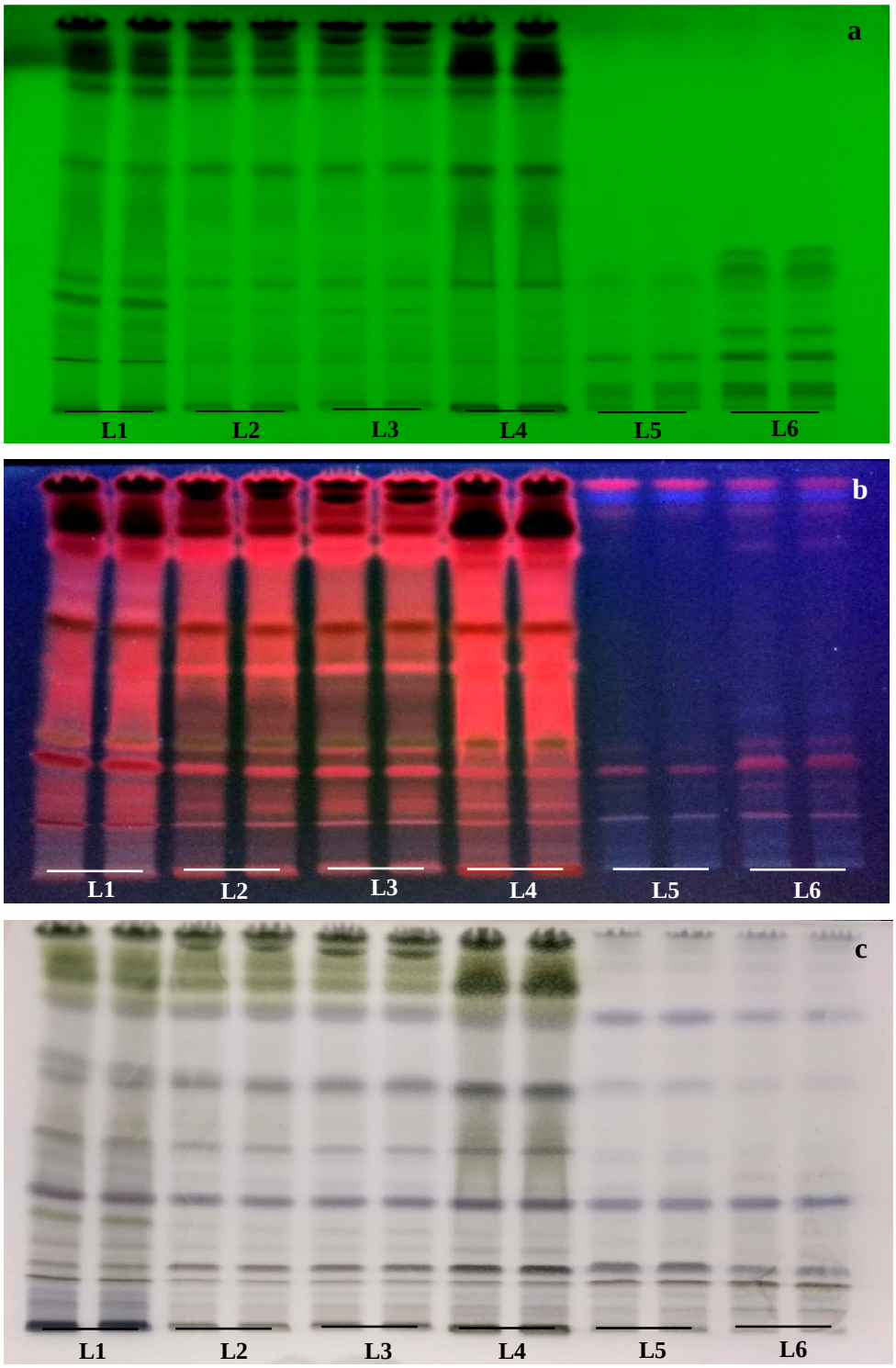

Figure 25. HPTLC fingerprints of ethyl acetate extract for different samples of *L. reticulata*: (a) UV 254 nm, (b) UV 366 nm and (c) white light after derivatization.

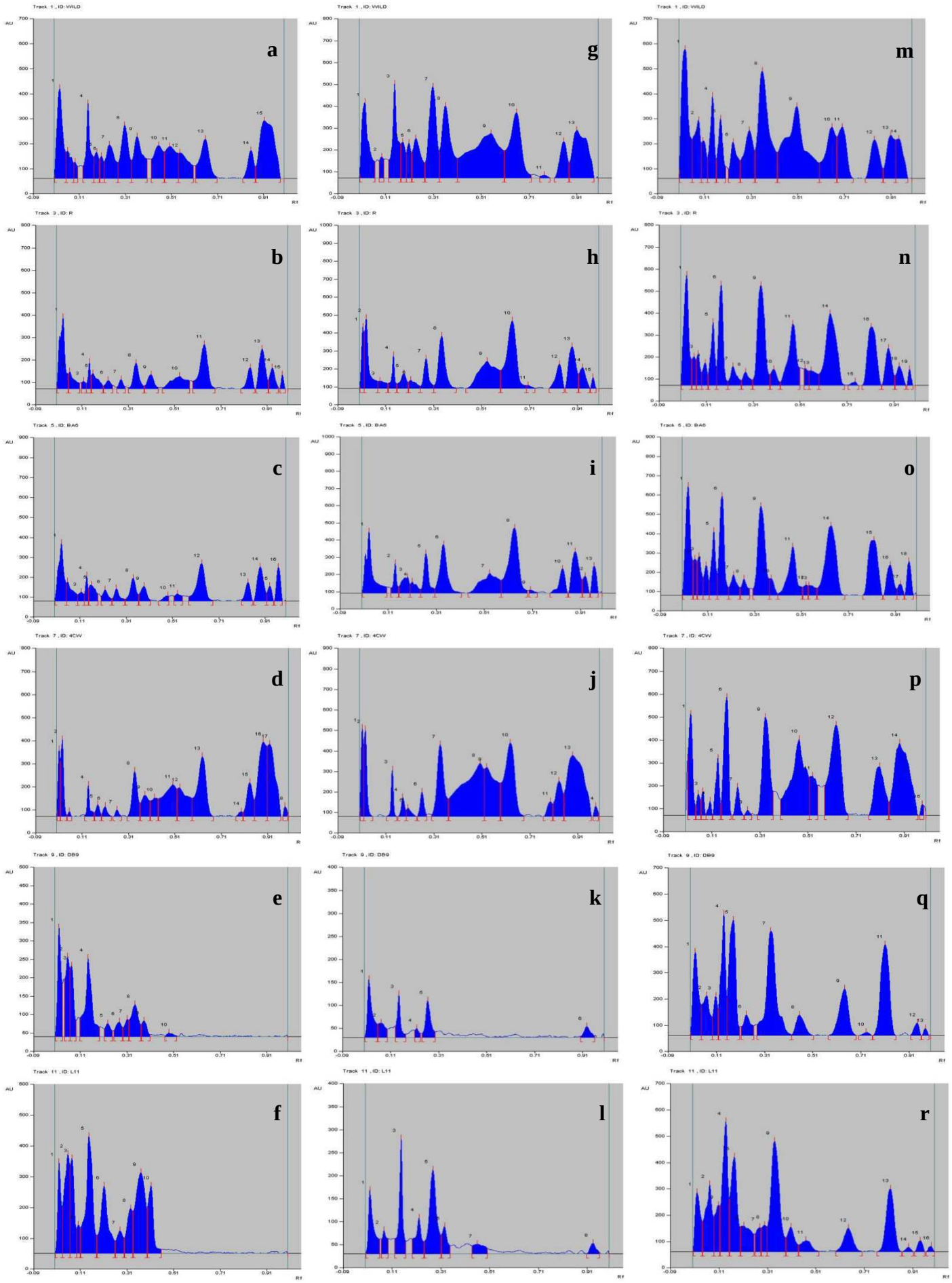

Figure 26. Chromatograms of different samples for ethyl acetate extract in *L. reticulata*: (a-f) 254 nm, (g-l) 366 nm and (m-r) 525 nm.

Table 12. Peak areas of different samples at 366 nm for ethyl acetate extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	-	-	3610.4	5.14	-	-	4531.3	4.41	-	-	-	-
0.02	10181.3	8.65	-	-	-	-	4723.1	4.60	2528.8	33.55	2851.7	16.21
0.03	-	-	6795.0	9.67	10188.9	14.44	-	-	-	-	-	-
0.07	-	-	-	-	-	-	-	-	947.0	12.57	-	-
0.08	-	-	-	-	-	-	-	-	-	-	1003.4	5.70
0.09	1688.7	1.43	1293.2	1.84	-	-	-	-	-	-	-	-
0.14	-	-	2197.7	3.13	2197.0	3.11	2017.5	1.96	1347.8	17.88	-	-
0.15	9198.9	7.82	-	-	-	-	-	-	-	-	3547.3	20.16
0.18	2922.9	2.48	-	-	-	-	1290.0	1.26	-	-	-	-
0.19	-	-	2107.9	3.00	3004.6	4.26	-	-	-	-	-	-
0.20	-	-	-	-	-	-	757.8	0.74	-	-	-	-
0.21	2741.2	2.33	1349.9	1.92	1723.5	2.44	-	-	-	-	-	-
0.22	-	-	-	-	-	-	-	-	331.2	4.39	1894.2	10.76
0.24	6011.4	5.11	-	-	-	-	-	-	-	-	-	-
0.26	-	-	-	-	-	-	1928.6	1.88	1747.3	23.18	-	-
0.27	-	-	-	-	4645.6	6.58	-	-	-	-	-	-
0.28	-	-	3352.8	4.77	-	-	-	-	-	-	5503.4	31.27
0.31	13216.0	11.23	-	-	-	-	-	-	-	-	-	-
0.33	-	-	-	-	-	-	-	-	-	-	1243.8	7.07
0.34	-	-	8153.8	11.60	8728.5	12.37	10186.2	9.91	-	-	-	-
0.36	12814.8	10.89	-	-	-	-	-	-	-	-	-	-
0.46	-	-	-	-	-	-	-	-	-	-	1006.4	5.72
0.50	-	-	-	-	-	-	22604.2	21.99	-	-	-	-
0.53	-	-	11324.9	16.11	8073.8	11.44	11453.8	11.14	-	-	-	-
0.55	25105.5	21.33	-	-	-	-	-	-	-	-	-	-
0.63	-	-	-	-	-	-	15455.0	15.04	-	-	-	-

0.64	-	-	15651.7	22.27	16032.2	22.72	-	-	-	-	-	-
0.66	14791.3	12.57	-	-	-	-	-	-	-	-	-	-
0.70	-	-	301.9	0.43	382.4	0.54	-	-	-	-	-	-
0.77	351.0	0.30	-	-	-	-	-	-	-	-	-	-
0.80	-	-	-	-	-	-	1511.5	1.47	-	-	-	-
0.83	-	-	-	-	-	-	5058.2	4.92	-	-	-	-
0.84	-	-	3468.0	4.93	3532.0	5.01	-	-	-	-	-	-
0.86	5058.5	4.30	-	-	-	-	-	-	-	-	-	-
0.89	-	-	7015.9	9.98	7429.8	10.53	20591.1	20.04	-	-	-	-
0.91	13613.2	11.57	-	-	-	-	-	-	-	-	-	-
0.93	-	-	2993.3	4.26	1841.9	2.61	-	-	634.2	8.42	547.2	3.11
0.97	-	-	-	-	2770.6	3.93	-	-	-	-	-	-
0.98	-	-	665.2	0.95	-	-	663.3	0.65	-	-	-	-

Table 13. Peak areas of different samples at 525 nm for ethyl acetate extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	-	-	-	-	-	-	7526.4	6.70	7339.3	9.42	5125.8	6.69
0.03	15704.7	12.69	10791.7	11.08	12160.2	11.35	-	-	-	-	-	-
0.05	-	-	-	-	-	-	1405.0	1.25	-	-	-	-
0.06	-	-	2095.4	2.15	2738.6	2.56	-	-	-	-	-	-
0.07	-	-	2136.0	2.19	3462.6	3.23	1193.5	1.06	4649.4	5.97	6954.5	9.08
0.08	6314.6	5.10	-	-	-	-	-	-	-	-	-	-
0.10	2973.3	2.40	1858.0	1.91	2674.8	2.50	722.9	0.64	-	-	-	-
0.11	-	-	-	-	-	-	-	-	2557.5	3.28	3311.1	4.32
0.13	-	-	-	-	-	-	3713.4	3.31	-	-	-	-
0.14	6013.0	4.86	4888.7	5.02	5796.9	5.41	-	-	8569.0	11.00	11074.9	14.46
0.17	-	-	8824.8	9.06	10615.2	9.91	10313.7	9.18	-	-	9592.7	12.52
0.18	4791.1	3.87	-	-	-	-	-	-	12235.0	15.71	-	-
0.21	-	-	-	-	-	-	-	-	-	-	4183.6	5.46
0.22	-	-	2327.3	2.93	2906.0	2.71	1960.2	1.74	-	-	-	-
0.23	4217.4	3.41	-	-	-	-	-	-	2487.3	3.19	-	-
0.26	-	-	-	-	1979.4	1.85	330.0	0.29	-	-	-	-
0.28	-	-	1639.1	1.68	-	-	-	-	-	-	1832.6	2.39
0.30	7399.7	5.98	-	-	-	-	-	-	-	-	2114.9	2.76
0.33	-	-	-	-	-	-	13278.2	11.82	16421.9	21.09	-	-
0.34	-	-	14359.8	14.75	14825.6	13.84	-	-	-	-	14828.9	19.36
0.36	19711.9	15.93	-	-	-	-	-	-	-	-	-	-
0.38	-	-	-	-	1617.7	1.51	-	-	-	-	-	-
0.40	-	-	1879.3	1.93	-	-	-	-	-	-	2825.0	3.69
0.45	-	-	-	-	-	-	-	-	2758.3	3.54	-	-
0.47	-	-	-	-	8806.8	8.22	19396.8	17.27	-	-	1831.2	2.39
0.48	-	-	9745.6	10.01	-	-	-	-	-	-	-	-

0.50	23203.2	18.75	-	-	-	-	-	-	-	-	-	-
0.53	-	-	1429.3	1.47	1027.3	0.96	4769.1	4.25	-	-	-	-
0.54	-	-	-	-	1453.9	1.36	-	-	-	-	-	-
0.56	-	-	2160.7	2.22	-	-	-	-	-	-	-	-
0.63	-	-	-	-	16841.2	15.72	18274.1	16.27	-	-	-	-
0.64	-	-	15173.4	15.58	-	-	-	-	6794.9	8.73	3408.6	4.45
0.66	8967.4	7.25	-	-	-	-	-	-	-	-	-	-
0.70	7556.9	6.11	-	-	-	-	-	-	-	-	-	-
0.73	-	-	-	-	-	-	-	-	293.8	0.38	-	-
0.74	-	-	347.8	0.36	-	-	-	-	-	-	-	-
0.81	-	-	10021.8	10.29	-	-	9089.3	8.09	12424.2	15.95	-	-
0.82	-	-	-	-	11683.8	10.91	-	-	-	-	7968.1	10.40
0.84	5911.7	4.78	-	-	-	-	-	-	-	-	-	-
0.88	-	-	4598.9	4.72	-	-	-	-	-	-	-	-
0.89	-	-	-	-	4430.2	4.14	19821.5	17.64	-	-	343.1	0.45
0.91	6000.7	4.85	-	-	-	-	-	-	-	-	-	-
0.93	-	-	2222.1	2.28	1117.1	1.04	-	-	-	-	-	-
0.94	4974.7	4.02	-	-	-	-	-	-	975.6	1.25	862.2	1.13
0.97	-	-	871.5	0.90	2996.6	2.80	-	-	-	-	-	-
0.98	-	-	-	-	-	-	549.6	0.49	369.1	0.47	341.3	0.45

4.2.1.3 Methanol Extract

Samples were extracted using methanol and band separation was performed in mobile phase i.e. toluene:ethyl acetate:formic acid:methanol (6.0:2.7:0.6:0.7 v/v). Like previous solvents, in this extract also the difference in banding pattern and chromatograms was recorded when observed and scanned at different wavelengths.

- **254 nm**

All samples showed variation in profiling when observed in 254 nm (Fig. 27a). Similarly scanning generated almost similar peaks i.e. 10-11 for samples L1-L4. The maximum area was 16015.3 of a peak at Rf 0.22 in L1 sample and 23160.1, 17714.1 and 10089.7 were for peak at Rf 0.03 for L2-L4 (Fig. 28a-d). L5 sample showed total 12 peaks and at Rf 0.03 it was observed the maximum area of peak (6252.0) (Fig. 28e). L6 samples appeared with same 7 peaks and highest peak area 19201.8 was obtained at Rf 0.66 (Fig. 28f). Chromatograms resulted with different pattern at respective Rf and when all the samples were compared, it identified presence of some distinct peaks. Peak at Rf 0.02 was present in all the samples (L1-L6) while at Rf 0.05, 0.22 and 0.63 represented unique peaks in L1 sample. In L5 sample Rf (0.25) a distinct peak was recorded and one another peak at Rf 0.66 was present in L5 and L6 samples. Some of peaks at Rf (0.84 and 0.93) were seen in all the *in vitro* samples (L2-L6) (Table 14).

- **366 nm**

Changing the UV wavelength to 366 nm also revealed change in banding pattern when all samples were compared (Fig. 27b). L1 sample reported 9 peaks and maximum area of 22592.1 on the chromatogram at Rf (0.22) (Fig. 28g). Whereas in samples L2, L3, L4 and L6 total 9, 12, 9 and 6 peaks were appeared and highest peak areas (15780.4, 9934.4, 6951.2 and 6444.6) were observed at same Rf 0.03 (Fig. 28h, i, j, l). Whereas L5 sample showed 5 peaks of which the highest area of 4756.7 was for a peak at Rf 0.52 (Fig. 28k). Chromatograms revealed that some distinct peaks were found i.e. at Rf (0.03) peak present in only *in vitro* samples (L2-L6) therefore this peak was unique. Same way some peaks noted at Rf (0.22, 0.37 and 0.50) were only present in *in vivo* sample and absent in all *in vitro* samples. Similarly, few peaks showed clear difference between callus culture samples and other *in vitro/in vivo* samples, whereas *in vitro* shoot samples also showed unique peaks at Rf (0.54, 0.58 and 0.97) (Table 15).

Table 14. Peak areas of different samples at 254 nm for methanol extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	10594.7	19.08	-	-	17714.1	40.41	-	-	-	-	-	-
0.03	-	-	23160.1	58.74	-	-	10089.7	28.51	6252.0	17.00	5895.7	9.93
0.05	5284.0	9.52	-	-	-	-	-	-	-	-	-	-
0.09	-	-	-	-	-	-	213.0	0.60	-	-	-	-
0.10	-	-	-	-	1367.4	3.12	-	-	-	-	-	-
0.13	-	-	-	-	1171.8	2.67	-	-	-	-	-	-
0.16	-	-	2451.3	6.22	-	-	-	-				
0.18	-	-	-	-	-	-	-	-	1860.1	5.06	940.6	1.58
0.19	-	-	-	-	2961.2	6.76	2486.4	7.03	-	-	-	-
0.22	16015.3	28.84	-	-	-	-	-	-	-	-	-	-
0.25	-	-	-	-	-	-	-	-	642.1	1.75	-	-
0.28	-	-	2317.0	5.88	-	-	-	-			-	-
0.29	-	-	-	-	-	-	-	-	374.5	1.02	-	-
0.31	-	-	-	-	-	-	5367.5	15.17	-	-	-	-
0.32	-	-	-	-	5180.0	11.82	-	-	-	-	-	-
0.37	6804.6	12.26	-	-	-	-	-	-	3509.5	9.54	4408.9	7.42
0.38	-	-	546.4	1.39	-	-			-	-	-	-
0.39	-	-	-	-	-	-	2441.7	6.90	-	-	-	-
0.40	-	-	583.6	1.48	-	-	-	-	-	-	-	-
0.41	-	-	-	-	3447.4	7.86	-	-	-	-	-	-
0.42	-	-	-	-	-	-	-	-	2761.9	7.51	-	-
0.47	2652.7	4.78	-	-	-	-	-	-	-	-	-	-
0.48	-	-	987.2	2.50	-	-	-	-	808.2	2.20	-	-
0.49	-	-	-	-	-	-	1533.8	4.33	-	-	-	-
0.50	-	-	-	-	1320.9	3.01	-	-	-	-	16130.0	27.16
0.53	-	-	-	-	-	-	-	-	5250.6	14.28	-	-

0.54	3036.6	5.47	3199.8	8.12	-	-	3333.8	9.42	-	-	-	-
0.55	-	-	-	-	2939.6	6.71	-	-	-	-	-	-
0.59	5926.6	10.67	417.9	1.06	651.6	1.49	539.2	1.52	399.8	1.09	-	-
0.63	1501.5	2.70	-	-	-	-	-	-	-	-	-	-
0.66	-	-	-	-	-	-	-	-	3497.6	9.51	19201.8	32.34
0.72	444.5	0.80	-	-	-	-	-	-	-	-	-	-
0.84	-	-	-	-	-	-	987.4	2.79	2223.0	6.04	2415.1	4.07
0.85	-	-	506.0	1.28	480.1	1.10	-	-	-	-	-	-
0.88	590.5	1.06	-	-	-	-	-	-	-	-	-	-
0.93	-	-	-	-	-	-	-	-	9202.2	25.02	10387.8	17.49
0.94	-	-	5256.5	13.33	6600.2	15.06	8393.5	23.72	-	-	-	-
0.96	2673.7	4.82	-	-	-	-	-	-	-	-	-	-

Table 15. Peak areas of different samples at 366 nm for methanol extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	4015.7	6.73	-	-	3087.0	7.88	-	-	-	-	-	-
0.02	8833.3	14.81	-	-	-	-	-	-	-	-	-	-
0.03	-	-	15780.4	43.50	9934.4	25.36	6951.2	20.42	3721.1	26.74	6444.6	25.35
0.16	-	-	4748.2	13.09	-	-	-	-	-	-	-	-
0.17	-	-	-	-	-	-	-	-	306.7	2.20	-	-
0.19	-	-	-	-	-	-	5540.4	16.28	-	-	-	-
0.20	-	-	-	-	5832.5	14.89	-	-	-	-	-	-
0.22	22592.1	37.89	-	-	-	-	-	-	-	-	-	-
0.24	-	-	-	-	-	-	-	-	-	-	-	-
0.28	-	-	2803.8	7.73	-	-	-	-	-	-	-	-
0.30	185.3	0.31	-	-	-	-	-	-	-	-	-	-
0.31	-	-	-	-	-	-	5048.9	14.83	-	-	-	-
0.32	-	-			5153.9	13.16	-	-	-	-	-	-
0.37	5142.1	8.62	-	-	-	-	-	-	-	-	-	-
0.40	-	-	-	-	178.1	0.45	-	-	-	-	3705.6	14.58
0.42	-	-	1285.3	3.54			-	-	1942.7	13.96	-	-
0.43	-	-	-	-	-	-	-	-	-	-	2685.6	10.56
0.44	-	-	-	-	-	-	1532.0	4.50	-	-	-	-
0.45	-	-	-	-	1256.8	3.21	-	-	-	-	-	-
0.47	-	-	1261.7	3.48	-	-	2471.8	7.26	-	-	-	-
0.48	-	-	-	-	1511.6	3.86	-	-	-	-	-	-
0.49	-	-	-	-	-	-	-	-	-	-	3771.9	14.84
0.50	3765.3	6.31	-	-	-	-	-	-	-	-	-	-
0.52	-	-	-	-	-	-	-	-	4756.7	34.18	4510.6	17.74
0.54	2832.1	4.75	6065.8	16.72	-	-	6946.6	20.41	-	-	-	-
0.55	-	-	-	-	5771.1	14.73	-	-	-	-	-	-

0.58	-	-	959.9	2.65	-	-	1473.8	4.33	-	-	-	-
0.59	11042.6	18.52	-	-	1481.2	3.78	-	-	-	-	-	-
0.79	-	-	-	-	459.9	1.17	-	-	-	-	-	-
0.93	-	-	-	-	-	-	-	-	3189.0	22.92	4303.0	16.93
0.94	-	-	-	-	2437.5	6.22	2599.6	7.64	-	-	-	-
0.95	-	-	2115.3	5.83	-	-	-	-	-	-	-	-
0.97	1216.7	2.04	1255.5	3.46	2070.5	5.29	1477.2	4.34	-	-	-	-

- **525 nm**

Fingerprint formed after derivatization showed almost pattern in L1 and L5 which were different from rest of the samples (Fig. 27c). Different profile with number of peaks and areas were recorded after scanning and it was observed that L1 and L2 samples recorded different number of peaks (14 and 12). The maximum areas (7463.7 and 26272.7) were recorded for peak at same Rf (0.05) but vast difference was observed in them (Fig. 28m, n). L3 and L4 samples showed peaks (16 and 14) and highest areas were at same Rf 0.08 but slight variation was noted i.e. 13870.0, 15250.3 (Fig. 28o, p). L5 sample noted 17 peaks and peak present at Rf (0.42) had maximum area was 13093.9 (Fig. 28q). Whereas L6 sample showed total 15 peaks and area 22886.5 at Rf 0.51 (Fig. 28r). There was few unique peaks were found from the chromatograms of all the samples. Peaks at Rf (0.13 and 0.69) were only present in callus cultures (L5 and L6) whereas in L1 sample distinct peaks were observed at Rf (0.20, 0.78 and 0.85). Similarly peak at Rf (0.33) was present in all the samples while sample L6 also identified unique peaks at various Rf (0.73, 0.83, 0.96 and 0.97) (Table 16).

- It was concluded from fingerprint analysis that all the selected *in vitro* samples (shoot and callus) were able to synthesize metabolites. The biosynthetic potentency of *in vitro* shoot cultures was at par with *in vivo* shoots, whereas callus cultures synthesized comparatively less metabolites. Thus, it was concluded that *in vitro* nodal derived cultures were chemically similar to *in vivo* shoots.

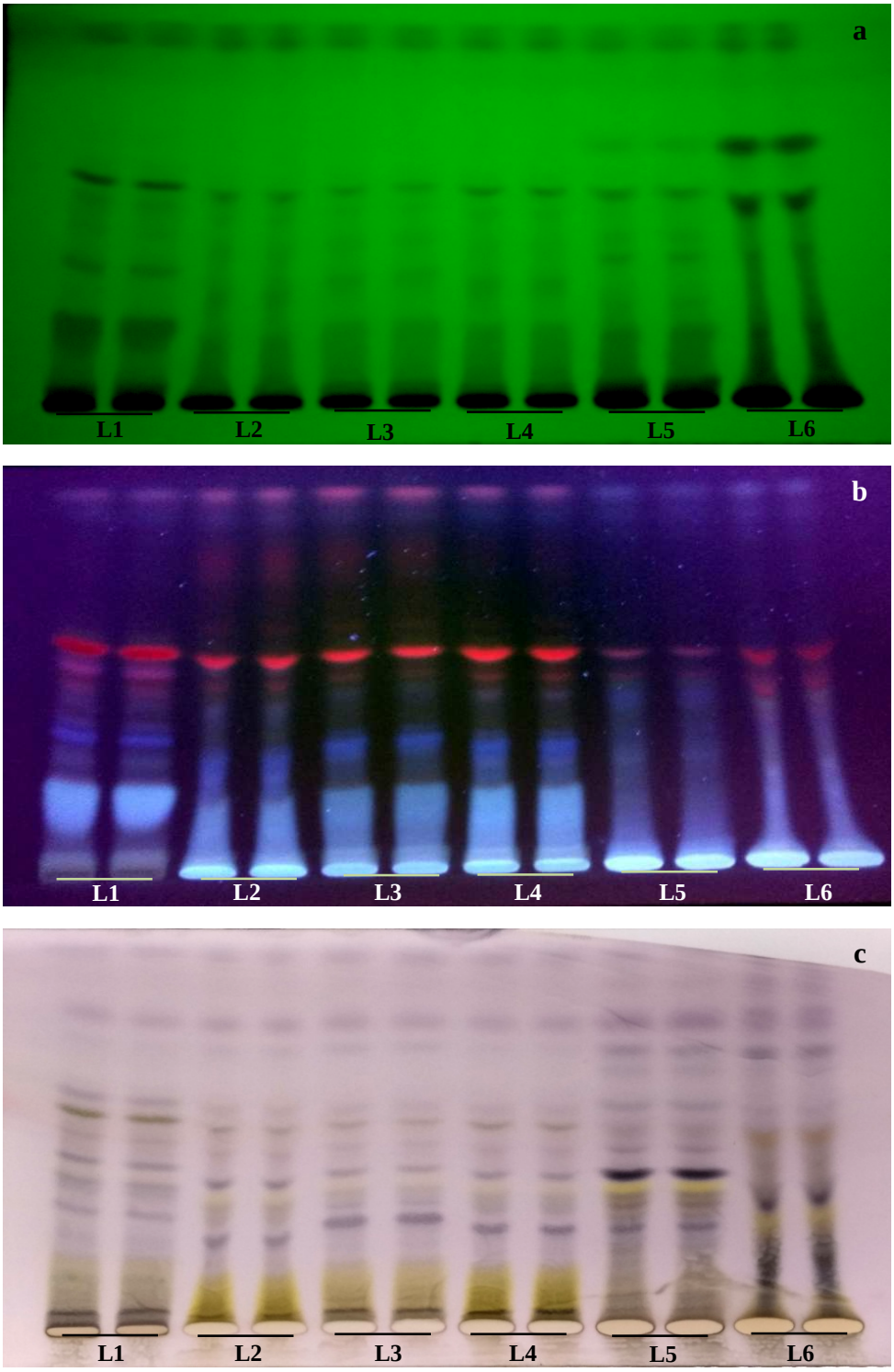

Figure 27. HPTLC fingerprints of methanol extract for different samples of *L. reticulata*: (a) UV 254 nm, (b) UV 366 nm and (c) white light after derivatization.

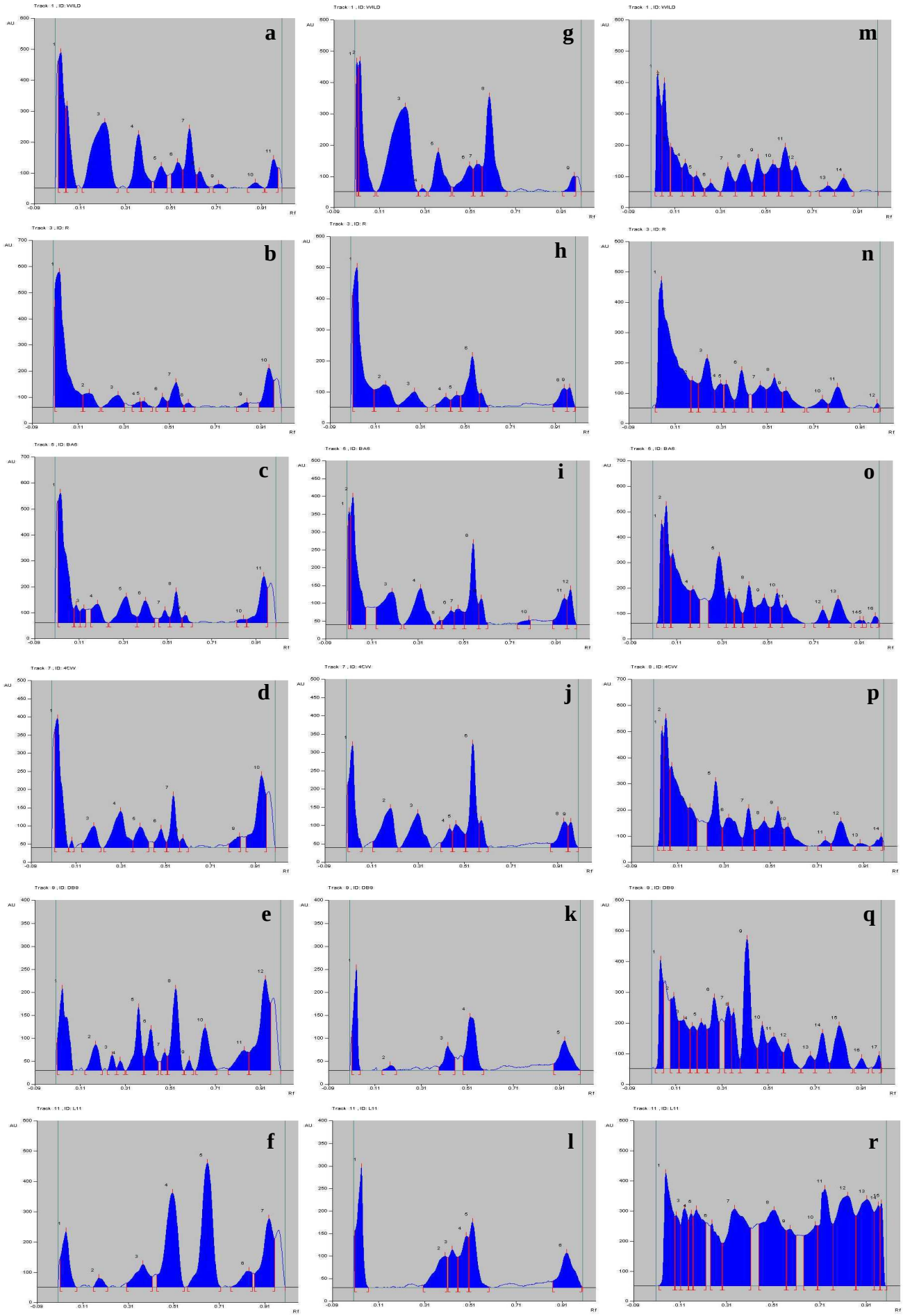

Figure 28. Chromatograms of different samples for methanol extract in *L. reticulata*: (a-f) 254 nm, (g-l) 366 nm and (m-r) 525 nm.

Table 16. Peak areas of different samples at 525 nm for methanol extracts.

Rf value	L1		L2		L3		L4		L5		L6	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.03	7199.1	14.75	-	-	-	-	-	-	-	-	-	-
0.04	-	-	-	-	5843.8	8.06	5386.2	7.57	6230.4	7.58	-	-
0.05	-	-	26272.7	45.04	-	-	-	-	-	-	12533.3	7.21
0.06	7463.7	15.29	-	-	9826.2	13.55	9737.7	13.68	-	-	-	-
0.08	-	-	-	-	-	-	15250.3	21.43	-	-	-	-
0.09	4912.2	10.07	-	-	13870.0	19.13	-	-	-	-	4900.1	2.82
0.10	-	-	-	-	-	-	-	-	6770.8	8.23	-	-
0.13	-	-	-	-	-	-	-	-	-	-	6869.2	3.95
0.14	-	-	-	-	-	-	-	-	6189.6	7.53	-	-
0.16	2970.6	6.09	-	-	-	-	4227.6	5.94	-	-	3937.1	2.26
0.18	-	-	2500.2	4.29	4476.2	6.17	-	-	3464.2	4.21	11298.0	6.50
0.20	1453.8	2.98	-	-	-	-	-	-	-	-	-	-
0.22	-	-	-	-	-	-	-	-	5300.7	6.44	-	-
0.25	-	-	6886.2	11.81	-	-	-	-	-	-	8010.4	4.61
0.27	787.5	1.61	-	-	-	-	8283.2	11.64	8021.6	9.75	-	-
0.30	-	-	-	-	11133.5	15.35	-	-	-	-	-	-
0.31	-	-	2317.9	3.97	-	-	-	-	-	-	-	-
0.33	-	-	2222.9	3.81	-	-	6182.7	8.69				
0.34	2521.5	5.17	-	-	3382.5	4.66	-	-	5074.5	6.17	22291.6	12.82
0.36	-	-	-	-			-	-	3751.2	4.56	-	-
0.37	-	-	-	-	2334.1	3.22	-	-	-	-	-	-
0.40	-	-	4012.5	6.88	-	-	-	-	-	-	-	-
0.41	-	-	-	-	-	-	4026.9	5.66	-	-	-	-
0.42	3864.6	7.92	-	-	-	-	-	-	13093.9	15.92	-	-
0.43	-	-	-	-	4309.8	5.94	-	-	-	-	-	-
0.47	3387.1	6.94	-	-	-	-	-	-	-	-	-	-

0.48	-	-	3381.5	5.80	-	-	4495.5	6.32	4034.7	4.91	-	-
0.49	-	-	-	-	4360.6	6.01	-	-	-	-	-	-
0.51	-	-	-	-	-	-	-	-	-	-	22886.5	13.16
0.53	-	-	-	-	-	-	-	-	4890.4	5.95	-	-
0.54	3993.8	8.18	4348.9	7.46	-	-	4772.3	6.71	-	-	-	-
0.55	-	-	-	-	3670.8	5.06			-	-	-	-
0.58	-	-	-	-			3005.0	4.22	-	-	6690.9	3.85
0.59	5147.8	10.55	2297.1	3.94	2842.5	3.92	-	-	2550.6	3.10	-	-
0.64	2878.7	5.90	-	-	-	-	-	-	-	-	-	-
0.69	-	-	-	-	-	-	-	-	1292.5	1.57	9684.2	5.57
0.73	-	-	-	-	-	-	-	-	-	-	14744.8	8.48
0.74	-	-	-	-	-	-	650.6	0.91	3683.0	4.48	-	-
0.75	-	-	1127.5	1.93	1531.0	2.11	-	-	-	-	-	-
0.78	621.9	1.27	-	-	-	-	-	-	-	-	-	-
0.81	-	-	2761.8	4.73	-	-	3918.7	5.51	6295.5	7.65	-	-
0.82	-	-	-	-	3992.2	5.51	-	-	-	-	-	-
0.83	-	-	-	-	-	-	-	-	-	-	22792.4	13.11
0.85	1598.3	3.28	-	-	-	-	-	-	-	-	-	-
0.90	-	-	-	-	-	-	348.1	0.49	-	-	-	-
0.91	-	-	-	-	254.1	0.35	-	-	860.6	1.05	18345.8	10.55
0.93	-	-	-	-	120.5	0.17	-	-	-	-	-	-
0.96	-	-	-	-	-	-	-	-	-	-	5667.5	3.26
0.97	-	-	-	-	-	-	-	-	-	-	3203.8	1.84
0.98	-	-	203.6	0.35	563.2	0.78	-	-	-	-	-	-
0.99	-	-	-	-	-	-	883.0	1.24	741.7	0.90	-	-

HPTLC fingerprint of different samples revealed that the culture type and PGRs affected metabolite synthesis. All the extracts at different wavelengths showed that the profile of shoot cultures were at par with *in vivo* shoots, but the number of bands were less in callus samples. This is in accordance with the report on callus and shoot cultures of *Oroxylum indicum* where the fingerprint changesd between *in vitro* cultures and also in comparison to *in vivo* plant (Rojsanga et al., 2017). Similarly variation was observed between three extract for fingerprint of callus and *in vivo* samples and same is reported in *Gyrinops walla* (Selvaskanthan et al., 2020). They have also observed that when 2,4-D was present in the medium it failed to increase number of bands as well as decrease the intensity of bands in callus samples, which is in line with present result. Recently, Buranasudja et al. (2021) documented that HPTLC profiling of callus culture of *Centella asiatica* was different as compared to mother plant. Possible explanation to this in direct regeneration there is a definite organization, as the cells are oriented towards a specific biological development, which leads to a uniform biosynthesis of secondary metabolites. On the other hand, calli have an unorganized constitution, where cells might not be active and hence are oriented towards a defined development and secondary metabolite production (Mukherjee et al., 2020). The same phenomenon has been reported by Jain et al. (2011) in *Withania somnifera* in which the metabolites synthesis takes place in specific tissues or organs and are lacking in callus tissue. Similarly in *Picrorhiza kurroa in vitro* cultures, only shoots had the potential to synthesize picroside production but callus cultures failed to synthesize (Sood and Chauhan, 2009). Similarly effect of different regeneration pathways showed variation in fingerprint in *Curculigo orchioides* (Alagar et al., 2014), *Curcuma longa* (Kharade et al., 2014), *Celastrus paniculatus* (Anusha et al., 2016) and *Crataeva tapia* (Sharma et al., 2016). Another reason for change in HPTLC fingerprint is because of influence of PGRs like cytokinins and auxins on biosynthesis of different groups of secondary metabolites (Ekiert et al., 2008; El Tahchy et al., 2011; Grover et al., 2012; Joshaghani et al., 2014; Thiruvengadam and Chung, 2015).

HPTLC fingerprint with a visible pattern of bands and is used to determine the number of components in a mixture (Reich and Schibli, 2006). However in *L. reticulata* variation in number of bands was observed among the different extracts, it might be due to efficacy of solvents to extract group of metabolites and same is observed in *Amsonia orientalis* extracts where number of bands varies between polar, mid-polar and non-polar solvents (Acemi et al., 2020). This technique has also been used to assess the biosynthetic potential of *in vitro* samples of other Asclepiadaceae members like *Ceropegia juncea* (Nikam and Savant, 2009),

Hemidesmus indicus (Pathak et al., 2017) and *Tylophora indica* (Patel et al., 2021) as well as other plants like *Asparagus adscendens* (Mehta and Subramanian, 2005), *Bacopa monnieri* (Srivastava and Shrivastava, 2008) and *Chlorophytum borivilianum* (Basu and Jha, 2013).

4.2.2 Quantitative Analysis

The metabolite selected for the present study was *p*-coumaric acid therefore initially a standard curve of *p*-coumaric acid was prepared and method validation study for HPTLC was performed. In the further experimental work, quantity of *p*-coumaric acid was assessed using different samples.

4.2.2.1 Standard Curve for p-Coumaric Acid and HPTLC Method Validation

Linear relationship was observed by plotting a six point calibration curve of standard (*p*-coumaric acid) concentration against peak areas. *p*-coumaric acid showed linear response in the concentration range of 100-600 µg/spot (Fig. 29). The corresponding linear regression equation was y = 19.969x + 1343 with correlation coefficient (R^2) of 0.998 for *p*-coumaric acid. Residual analysis was performed to ascertain linearity. The slope and intercept of the curve were also determined and it was 1996 and 1343 respectively (Table 18).

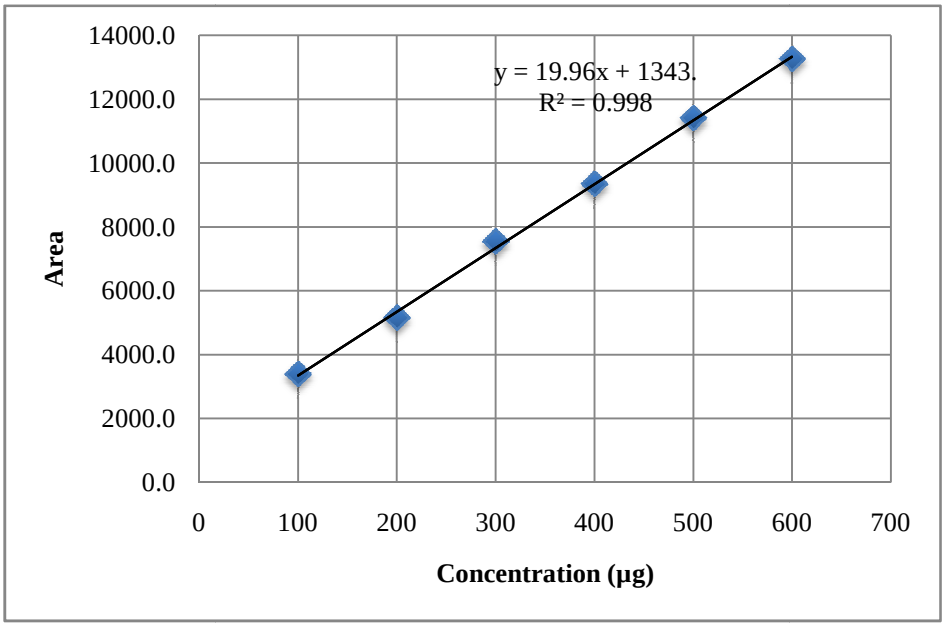

Figure 29. Standard curve of *p*-coumaric acid.

HPTLC method was validated according to International Conference on Harmonisation (ICH) guidelines (ICH, 1997) for linearity, limit of detection (LOD) and limit of quantification (LOQ), precision and specificity.

- **Limit of Detection (LOD) and Limit of Quantification (LOQ)**

The signal-to-noise ratios of 3:1 and 10:1 were considered as LOD and LOQ. The LOD and LOQ were found to be 10 µg/spot and 100 µg/spotrespectively (Table 18).

- **Precision**

The results of the repeatability and intermediate precision analysis are shown in Table 18. The repeatability of the method was tested by analysing a definite amount (400 µg/spot) of the standard for six times after application on TLC plate, which resulted in RSD of 1.7%. The intra and inter-day variation of nine replicates for the determination of *p*-coumaric acid was carried out at three different concentration levels of 200, 400 and 600 µg/spot (Table 17).

Table 17. Intra-day and inter-day precisions for method validation.

Sr. No.	Standard concentration (µg)	Intra-day CV (%)	Inter-day CV (%)
1	200	1.9	2.0
2	400	1.7	1.3
3	600	1.5	2.1
Average	-	**1.7**	**1.8**

- **Specificity**

To validate the method for specificity, sample and standard were subjected to TLC analysis. Chromatogram showed the band corresponding to the standard *p*-coumaric acid and at the same Rf (0.23) in the sample. Further spectral analysis (λ_{max} 254 nm) was done for confirmation and it revealed that the peaks obtained from both standard and test samples were identical, as they had similar pattern (Table 18).

Table 18. HPTLC method validation parameters for *p*-coumaric acid.

Parameters	Results
Linearity range (µg/spot)	100-600 µg
Linear regression equation	y = 19.969x + 1343
Correlation coefficient (R^2)	0.998
Standard error of slop	1996
Standard error of intercept	1343
Limit of detection (LOD)	10 µg
Limit of quantification (LOQ)	100 µg
Precision (%RSD)	
Repeatability (n = 6)	1.9
Intra-day precision (n = 9)	1.7
Inter-day precision (n = 9)	1.8
Specificity	Specific

4.2.2.2 Quantification of p-Coumaric Acid

Methanol extract of different samples when separated on TLC plate, presence of *p*-coumaric acid was confirmed as band in all the samples (Fig. 30a) and by scanning it generated chromatograms (Fig. 30b). Further spectral analysis was done for confirmenation of *p*-coumaric acid with respect to standard (Fig. 30c). The area under the curve (AUC) obtained after scanning at 254 nm was different for all the samples. It was recorded that for *in vivo* and *in vitro* shoot samples (L1-L4) band on TLC plate were at similar Rf (0.23) to standard and the corresponding peak of sample to standard also resulted after densitomatric scanning. Further when AUC were recorded, it revealed that the content of *p*-coumaric acid in sample L1-L4 were in range of LOD but out of range for LOQ. On the other hand, maximum quantity was recorded in callus samples L6 i.e. 78.08 ± 2.20 mg/gm, whereas it was less in sample L5 i.e. 16.22 ± 0.09 mg/gm (Table 19). It was accomplished that callus culture (L6) were developed in medium supplemented with BA (20 µM) + NAA (0.5 µM) derived through leaf explants gave higher content of *p*-coumaric acid. Therefore this callus culture was beneficial for the quantification or elicitation of *p*-coumaric acid.

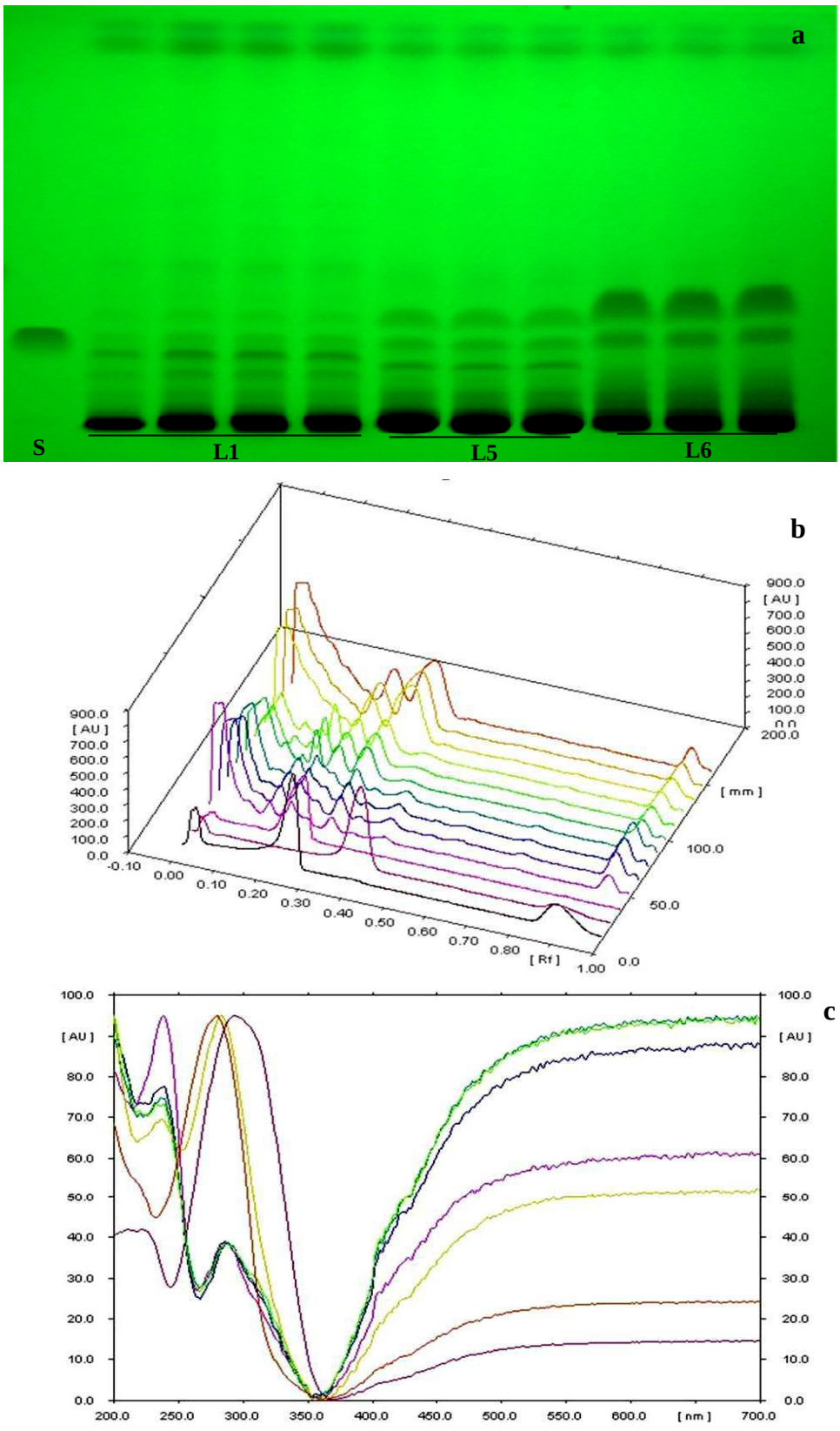

Figure 30. Quantification of *p*-coumaric acid using HPTLC in samples of *L. reticulata*: (a) TLC plate under UV 254 nm containing standard (S), *in vivo* shoots (L1), callus from BA (15 µM) + 2,4-D (1.0 µM) (L5) and callus from BA (20 µM) + NAA (0.5 µM) (L6) (b) densitometric scanning of plate at UV 254 nm and (c) spectra of samples at UV 254 nm.

Table 19. Quantification of *p*-coumaric acid in *in vivo* and *in vitro* samples of *L. reticulata* using HPTLC.

Sample	Source	Medium	*p*-Coumaric acid quantity (mg/g)*
L1	*In vivo* shoots	-	NQ
L2	Nodal derived shoots	BA (5 µM) + Kn (5 µM)	NQ
L3	Nodal derived shoots	BA (10 µM) + $AdSO_4$ (10 µM)	NQ
L4	Nodal derived shoots	CW (4%)	NQ
L5	Leaf derived callus	BA (15 µM) + 2,4-D (1.0 µM)	16.22 ± 0.09 b
L6	Leaf derived callus	BA (20 µM) + NAA (0.5 µM)	78.08 ± 2.20 a

NQ- Not Quantifiable. *Values represent mean ± SE. Means (n = 3) followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test.

- Thus it was inferred that although HPTLC profiles of shoot cultures were at par with *in vivo* shoots, selected metabolite wasn't quantifiable in any of these shoot samples, and it was only present in detectable amount in callus cultures. Nevertheless callus from both the media synthesized *p*-coumaric acid, it was maximum in leaf callus derived from BA (20 µM) + NAA (0.5 µM), thus this media was further taken up for elicitation study.

Shoot and callus cultures derived from media having different PGR composition were analyzed for *p*-coumaric acid content and it was observed that amongst all the cultures it was quantifiable only in callus cultures and not in shoot cultures. This is in corroboration with response in *Larrea divaricata* (Palacio et al., 2012) and *Aronia melanocarpa* (Szopa et al., 2013) where *p*-coumaric acid synthesized only in undifferentiated cultures and not in shoot cultures. Similarly Panwar and Guru (2011) depicted that alkaloid contents in *in vitro* leaves yielded less reserpine, whereas stem and leaf-derived calli yielded higher reserpine. Moreover, higher essential oil synthesis is reported in callus cultures of *Curcuma longa* as compared to mother plants (Kuanar et al., 2012). The elevated content of targeted metabolite in callus cultures makes these cultures a promising source for production of valuable secondary metabolites from different important medicinal plants as compared to *in vivo* plants (Murthy et al., 2014). Callus cultures are reported to be good source for synthesis of pharmaceutically important secondary metabolites e.g. asciaticoside in *Centella asiatica* (Gandi and Giri, 2013), picroside-II in *Picrorhiza kurroa* (Rehman et al., 2014), β-sitosterol

in *Crataeva tapia* (Sharma et al., 2016), berberine in *Tinospora cordifolia* (Mittal and Sharma, 2017) and baicalein in *Oroxylum indicum* (Faraz et al., 2020).

Apart from culture type, another factor which affects secondary metabolite synthesis is PGRs as they not only enhance the growth and differentiation of cultures but also alters the synthesis of secondary metabolites (Dornenburg and Knorr, 1995). Cytokinins of the medium are known to repress transporters of macronutrients such as nitrate, ammonium, sulphate, phosphate, and also regulate the expression of *PAL* gene of phenylpropanoid pathway (Sakakibara et al. 2006). Similarly the type of auxins also affects metabolite synthesis as it is well documented that auxin response factors (ARFs) interacts with auxin regulatory genes related to several physiological and biochemical processes, and in turn affects secondary metabolite synthesis (Chapman and Estelle, 2009). Ahmad et al. (2019) observed that synthesis of *p*-coumaric acid is affected by PGRs of the medium in *Ipomoea turbinate*. When *p*-coumaric acid was analyzed in cultures of *L. reticulata*, higher content was recorded in callus grown in presence of BA and NAA as compared to medium supplemented with BA and 2,4-D. The amount of harmaline and harmine was more in presence of BA-NAA as compared to BA-2,4-D where as contrary results are reported for diosgenin accumulation in callus of *Tribulus terrestris* (Nikam et al., 2009). Study on *Echinacea purpurea* also suggest that phenolics in callus cultures is affected by combinations of cytokinin with either NAA or 2,4-D (Ramezannezhad et al., 2019). Recently in callus of *Amsonia orientalis* it has been observed that the metabolite content is more in presence of cytokinin with auxin (Selvaskanthan et al., 2020). Beneficial effect of BA-NAA combination on accumulation of *p*-coumaric acid in *Hypericum perforatum* cvs. Helos (Kwiecień et al., 2015) as well as in other metabolites like phenolic acids in *Ruta graveolens* (Ekiert et al., 2008), lanatoside C and digoxin in *Digitalis ferruginea* (Verma et al., 2014) and cucurbitacins E and I in *Ecballium elaterium* (El-Mekkawy et al., 2018) is well reported. Generally 2,4-D is known to adversely affect metabolite production because of its less efficiency for triggering secondary metabolism (Mantell and Smith, 1983; Misawa, 1985). In line with present results, stimulatory effect of NAA and adverse effect of 2,4-D on production of metabolites through callus cultures is reported in other Asclepiadaceae members i.e. *Hemidesmus indicus* (Misra et al., 2005) and *Ceropagea juncea* (Nikam and Savant, 2009).

4.2.3 Elicitation of *p*-Coumaric Acid

Callus cultures were developed in presence of BA (20 µM) + NAA (0.5 µM) for the elicitation of *p*-coumaric acid using two elicitors i.e. yeast extracts and salicylic acid in different concentrations. Cultures were developed and growth was also monitored weekly from induction to declining stage. Time interval needs to be assessed for sufficient callus biomass and therefore growth curve was prepared.

4.2.3.1 Callus Growth Determination

The present study was carried out for the determination of the growth pattern of callus culture in *L. reticulata*. This study was conducted to identify the onset and duration of the various growth stages. Initially callus growth progressed slowly, but as time passed, growth proceeded rapidly then reached to a steady state and then declined. Initially there was slow growth period which was up to three weeks, then callus started to proliferate by four weeks and maximum biomass was occurred at five weeks. Successively growth deceleration occurred from the beginning of the sixth week. This study showed that sufficient biomass of callus was obtained during four to five weeks (Fig. 31).

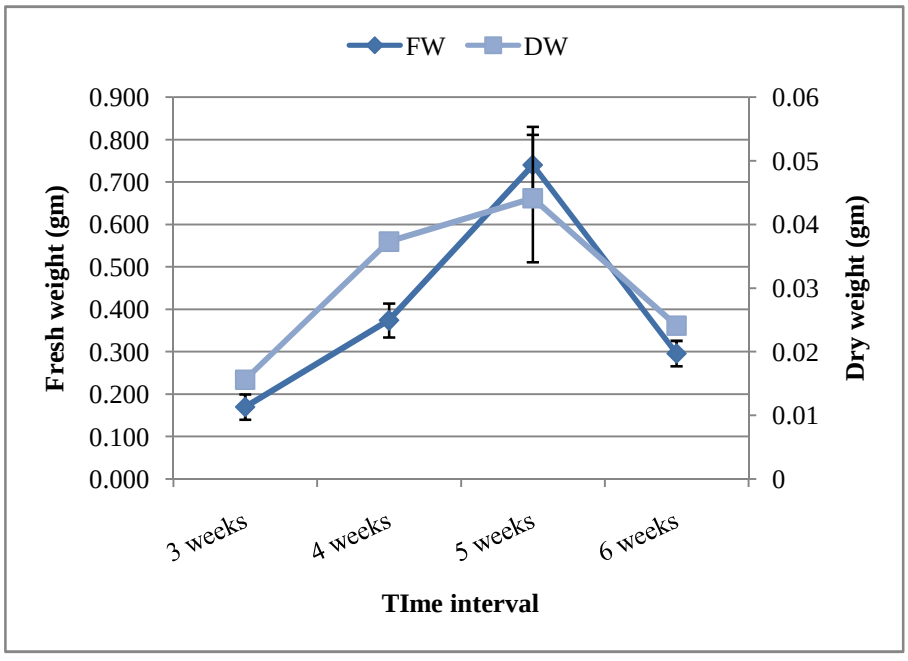

Figure 31. Growth determination of callus till six weeks.

Each line shows the mean values (n = 5) and error bar as standard error.

4.2.3.2 p-Coumaric Acid Content in Control Callus Culture

According to the growth curve study, the cultures grown in absence of elicitors served as control and were harvested at four and five weeks. Callus proliferated in the medium and the biomass in terms of FW (0.374 ± 0.04 gm) and DW (0.039 ± 0.00 gm) was recorded at four weeks and reached maximum in five weeks i.e. FW (0.740 ± 0.09 gm) and DW (0.051 ± 0.00 gm) (Fig. 32). HPTLC analysis of control samples detected the presence of *p*-coumaric acid only in five weeks old sample and not in sample of four weeks. Densitometric scanning at 254 nm of the five week old sample confirmed that the peak corresponded to the standard. When area under curve (AUC) for five weeks old sample was obtained, it was out of the range of LOQ (Fig. 33a, b) (Table. 20).

4.2.3.3 Effect of Yeast Extract and Salicylic Acid on Callus Biomass and p-Coumaric Acid Content

As *p*-coumaric acid was not detected in control sample, in this experiment the explants were treated with YE and SA elicitors. Leaf explants were placed in the medium supplemented with YE/SA and allowed to grow till five weeks. The presence of YE (25-200 mg/l) in the medium facilitated callus proliferation in almost all the concentrations within five weeks as the biomass of shoots (FW and DW) increased. Further analyzing the samples on TLC plates confirmed band of *p*-coumaric acid in all the concentrations as a peak after densitometric scanning at 254 nm. After scanning variation was observed in content of *p*-coumaric acid among all the concentrations and weeks.

- **25 mg/l**

When 25 mg/l of YE was added to the medium callus biomass increased as their FW (0.597 ± 0.04 gm) and DW (0.051 ± 0.00 gm) at four weeks was higher than control. Whereas when callus that was harvested in five weeks the FW (0.710 ± 0.19 gm) and DW (0.040 ± 0.01 gm) was less as compared to five weeks old control sample (Fig. 32). *p*-Coumaric acid quantity was analyzed after scanning at 254 nm, it was observed that peak of *p*-coumaric acid in samples was corresponding to the standard. The content of metabolite was not quantifiable in four weeks sample, and it was able to quantified (22.55 ± 0.36 mg/gm) when time interval was increased for treatment to the cultures till five weeks (Fig. 33c, d) (Table 20).

- **50 mg/l**

YE concentration was increased in the medium to 50 mg/l, and the biomass i.e. FW (0.452 ± 0.03 gm) and DW (0.038 ± 0.00 gm) which was noted in five weeks was less as compared to 25 mg/l concentration of YE but it was higher than control. The callus became proliferative and that can seen from biomass as FW (0.800 ± 0.08 gm) and DW (0.046 ± 0.00 gm) was recorded by fourth week (Fig. 32). But at this concentration, YE failed to trigger the synthesis of *p*-coumaric acid and hence it coulden't be quantified after four weeks. During the fifth week this elicitor was able to elicit the content of *p*-coumaric acid to 22.95 ± 0.81 mg/gm which was almost identical to the content obtained at 25 mg/l (Fig. 33c, d) (Table 20).

- **100 mg/l**

Maximum callus biomass was recorded when medium was supplemented with 100 mg/l of YE for both four and five weeks i.e. FW (0.649 ± 0.09 gm) and DW (0.050 ± 0.01 gm), FW (0.927 ± 0.09 gm) and DW (0.077 ± 0.00 gm) respectively (Fig. 32). However, the densitometric scanning showed that *p*-coumaric acid was quantified only in four weeks i.e. 10.72 ± 1.11 mg/gm, which was less as compared to the lower YE concentrations (25 and 50 mg/l). Increase in YE concentration inhibited the synthesis and by five weeks it was not quantified (Fig. 33e,f) (Table 20).

- **200 mg/l**

Further increasing the concentration to 200 mg/l, slightly decreased the biomass i.e. FW (0.574 ± 0.09 gm) and DW (0.041 ± 0.01 gm) at four weeks. A slight increase in FW (0.726 ± 0.06 gm) and DW (0.064 ± 0.01 gm) was noted at five weeks (Fig. 32). At this concentration *p*-coumaric acid was present in both weeks which was not observed in any of the earlier YE concentrations tried. Callus cultures at four weeks were able to synthesize maximum content of p-coumaric acid i.e. 88.17 ± 2.72 mg/gm, which was 3.91 fold higher than 25 mg/l. When cultures were assessed for five weeks it was revealed that the content decreased to 41.44 ± 0.59 mg/gm which was nearly half as compared to the earlier weeks (Fig. 33e, f) (Table 20).

- Thus, it could be concluded from the elicitation experiment that yeast extract was beneficial for callus biomass as well as for eliciting *p*-coumaric acid content within four weeks.

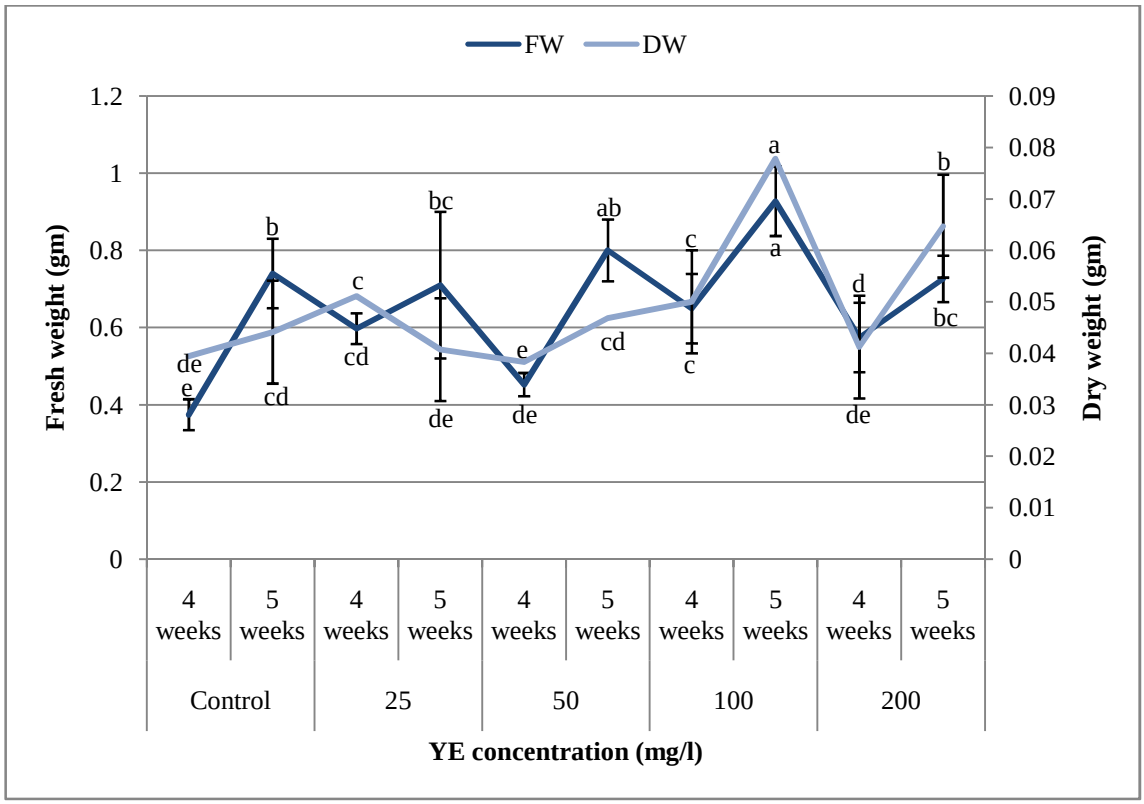

Figure 32. Effect of yeast extract on callus biomass derived through leaf explants of *L. reticulata*.

Each line shows the mean values (n = 5) and error bar as standard error. Means followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Table 20. Effect of different concentrations of YE on *p*-coumaric acid content.

YE concentrations (mg/l)	*p*-Coumaric acid (mg/gm)*	
	4 weeks	5 weeks
Control	ND	NQ
25	NQ	22.55 ± 0.36 bc
50	NQ	22.95 ± 0.81 bc
100	10.72 ± 1.11 c	NQ
200	88.17 ± 2.72 a	41.44 ± 0.59 b

ND- Not Detectable, NQ- Not Quantifiable

*Values represent mean ± SE. Means (n = 3) followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test.

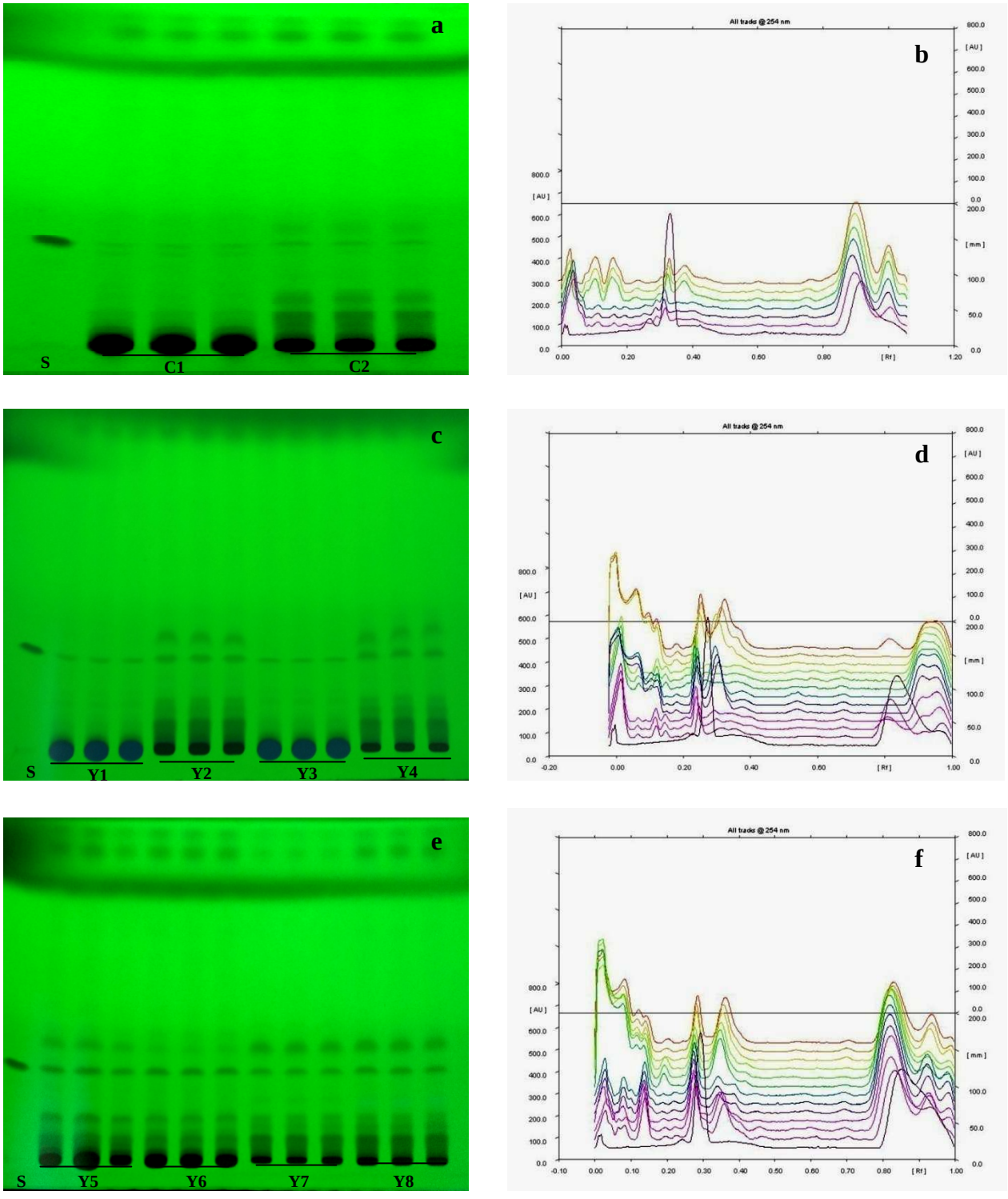

Figure 33. TLC plates and chromatograms for methanol extract of callus samples: (a, b) Control samples after 4 weeks- C1 and 5 weeks- C2, (c, d) YE (25 mg/l) treated callus samples after 4 weeks-Y1 and 5 weeks- Y2 and YE (50 mg/l) after 4 weeks- Y3 and 5 weeks- Y4, (e, f) YE (100 mg/l) treated callus samples after 4 weeks-Y5 and 5 weeks- Y6 and YE (200 mg/l) after 4 weeks- Y7 and 5 weeks- Y8. S- *p*-coumaric acid standard. Scanning of plates was done at 254 nm.

Similarly when explants were treated with salicylic acid, all explants turned brown within a week and gradually became black. Presence of different concentrations of SA (25-200 µM) in the medium had an adverse effect on leaf explant, as they failed to survive even after repeatedly performing the same experiment three times.

- *L. reticulata* callus cultures had the potency to synthesize the targeted compound and the same could be enhanced by treating the cultures with YE elicitor.

In recent years, callus cultures have gained commercial potential for the manufacture of pharmaceutically important secondary metabolites (Ogita, 2015; Wu et al., 2016). One of the advantage of callus culture is they are more reliable than collecting plant materials from wild for extracting the therapeutic metabolites (Efferth et al., 2019). Yeast extract, a biotic elicitor, has been used due to their ability to stimulate the defence mechanism, which leads to increase biosynthesis of valuable metabolites, but its optimal level may be different for each plant species (Abraham et al., 2011). Positive effect of YE on metabolites content is reported in *Lithospermum erythrorhizon* (Ogata et al., 2004), *Silybum marianum* (Sanchez-Sampedro et al., 2005), *Gymnema sylvestre* (Veerashree et al., 2012), *Artemisia monosperma* (Al-Gendy et al., 2016), *Hoppea fastigiata* (Moon and Mitra, 2016) and *Orthosiphon stamineus* (Razali et al., 2017). Thus in the present study, different concentrations of YE were utilized to treat leaf derived callus and its effect on *p*-coumaric acid was evaluated. The results suggested that lower concentration of YE (25 mg/l) facilitated maximum biomass in callus. This is in line with report of Vijayalakshmi and Shourie (2019) in which YE triggered biomass accumulation in callus cultures of licorice. Similarly, YE treatment increased biomass in comparison to control in *Morinda elliptica* (Chong et al., 2005) and *Hyoscyamus muticus* (Ibrahim et al., 2009). Generally YE has been used as a source of nutrient (Jonard, 1960), but it is also dependent on species as recently Chavan et al. (2021) reported that YE failed to increase biomass in callus cultures of *Salacia chinensis*.

HPTLC analysis of samples revealed that maximum production of *p*-coumaric acid was observed after treatment of callus with 200 mg/l of YE for four weeks. Similarly, YE significantly enhanced the production of different phenolic acids including *p*-coumaric acid in *Malus* × *domestica* (Cai et al., 2014). Younesikelaki et al. (2018) have suggested that addition of 100 mg/l YE to the medium resulted in maximum production of *p*-coumaric acid in *Althaea officinalis*. Up-regulation of *p*-coumaric acid has been also documented in *Glehnia*

littoralis after YE treatment (Ishikawa et al., 2007). YE also increased total phenolics in *Astragalus chrysochlorus* (Ozgur and Sule, 2009) and *Iphiona mucronata* (Al-Gendy et al., 2015). The synthesis of *p*-coumaric acid is regulated by the activity of phenylalanine ammonia-lyase (*PAL*), the first enzyme of phenylpropanoid pathway. The reports suggested that the expression of *PAL* rapidly increased after YE treatment and it precedes phenolic accumulation in cells (Sarkate et al., 2017). Till date there are many reports which have stated the beneficial effect of YE on production of other metabolites such as silymarin in *Silybum marianum* (Sampedro and Jorge, 2005), rosmarinic acid in *Lithospermum erythrorhizon* (Ogata et al., 2004) and *Orthosiphon aristatus* (Hunaefi and Smetanska, 2013), xanthone in *Hypericum perforatum* (Conceicao et al., 2006), psoralen in *Psoralea corylifolia* (Parast et al., 2011), vasicine in *Adhatoda vasica* (Bhambhani et al., 2012), biphenyl in *Sorbus aucuparia* (Qiu et al. 2012), carotenoid in *Cleome rosea* (Silva da Rocha et al., 2013), echitamine in *Alstonia scholaris* (Singh et al., 2015), chicoric acid and rosmarinic acid in *Ocimum basilicum* (Açıkgöz et al., 2020) and mulberroside A, oxyresveratrol and resveratrol in *Morus alba* (Inyai et al., 2021).

When *L. reticulata* leaf explants was treated with different concentrations of SA (25-200 μM), it adversly affected and they turned brown. Similarly SA negatively affected the growth of *Ginkgo biloba* (Kang et al., 2009) *Hypericum perforatum* (Gadzovska et al., 2013), and *Eurycoma longifolia* (Nhan and Loc, 2018) callus cultures. Another remarkable observation was found that when quantification was done in six weeks old callus culture grown on medium supplemented with BA+NAA, it was able to synthesize *p*-coumaric acid. Whereas at the time of elicitation, callus growth determination study evaluated that callus growth was declined in the six weeks and *p*-coumaric acid was not quantified when analysed in four and five weeks old callus cultures. This might be due to seasonal variation and in support of present results, variation in *p*-coumaric acid content is well documented in many plant species such as *Sasa argenteastriatus* (Ni et al., 2012), *Sasa quelpaertensis* (Ko et al., 2018) and *Convolvulus althaeoides* (Hrichi et al., 2020). It can be explained by the fact that every season have different quantum of water, temperature, and radiation. These climate changes not only alter abiotic factors but also manipulate different biotic factors of the surrounding environment, which exert plethora of abiotic and biotic stress on the plants and leads to biosynthesis of specific secondary metabolites for either direct defense or induction of signaling in response to abiotic/biotic stress condition (Singh et al., 2019).

Tylophora indica (Burm. F.) Merrill

Another important Asclepiadaceae member was taken up for the experimental work and the detailed results are summarized below.

4.3 Establishment of Cultures

Leaf and nodal explants were utilized for the development of *in vitro* cultures in MS media fortified with different PGRs.

4.3.1 Leaf Explant

In the first experiment leaves were collected from Botanical Garden which were used as an explant for establishing *in vitro* cultures. Leaf explants were evaluated for morphogenic response using cytokinins (5-20 µM) and auxins (0.1-1.0 µM), and observations revealed that PGRs individually or in combinations differentiated only callus, indirect shoots, direct and indirect somatic embryos.

4.3.1.1 Effect of Individual PGRs

Explants were placed in MS basal medium supplemented with sucrose (3%) which served as control. Leaf explants showed swelling in 90% cultures within four weeks but it failed to show any morphogenic response till eight weeks. Thus, MS medium was supplemented with individual cytokinins and auxins which were tried to induce response in explants.

- **Individual cytokinins (BA and Kn)**

Cytokinins are known to differentiate shoots from leaf explant. Therefore, the media was supplemented with BA/Kn in a concentration range of 5-20 µM. BA failed to evoke any significant response except 10 µM which differentiated greenish, compact callus from the cut margins of leaf between third and fourth week. Subculture was done after four weeks and the callus further proliferated till eight weeks (Fig. 34a). Increasing the concentrations further inhibited the response for induction of callus. When individual Kn (5-20 µM) was tried it only resulted in swelling of leaf explants in all the concentrations (Fig. 34b) and turned brown after four weeks and failed to survive in further weeks.

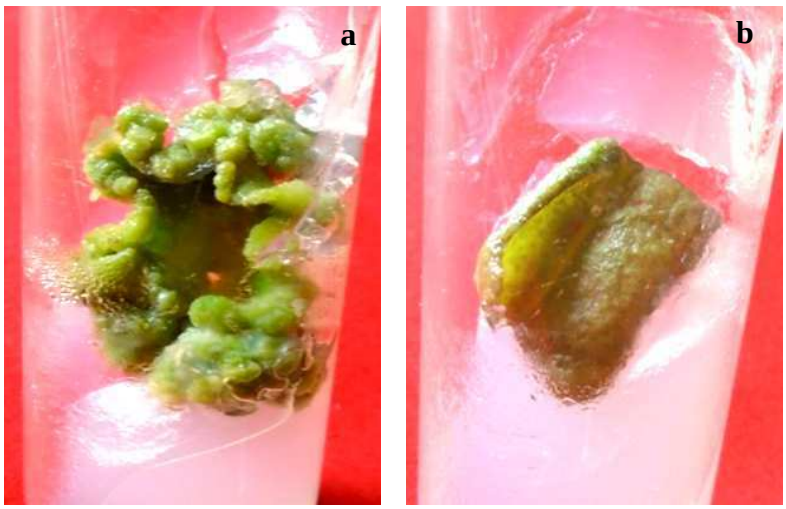

Figure 34. Effect of individual cytokinins on leaf explants of *T. indica*: (a) Differentiation of greenish compact callus in BA (10 µM) after eight weeks and (b) swelling of explant in presence of Kn within four weeks.

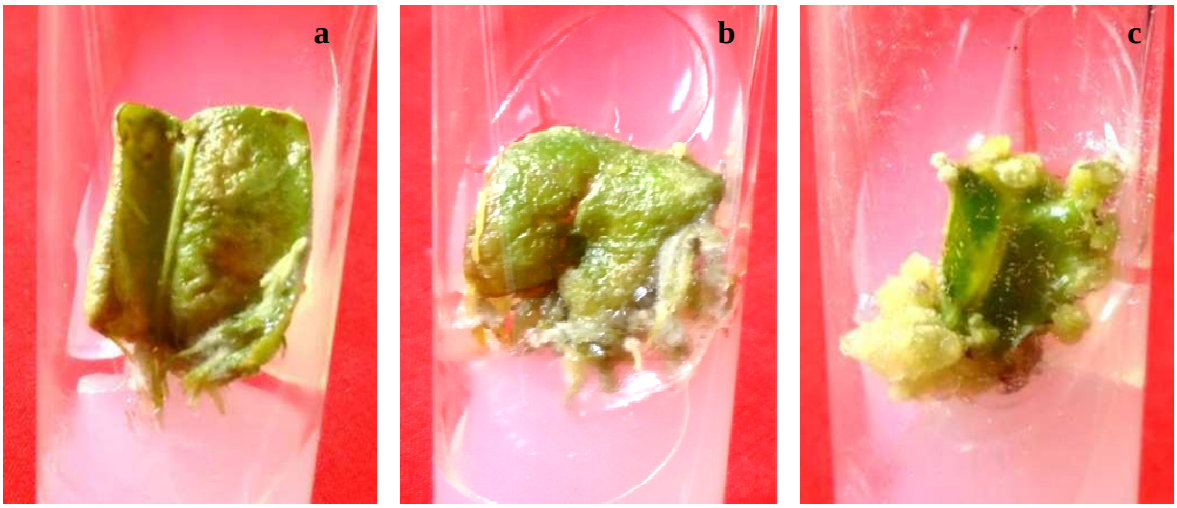

Figure 35. Effect of individual auxins on leaf explants of *T. indica* at the end of eight weeks: (a) Explant differentiated roots in presence of IAA (1.0 µM), (b) induction of roots from margins of explant in presence of NAA and (c) white, semi-friable callus observed at 2,4-D (1.0 µM).

- **Individual auxins (IAA, NAA and 2,4-D)**

The MS medium was fortified with different auxins like IAA/NAA/2,4-D individually in a range of 0.1-1.0 µM to analyze their effect on explant. Presence of IAA resulted in only swelling of the explants till four weeks and after subculture, roots differentiated from the proximal end of the lamina at 1 µM by the end of eight weeks (Fig. 35a). Similar response was obtained when IAA was replaced with NAA as only swelling of explants was observed and thin feathery roots were formed from periphery in all concentrations (Fig. 35b). Whereas in presence of 2,4-D at lower levels (0.1 and 0.5 µM) induced swelling of explants which turned black within eight weeks. This morphogenic response changed at 1 µM as white, semi-friable callus was formed, which proliferated and covered the entire lower lamina by end of eight weeks (Fig. 35c).

- In presence of individual cytokinins like BA and auxin 2,4-D, leaf explants formed callus, but rest of the PGRs failed to induce any morphogenic response.

4.3.1.2 Synergistic Effect of PGRs

The synergistic effect of cytokinins and cytokinins with auxins was also assessed, and varied morphogenic responses were observed which have been discussed below as per the response.

A. Effect of PGRs on Callus Induction

Few synergistic combinations of cytokinins and cytokinins with auxins which are described below resulted in only callus differentiation.

- **Kn and $AdSO_4$**

As Kn individually induced only swelling of explants, $AdSO_4$ which is known to help in shoot regeneration both were combined and their effect on explants was assessed. A combined effect resulted with formation of off-white compact callus from the periphery of explants in few concentrations (Fig. 36a). The other concentrations showed only swelling of explants till eight weeks.

Figure 36. Synergistic effect of PGRs on leaf explants of *T. indica* at the end of eight weeks: (a) Formation of compact callus in Kn (15 µM) + $AdSO_4$ (20 µM), (b) proliferative callus covering entire lamina in presence of BA (10 µM) + 2,4-D (0.5 µM) and (c) greenish compact callus in medium supplemented with Kn (15 µM) + 2,4-D (1.0 µM).

- **BA/Kn and 2,4-D**

When BA was combined with 2,4-D, lower concentrations of both PGRs formed callus but response was less. When BA at 10 µM was combined with various concentrations of 2,4-D, at 0.5 µM it differentiated callus in 70% cultures. A fast growing white compact callus was observed which covered the whole explants by end of eight weeks (Fig. 36b). Further increase in BA levels also formed callus with same morphology, but the proliferation rate and response gradually decreased.

BA was replaced by Kn and when combined with 2,4-D it also resulted in formation of callus, which was greenish-white, compact. In different concentrations there was variation in the rate of proliferation and percent response. Highest proliferation was observed in medium supplemented with Kn (15 µM) and 2,4-D (1.0 µM) in 60% cultures as it covered the entire lower lamina of leaf explants at the end of eight weeks (Fig. 36c).

- It was observed that few combinations differentiated only callus, among them BA with 2,4-D showed significant proliferation of callus derived through leaf explants.

B. Effect of PGRs on Indirect Shoot Regeneration

Among all synergistic combinations, the following three combinations regenerated indirect shoots from leaf explants.

- **BA and Kn**

MS medium fortified with combinations of BA and Kn exerted a strong influence on indirect shoot regeneration. This combination allowed multiple shoots to regenerate, when BA (5 µM) was with different concentrations of Kn (5-20 µM), the shoot number steadily increased and reached to 7.60 ± 0.73 at 20 µM with 50% response. Increasing the level of BA to 10 µM failed to enhance the shoot number at low levels of Kn but at 15 µM there was a sudden increase in number of shoots (22.50 ± 0.81) which reached to a length of 3.87 ± 0.53 cm by the end of eight weeks in 80% cultures (Table 21). In this combination it was observed that a greenish, nodular callus started to develop within a week which proliferated and covered the abaxial surface of leaf. By second week it differentiated large number of shoot buds (Fig. 37a) and at the end of four weeks few shoot buds grew into small shoots (Fig. 37b). After transfering to a fresh medium having same concentration of PGRs, it helped to enhance the number of shoot buds. Simultaneously multiplication of shoots was observed by six weeks

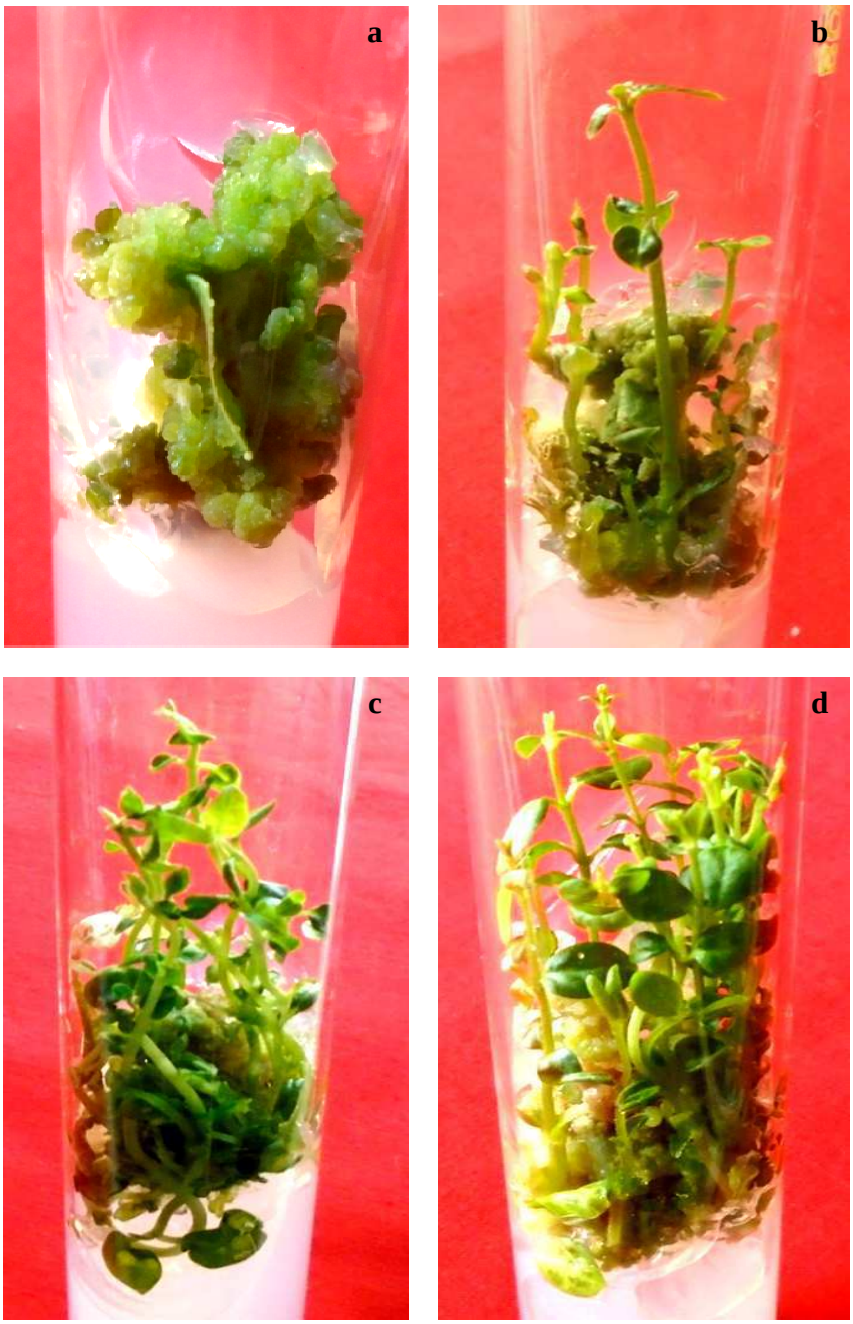

Figure 37. Synergistic effect of BA (10 µM) and Kn (15 µM) on optimum shoot regeneration from leaf explants of *T. indica*: (a) Induction of shoot buds on the surface of callus within two weeks, (b) emergence of small shoots by fourth week, (c) multiplication of shoots during sixth week and (d) elongation of shoots at the end of eight weeks.

(Fig. 37c) and further elongation of shoots was observed at eight weeks (Fig. 37d). Further increase in BA level to 15 µM and 20 µM showed a similar morphogenic response in presence of different concentrations of Kn but the shoot number declined and a maximum of 11.14 ± 1.04 number of shoots was achieved at 20 µM of both PGRs in 70% cultures.

Thus BA at 10 µM and Kn at 15 µM proved to have a synergistic effect on shoot formation and hence this combination was optimum out of all the other concentration tried.

- **BA and $AdSO_4$**

$AdSO_4$ is another type of cytokinin known to enhance the number of shoots and hence its synergistic combination with BA was evaluated for multiple shoot induction. When lower level of BA (5 µM) was coupled with different concentrations of $AdSO_4$, it inhibited the organogenic response till 15 µM of $AdSO_4$. It improved at 20 µM and formed 8.00 ± 0.63 shoots in 50% cultures with maximum 5.64 ± 0.91 cm length among all combinations. In a week, greenish nodular compact callus was differentiated from cut margin of explants and it proliferated on to the upper surface and covered the entire lamina within two weeks. Simultaneously the callus differentiated shoot buds by end of four weeks. This callus was transferred to fresh medium of same combination and it aided shoot buds to elongate into shoots. By the end of eight weeks as large number of shoots elongated and new small shoots were observed to differentiate at their base (Fig. 38a). Enhancement in BA level to 10 µM was able to regenerate only few shoots with 10-20 µM of $AdSO_4$. But increase in the level of both cytokinins failed to improve the number of shoots or percent response (Table 21).

- **BA and IAA**

Synergism of cytokinins and auxins are also known to contribute in development of shoots and thus a combined effect of BA (5-20 µM) and IAA (0.1-1.0 µM) on morphogenesis was evaluated. Their presence in the medium induced friable callus which simultaneously differentiated both shoots as well as SEs (discussed later). It was observed that lower concentration of BA (5 µM) with all IAA concentrations failed to form shoots. When BA was increased to 10 µM, callus differentiated multiple shoots (9.88 ± 0.86) with a length of 2.46 ± 0.15 cm, when IAA was at 0.5 µM in 80% cultures. In this combination, callus induction was observed within a week and it proliferated as greenish-white, semi-friable during second week. Callus grew in further weeks and simultaneously shoot buds differentiation occurred by four weeks. After subculture, shoot buds elongated and developed into shoots between six to

Figure 38. Synergistic effect of PGRs on indirect shoot regeneration from leaf explants of *T. indica* at the end of eight weeks: (a) Development of multiple shoots in presence of BA (5 µM) + $AdSO_4$ (20 µM) and (b) shoot formation in BA (10 µM) + IAA (0.5 µM).

seven weeks and at the end of eight weeks healthy multiple shoots were formed (Fig. 38b). At higher concentrations of BA (15-20 µM), the shoot number and percent response both gradually decreased (Table 21). Shoot number was comparatively less in this combination than optimum which may be due to simultaneous formation of somatic embryos.

- It was inferred that leaf explants of *T. indica* induced multiple shoots through indirect organogenesis in medium supplemented with both: combinations of cytokinins and cytokinin-auxin. But cytokinin combination like BA-Kn proved to be better for indirect shoot regeneration.

Table 21. Effect of different PGRs on shoot regeneration from leaf explants of *T. indica* (8 weeks).

PGRs (µM)				No. of shoots/explant*	Shoot length (cm)*	Response (%)
BA	Kn	$AdSO_4$	IAA			
0	0	0	0	0 f	0 f	0
5	5	0	0	2.75 ± 0.41 e	2.34 ± 0.22 cde	40
5	10	0	0	3.60 ± 0.67 de	3.04 ± 0.34 bcd	50
5	15	0	0	5.00 ± 0.63 cde	3.72 ± 0.49 b	50
5	20	0	0	7.60 ± 0.73 bcde	3.90 ± 0.40 b	50
10	5	0	0	1.67 ± 0.27 e	3.66 ± 0.53 b	30
10	10	0	0	3.50 ± 0.56 de	2.95 ± 0.48 bcd	40
10	15	0	0	22.50 ± 0.81 a	3.87 ± 0.53 b	80
10	20	0	0	7.83 ± 1.07 bcd	2.83 ± 0.33 cd	60
15	5	0	0	5.20 ± 0.77 cde	2.16 ± 0.24 cde	50
15	10	0	0	2.20 ± 0.44 e	2.95 ± 0.26 bcd	50
15	15	0	0	2.75 ± 0.41 e	2.72 ± 0.41 cd	40
15	20	0	0	7.20 ± 1.31 bcde	3.06 ± 0.38 bcd	50
20	5	0	0	9.86 ± 1.20 b	3.21 ± 0.49 bc	70
20	10	0	0	8.17 ± 1.09 bcd	3.07 ± 0.45 bc	60
20	15	0	0	9.43 ± 1.45 bc	3.53 ± 0.59 b	70
20	20	0	0	11.14 ± 1.04 b	3.36 ± 0.57 bc	70
5	0	5	0	0 f	0 f	0

5	0	10	0	0 f	0 f	0
5	0	15	0	0 f	0 f	0
5	0	20	0	8.00 ± 0.63 bcd	5.64 ± 0.91 a	50
10	0	5	0	4.00 ± 0.94 de	1.97 ± 0.18 de	30
10	0	10	0	0 f	0 f	0
10	0	15	0	2.25 ± 0.41 e	1.92 ± 0.36 de	40
10	0	20	0	4.67 ± 1.36 de	2.39 ± 0.29 cde	20
15	0	5	0	5.33 ± 1.66 cde	2.82 ± 0.51 cd	30
15	0	10	0	5.00 ± 1.41 cde	1.89 ± 0.21 de	20
15	0	15	0	0 f	0 f	0
15	0	20	0	0 f	0 f	0
20	0	5	0	0 f	0 f	0
20	0	10	0	0 f	0 f	0
20	0	15	0	3.00 ± 0.00 de	1.23 ± 0.12 e	10
20	0	20	0	0 f	0 f	0
5	0	0	0.1	0 f	0 f	0
5	0	0	0.5	0 f	0 f	0
5	0	0	1.0	1.83 ± 0.28 e	1.82 ± 0.17 de	60
10	0	0	0.1	9.50 ± 0.57 bc	3.25 ± 0.38 bc	60
10	0	0	0.5	9.88 ± 0.86 b	2.46 ± 0.15 cd	80
10	0	0	1.0	4.00 ± 1.25 de	3.09 ± 0.35 bc	30
15	0	0	0.1	1.90 ± 1.08 e	1.86 ± 0.32 de	30
15	0	0	0.5	5.00 ± 0.94 cde	1.89 ± 0.22 de	40
15	0	0	1.0	1.50 ± 0.35 e	1.43 ± 0.03 e	20
20	0	0	0.1	6.00 ± 1.41 bcde	1.85 ± 0.24 de	20
20	0	0	0.5	2.67 ± 0.30 e	2.46 ± 0.48 cd	60
20	0	0	1.0	2.33 ± 0.72 e	2.43 ± 0.22 cd	30

*Values represent mean ± SE. Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

In vitro regeneration is mainly divided in three phases: competition, dedifferentiation and redifferentiation (Sugiyama, 1999) and it is observed that different PGRs composition have a significant influence on leaf explants/callus ability to become organogenic and/or

embryogenic (Jimenez, 2005). Both rely on totipotency of somatic cells, which depend on perception of phytohormones, undergo proliferation, acquisition of organogenic or embryogenic competence and finally resulting in organ initiation and development (Karami et al., 2009; Yang and Zhang, 2010; Duclercq et al., 2011). Previously in many species simultaneous organogenesis and embryogenesis has been reported e.g. *Valeriana edulis* (Castillo-España et al., 2000), *Primulina tabacum* (Ma et al., 2010; Yang et al., 2012), *Miscanthu* × *giganteus* (Kim et al., 2010), *Metabriggsia ovalifolia* (Ma et al., 2011), *Crocus* sps. (Verma et al., 2016), *Passiflora edulis* (Huh et al., 2017), *Neolamarckia cadamba* (Li et al., 2019) and *Scaevola sericea* (Liang et al., 2020).

In organogenesis the explant has to undergo a number of steps, and it is critical that the explant is competent and exposed to stimuli sufficient to induce the differentiation and subsequent morphological development of plant tissues (Christianson and Warnick, 1985). This can be achieved by fortifying media with particular PGRs which triggers the entry of cell into cell cycle resulting in cell division, these getting organised into specific organ primordia like shoot buds, which will get differentiated into multiple shoots (Sugiyama, 1999; Phillips, 2004). Cytokinins are involved in various aspects of plant development (Samuelson and Larsson, 1993; Kiba et al., 2005; Sakakibara, 2006) as well as in signalling of iron, sulphur, and phosphorus (Franco-Zorrilla et al., 2002; Maruyama-Nakashita et al., 2004; Seguela et al., 2008). It is also documented that PGRs, particularly cytokinins, play a key role in the induction of nodular meristemoids which are dependent on a gene encoding a cytokinin receptor for activation of the nodule initiator (Murray et al., 2007; Fortes et al., 2010). However, in leaf explant of *T. indica* individual cytokinins induced only callus which failed to form shoots till eight weeks. This result is in corroboration with similar results observed in leaf explant of *Hemidesmus indicus*, another asclepiadaceae member where individually BA and Kn induced only callus (Pathak and Joshi, 2017). Similarly, in many species like *Stevia rebaudiana* (Janarthanam et al., 2009), *Tecomella undulata* (Patel and Patel, 2013) and *Biophytum sensitivum* (Kala et al., 2014) these PGRs formed only callus when added individually.

Whereas in previous report on *T. indica* individual cytokinins induced indirect organogenesis. Faisal and Anis (2003) reported callus formation in presence of 2,4,5-T (10 μM) and after subculturing on another medium containing 5 μM Kn, it differentiated 64.8 ± 0.74 shoots after 10 weeks of culture period. Later on Thomas and Phillip (2005) documented for the same plant that callus which was formed in presence of BA+2,4-D induced 66.7 shoots

after 90 days when transferred to TDZ (8 μM) supplemented medium. Similarly callus generated in BA+2,4-D combination differentiated 18.0 ± 0.4 shoots on medium fortified with only BAP (2.0 mg/l) (Sadguna et al., 2013). Whereas Kn (1 μM) has been documented to induce total 4.6 ± 1.1 shoots through direct organogenesis (Rathinavel and Sellathurai, 2010). Sahai et al. (2010) has also reported indirect organogenesis using two different media in *T. indica* as TDZ (2.5 μM) induced callus and when transferred to BA (5 μM), it formed 26.80 ± 0.97 shoots after 12 weeks. Whereas some researchers have suggested the use BA and IBA for shoot formation from leaf explant (Verma et al., 2010; Sharma et al., 2014). Contrary to above reports, in the present study optimum regeneration was achieved in combination of BA with Kn, their number was much higher as compared to earlier reports (Rathinavel and Sellathurai, 2010; Sahai et al., 2010a; Verma et al., 2010; Sadguna et al., 2013; Sharma et al., 2014; Soni et al., 2015). There are few reports which state high number of shoots, but it has been achieved in two different media such as one for callus induction and another for shoot differentiation and thus their culture period was lengthy (Faisal and Anis, 2003; Thomas and Phillip, 2005; Sahai et al., 2010). But in the present study indirect organogenesis occurred in the same medium till eight weeks which reduced the overall time period for propagation.

The observations in the present study revealed that lower BA (10 μM) and higher Kn (15 μM) level evoked optimum regeneration, which is in line with report of *Vernonia anthelmintica* in which optimum indirect organogenesis was observed in presence of BA (4 mg/l) and Kn (6 mg/l) (Rajan et al., 2020). The same combination of BA with Kn is recently documented in *Aloe vera* for maximum shoot formation (Singh et al., 2020). The synergistic effect of these two cytokinins has been previously documented for multiple shoot regeneration from leaf explant of many medicinal plants like *Stevia rebaudiana* (Sreedhar et al., 2008), *Withania somnifera* (Joshi and Padhya, 2010), *Gladiolus grandiflorus* (Shaheenuzzaman et al., 2011) and *Achyranthes aspera* (Sen et al., 2014). On the contrary Jain et al. (2011) found that leaves of *Withania coagulans* induced optimum number of shoots in medium having BA (22.2 μM) and Kn (2.3 μM), but they were stunted and had to be transferred to medium having BA (2.2 μM), Kn (2.3 μM) and phloroglucinol (3.9 μM) for elongation. Similarly BA-Kn proved to be beneficial in multiplication and elongation of *Enicostemma hyssopifolium* shoots (Seetharam et al., 2002).

Although many studies have suggested that BA and Kn induced optimum shoots, some reports have documented the addition of auxins like IAA or NAA for multiple shoot formation. The combination of BA-Kn-IAA is reported for optimum regeneration in *Gynura*

procumbens (Banu et al., 2017) and *Scoparia dulcis* (Gandhi et al., 2020); and combination of BA-Kn-NAA in *Punica granatum* (Soni and Kanwar, 2016), *Moringa oleifera* (Jun-Jie et al., 2017), *Brucea mollis* (Das et al., 2018) and *Isodon amethystoides* (Duan et al., 2019). The combination of BA with another cytokinin has been reported for optimum organogenesis in *Hibiscus syriacus* (Seo et al., 2017) and other plants (Beasley and Pijut, 2013; Lee and Pijut, 2017). *T. indica* regenerated optimum shoots in presence of cytokinins which may be due to the fact that the explants might contain adequate levels of endogenous auxin, as the same is also reported earlier (Bacchetta et al., 2003; Jin et al., 2014). Previous reports documented that the tissues undergo dedifferentiation and redifferentiation only when both exogenous and endogenous PGRs in the medium interact and differentiate shoots (Schwarz and Beaty, 1996; Huang et al., 2012).

When $AdSO_4$ was incorporated in the media along with BA/Kn, BA+$AdSO_4$ differentiated shoots and SEs simultaneously, whereas Kn+$AdSO_4$ failed to induce any morphogenic response which is in accordance with previous report on *Rosa hybrida* and *Mussaenda erythrophylla* (Das, 2010). Simultaneous shoot organogenesis and somatic embryogenesis has been also documented in *Valeriana jatamansi* (Chen et al., 2014) and *Metabriggsia ovalifolia* (Ouyang et al., 2016).

Another combination BA-IAA induced indirect organogenesis in *T. indica* whereas BA-NAA failed to induce shoot organogenesis, and this is in line with results reported by Faisal and Anis (2003) as they have also stated that NAA inhibits activity for shoot regeneration in *T. indica*. This is also in accordance with the results documented for leaf explant of *H. indicus* where BA-IAA combination favoured shoot formation and BA-NAA had adverse effect (Pathak and Joshi, 2017). Similarly leaf explants of *Anisochilus carnosus* when placed in the medium with BA-IAA proved to be advantageous over BA-NAA (Reshi et al., 2017). This combination has also proved beneficial in *Pityopsis ruthii* (Wadl et al., 2011), *Spilanthes acmella* (Singh and Chaturvedi, 2012) and *Bacopa monnieri* (Kumari et al., 2015). In earlier reports on leaf explant of *T. indica* it was documented that shoots regenerated in combination of BA and IAA (0.5 mg/l) (Haque and Ghosh, 2013; Soni et al., 2015), but in the present study this combination induced simultaneous formation of shoots and SEs. Whereas Banu et al. (2017) reported contrary results where shoot regeneration was observed from leaf explants of *Gynura procumbens* in presence of BA-NAA but changing auxin to IAA with BA failed to induce shoots. Similar contrary results were also reported in many studies where NAA along with different cytokinins was able to induce shoots but when IAA was combined with

cytokinins it failed to induce shoots (Sriskandarajah et al., 2004; Gehl et al., 2020). The reason is that induction of *in vitro* shoots depends on correct ratio of cytokinin to auxin (Thorpe, 1980) and changing the type of auxin is proved to be a critical factor for shoot organogenesis (Yancheva et al., 2003; Mahoney et al., 2018).

It was also observed that presence of BA with $AdSO_4$ or IAA in the medium simultaneously induced the callus to differentiate shoots and somatic embryos. It has been suggested that differential responses of explants obtained under involvement of different cytokinins may be a result of their varied translocation rates, differential uptake, various effects on metabolic processes, and ability to change the level of endogenous cytokinins (Rathore et al., 2016).

C. Effect of PGRs on Somatic Embryogenesis

When leaf explants were placed in the MS medium, the following combinations of PGRs induced somatic embryos as well.

- **BA and $AdSO_4$**

In this combination, at lower level of BA (5 µM) with various concentrations of $AdSO_4$ it was observed that at all levels SEs were formed. $AdSO_4$ with 5-15 µM concentrations, differentiated less embryos and the number enhanced to 8.50 ± 2.19 when level reached to 20 µM. As BA level increased to 10 µM, it failed to further enhance the number of SEs and only 9.50 ± 0.35 embryos were formed when $AdSO_4$ was at 10 µM. Callus induction was initiated from cut margin within a week and it was whitish-green and semi-friable. It proliferated towards lower lamina in further weeks and simultaneously differentiated globular embryos within four weeks. Further development of embryos to heart, torpedo and mature stages was achieved after subculture. They germinated into small plantlets and few remained in globular or elongated globular stage by end of eight weeks (Fig. 39a). Further increase in $AdSO_4$ level to 15 µM decreased the number of SEs to 5.50 ± 1.30. Raising level of BA showed inhibitory effect as only few SEs were differentiated and finally at 20 µM the callus completely lost the potency to form SEs (Table 22).

It was clear from the observations that leaf explants of *T. indica* were able to enter somatic embryogenesis simultaneously with organogenesis when cytokinins were added together in the medium. Hence both cytokinins (BA and Kn) combined with different auxins (IAA and NAA) to check their effect on somatic embryos.

- **BA and IAA**

Addition of BA and IAA together induced the leaf explants to differentiate shoots (above discussed) as well as somatic embryos. BA at low concentration (5-10 µM) with all concentrations of IAA induced only few embryos which could reach to only globular stage. When BA level reached to 15 µM with IAA to 1.0 µM i.e. 5.50 ± 1.77 embryos developed in 20% cultures. Further increasing the level of BA to 20 µM, maximum embryos (9.33 ± 2.42) were formed in presence of 0.5 µM of IAA but the response was very less (30%) (Table 22). The callus was greenish-white and friable in nature which initiated from periphery after two weeks and spread towards the lower lamina of leaf explants. Callus was less proliferative but was embryogenic as it differentiated number of globular embryos within four weeks. At the end of eight weeks many embryos get converted into cotyledonary stage and few embryos which were induced later remained in heart and torpedo stages by the end of eight weeks (Fig. 39b).

- **BA and NAA**

When BA was combined with another auxin NAA, it helped in enhancing the formation of the somatic embryos, but variation was observed with different concentrations. When both PGRs were at 5 µM it formed total of 14.67 ± 5.30 SEs in only 30% cultures and increasing the NAA levels, the number slightly decreased. The frequency increased to 16.75 ± 2.53 embryos (40% response) by raising the level of BA to 10 µM with NAA (0.1 µM) (Table 22). In this combination, a greenish-white callus differentiated on the abaxial surface within two weeks. It further proliferated and facilitated induction of globular embryos and they further developed after transferring into fresh medium of same composition by four weeks. Embryos germinated into plantlets at the end of eight weeks (Fig. 39c). At higher concentration of BA (15 µM) reduction in number of SEs and in % response was observed but BA at 20 µM with NAA (0.1 µM) the number reached to 14.50 ± 2.28 in 40% cultures. Observations revealed that low concentration of NAA (0.1 µM) was able to induce embryos with all levels of BA. Additionally, many combinations also induced shoot buds, but the percent response was less and they failed to develop into shoots even after subculture.

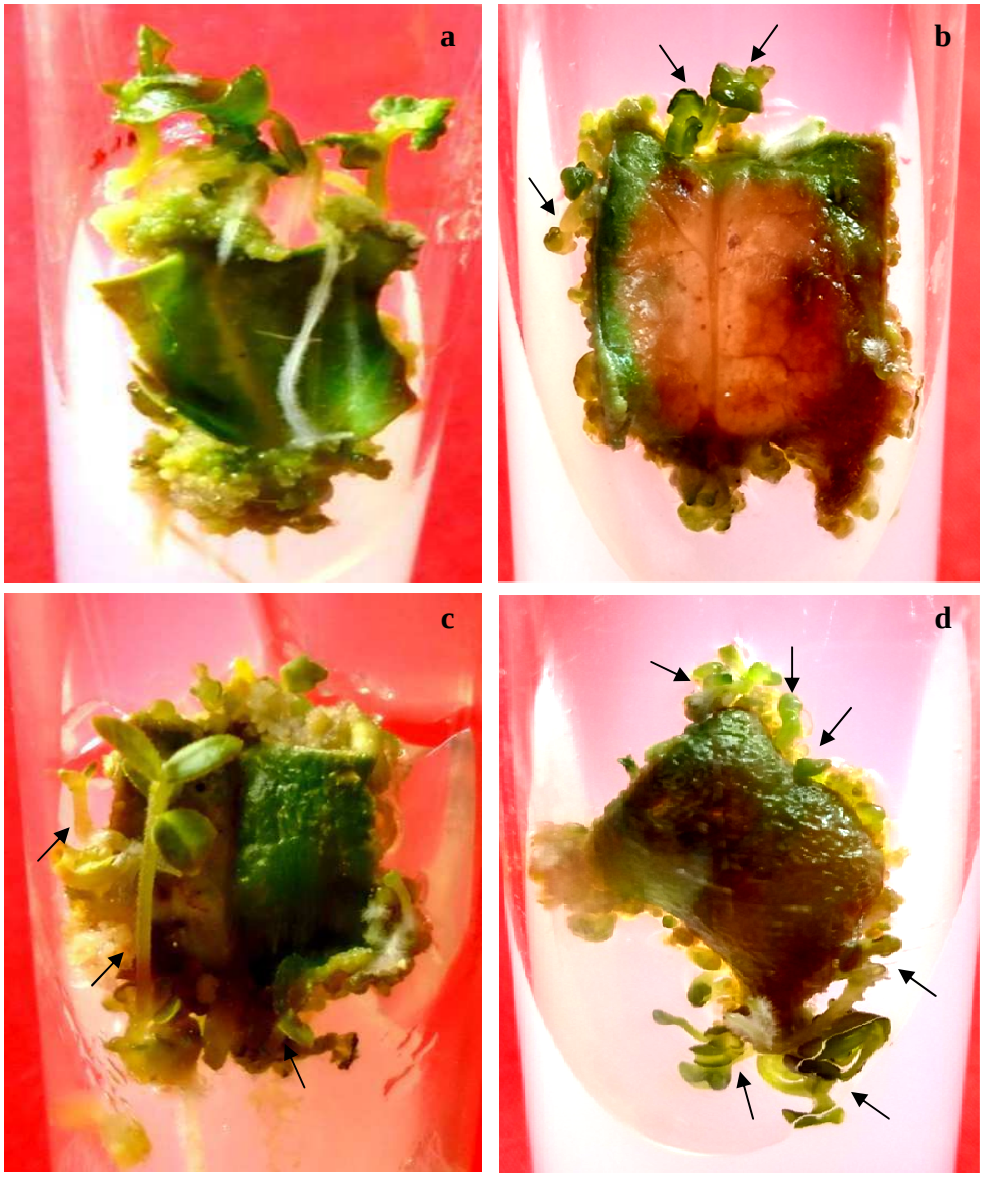

Figure 39. Differentiation of somatic embryos in different combinations of PGRs from leaf explants of *T. indica* at the end of eight weeks: (a) Germination of SEs into plantlets in presence of BA (10 µM) + $AdSO_4$ (10 µM), (b) matured embryos in BA (20 µM) and IAA (0.5 µM), (c) development of small plantlets in presence of BA (10 µM) and NAA (0.1 µM) and (d) different stages of SEs in medium containing Kn (5 µM) and NAA (1.0 µM).

Table 22. Effect of different PGRs on somatic embryogenesis from leaf explants of *T. indica* (8 weeks).

PGRs (µM)					No. of SEs/explant*	Response (%)
BA	Kn	$AdSO_4$	IAA	NAA		
0	0	0	0	0	0 i	0
5	0	5	0	0	3.75 ± 0.54 gh	40
5	0	10	0	0	1.25 ± 0.22 h	40
5	0	15	0	0	2.20 ± 0.66 h	50
5	0	20	0	0	8.50 ± 2.19 ef	40
10	0	5	0	0	3.67 ± 0.72 gh	30
10	0	10	0	0	9.50 ± 0.35 e	20
10	0	15	0	0	5.50 ± 1.30 g	40
10	0	20	0	0	2.00 ± 0.00 h	20
15	0	5	0	0	5.33 ± 1.19 g	30
15	0	10	0	0	1.50 ± 0.35 h	20
15	0	15	0	0	2.00 ±0.00 h	20
15	0	20	0	0	0 i	0
20	0	5	0	0	0 i	0
20	0	10	0	0	0 i	0
20	0	15	0	0	0 i	0
20	0	20	0	0	0 i	0
5	0	0	0.1	0	0 i	0
5	0	0	0.5	0	0 i	0
5	0	0	1.0	0	0 i	0
10	0	0	0.1	0	0 i	0
10	0	0	0.5	0	0 i	0
10	0	0	1.0	0	5.00 ± 1.70 g	30
15	0	0	0.1	0	0 i	0
15	0	0	0.5	0	0 i	0
15	0	0	1.0	0	5.50 ± 1.77 g	20
20	0	0	0.1	0	9.00 ± 0.71 ef	20
20	0	0	0.5	0	9.33 ± 2.42 e	30

20	0	0	1.0	0	5.75 ± 1.14 g	40
5	0	0	0	0.1	14.67 ± 5.30 c	30
5	0	0	0	0.5	6.33 ± 0.61 fg	60
5	0	0	0	1.0	12.00 ± 3.86 cd	40
10	0	0	0	0.1	16.75 ± 2.53 c	40
10	0	0	0	0.5	0 i	0
10	0	0	0	1.0	9.00 ± 0.50 ef	40
15	0	0	0	0.1	0 i	0
15	0	0	0	0.5	5.67 ± 0.98 g	30
15	0	0	0	1.0	2.33 ± 0.27 h	30
20	0	0	0	0.1	14.50 ± 2.28 c	40
20	0	0	0	0.5	6.25 ± 1.52 fg	40
20	0	0	0	1.0	4.50 ± 1.06 g	20
0	5	0	0.1	0	26.00 ± 1.13 a	100
0	5	0	0.5	0	10.90 ± 0.88 de	100
0	5	0	1.0	0	7.38 ± 1.31 f	80
0	10	0	0.1	0	9.88 ± 1.83 e	80
0	10	0	0.5	0	19.00 ± 2.13 b	90
0	10	0	1.0	0	10.63 ± 1.49 de	80
0	15	0	0.1	0	10.00 ± 1.75 de	70
0	15	0	0.5	0	8.57 ± 0.83 ef	70
0	15	0	1.0	0	6.22 ± 0.77 fg	90
0	20	0	0.1	0	5.63 ± 1.22 g	80
0	20	0	0.5	0	3.00 ± 0.64 gh	70
0	20	0	1.0	0	2.17 ± 0.28 h	60
0	5	0	0	0.1	8.50 ± 2.27 ef	60
0	5	0	0	0.5	6.43 ± 0.92 fg	70
0	5	0	0	1.0	24.38 ± 2.14 ab	80
0	10	0	0	0.1	10.40 ± 2.20 de	50
0	10	0	0	0.5	10.00 ± 0.94 de	30
0	10	0	0	1.0	12.50 ± 1.98 cd	100
0	15	0	0	0.1	24.40 ± 6.89 ab	50

0	15	0	0	0.5	23.44 ± 4.91 ab	90
0	15	0	0	1.0	22.00 ± 4.07 ab	70
0	20	0	0	0.1	19.20 ± 2.17 ab	100
0	20	0	0	0.5	18.86 ± 2.87 b	70
0	20	0	0	1.0	21.00 ± 3.13 ab	80

*Values represent mean ± SE. Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

- **Kn and IAA**

BA was replaced with Kn and combined with auxins IAA/NAA and their morphogenic response was assessed. Kn with IAA failed to induce shoots but showed pronounced result for somatic embryogenesis. Lower concentration of Kn (5 µM) and IAA (0.1 µM) proved significant to form a maximum number of SEs i.e. 26.00 ± 1.13 in 100% cultures at the end of eight weeks (Table 22). This concentration differentiated highest number of SEs from all combinations of PGRs tried above. Initially a white, friable callus differentiated which proliferated and covered the entire lower lamina of leaf explants. As this callus was embryogenic a large number of globular embryos induced between two to three weeks on the surface of callus cluster (Fig. 40a). In further weeks callus proliferated and simultaneously few embryos entered the further stages by forth week (Fig. 40b). This callus cluster when transferred to a fresh medium with the same concentration proved advantageous for further development of embryos into different stages like heart, torpedo and cotyledonary and few germinated into plantlets within six weeks (Fig. 40c). By the end of eight weeks large number of mature embryos started to grow into healthy plantlets (Fig. 40d). This combination also showed direct differentiation of SEs (Fig. 40e). When Kn concentration was kept constant and IAA level was increased to 0.5 and 1.0 µM gradual decline in the number of SEs occurred. Kn 10-20 µM when coupled with various levels of IAA, failed to improve the number, except Kn at 10 µM with IAA at 0.5 µM differentiated 19.00 ± 2.13 embryos in 90% cultures. In this combination the whole germination process of SE was carried out from globular stage to plantlet in the same medium and they developed significantly in healthy plantlets with strong roots by end of eight weeks (Fig. 40f).

In few combinations of Kn-IAA, direct SEs were also observed but the response for induction was recorded less (30-40%) i.e. in Kn (5 µM) + IAA (0.1 µM), Kn (10 µM) + IAA (1.0 µM) and Kn (15 µM) + IAA (1.0 µM).

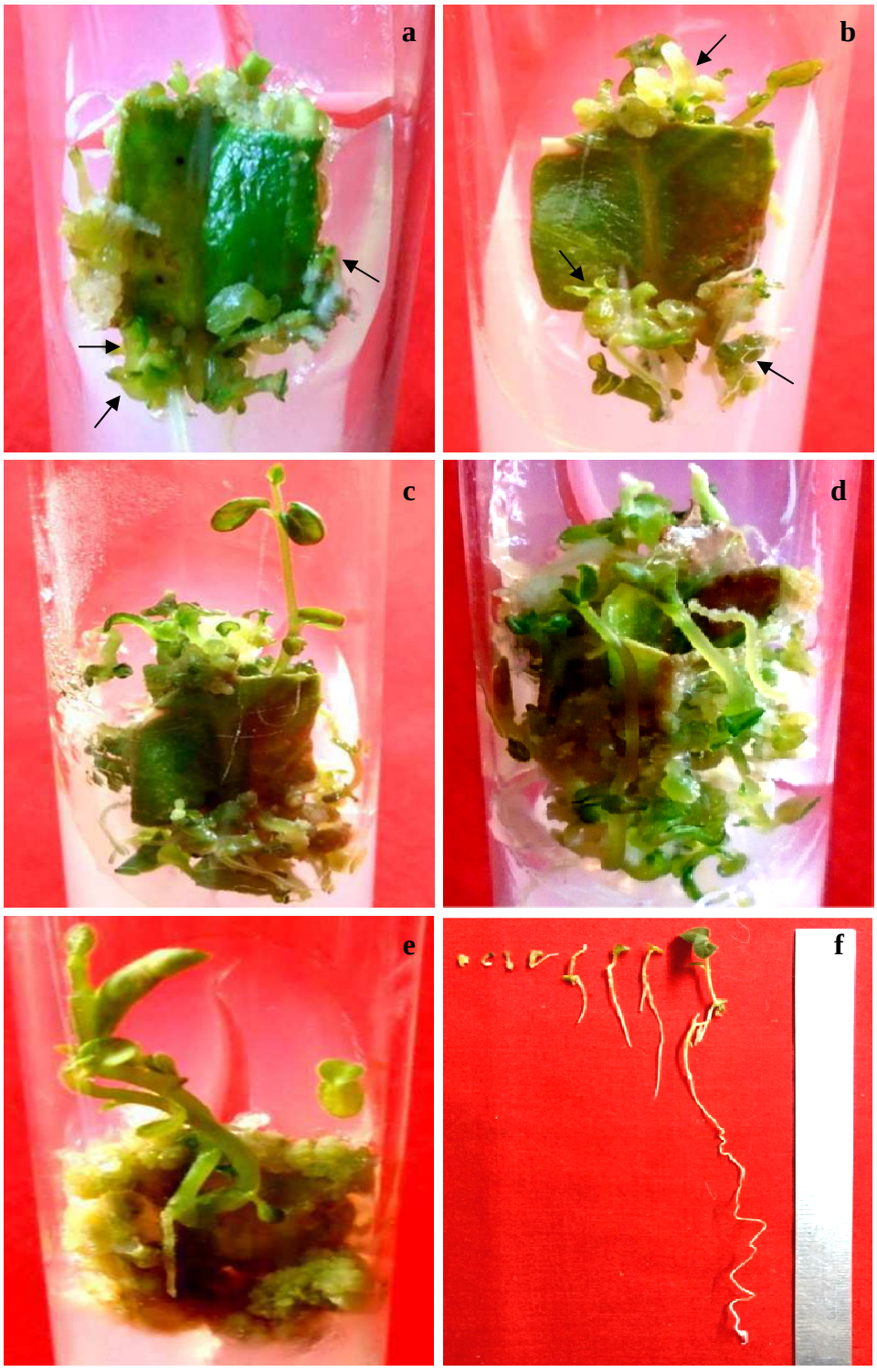

Figure 40. Somatic embryos from leaf explant of *T. indica* in MS + Kn (5 µM) + IAA (0.1 µM): (a-d) Indirect somatic embryogenesis- (a) SEs after two weeks, (b) heart and torpedo stages at four weeks, (c) germination of SEs into plantlets after six weeks, (d) healthy plantlets at the end of eight weeks, (e) germination of direct SEs into plantlet and (f) sequential developmental stages.

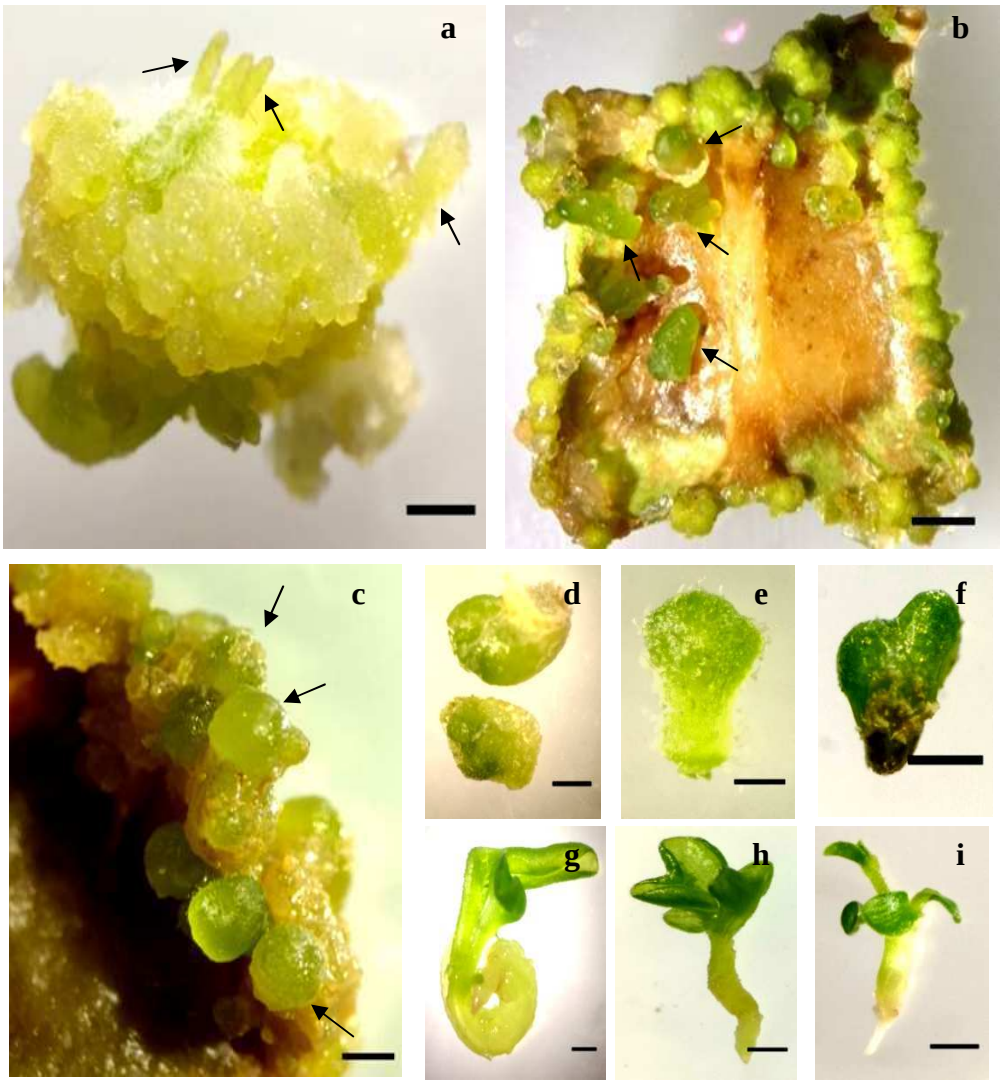

Figure 41. Microscopic observation of SEs formed in MS + Kn (5 µM) + IAA (0.1 µM) from leaf explant of *T. indica*: (a) Differentiation of SEs from callus, (b, c) direct differentiation of SEs, (d-i) stages of SEs- (d) globular, (e) elongated globular, (f) heart, (g) torpedo, (h) cotyledonary and (i) plantlet. (Bar = 0.5 mm)

Microscopy also helped in confirmation of indirect (Fig. 41a) and direct (Fig. 41b, c) somatic embryos from leaf explants. The growth of somatic embryos was asynchronous, so they were in different developmental stages on the same explants and all stages were separated from callus i.e. globular, elongated globular, heart, torpedo, cotyledonary and plantlet (Fig. 41d-i).

- **Kn and NAA**

Another auxin NAA was also tried with Kn, this combination also proved better for somatic embryos as all the concentrations formed embryos with high frequency. Lower concentration (5 µM) of Kn with NAA (0.1 and 0.5 µM) resulted in less number of embryos as well as percent response. This number enhanced to 24.38 ± 2.14 in 80% cultures in presence of NAA (1.0 µM). Callus induction was initiated from cut end of the explant after two weeks, but the growth of callus was very slow. A dual pathway of direct and indirect differentiation of embryo was observed as within four weeks. Different stages of embryos were observed within six weeks, then few embryos germinated into plantlets by the end of eight weeks (Fig. 39d). Further raising the Kn level to 10 µM with various levels of NAA, lowered down the number of embryos. Frequency of SEs was increased to 24.40 ± 6.89 again when Kn level enhanced to 15 µM with 0.1 µM NAA. Number significantly increased by increasing the concentration of Kn to 20 µM (Table 22).

- Kn with IAA and NAA showed significant result for somatic embryos induction and favoured maturation and conversion of embryos to plantlets in the same medium. Therefore they were beneficial for somatic embryogenesis from leaf explants in *T. indica*.

The process of morphogenesis depends on external and internal factors, such as exogenously applied PGRs and the ability of the plant tissue to perceive and respond to PGRs (Prakash and Gurumurthi, 2010; de Souza et al., 2019). Thus, *in vitro* cultures responses may vary because it depends on the PGRs applied (Magyar-Tábori et al., 2010; Lee and Pijut, 2017). The embryogenic commitment of the mature cells of the explant requires a dedifferentiation step in which the cells lose their specific destiny and become meristematic (Sugiyama, 1999), and initiate a new developmental fate for regeneration of embryos. Therefore, induction of embryogenic calli is the prerequisite for efficient somatic embryogenesis. It has been proved that under suitable inductive conditions, somatic cells may

undergo restructuring to generate embryogenic cells, which then go through a series of biochemical and morphological changes resulting in formation of a somatic embryo which regenerate into whole plant (Quiroz-Figueroa et al., 2006; Yang and Zhang, 2010).

Auxins have beneficial effect on stimulating the embryogenic competence of somatic tissues (Gaj, 2004; Lincy et al., 2009) whereas the combination of auxins and cytokinins are the key factors to determine the embryogenic response due to their pervasive participation in the cell cycle regulation and cell division (Francis and Sorrell, 2001). Embryogenic callus induction was found to be effective in the combined treatment of auxins and cytokinins (Ma and Xu, 2002; Hernández et al., 2003; Nath and Buragohain, 2005). In the present study, the callus differentiated shoots and somatic embryos simultaneously in presence of BA-IAA within eight weeks, this is contrary to previous report on same plant where either indirect organogenesis (Nema et al., 2011; Haque and Ghosh, 2013) or somatic embryogenesis (Manjula et al., 2000) has been reported in the same PGR combination. Whereas in *Solanum nigrum* maximum SEs were formed in presence of BAP and NAA and after they were transferred to BAP and IAA it resulted in highest frequency of embryo germination (Sharada et al., 2019). The results also confirmed that the mode of direct as well as indirect somatic embryogenesis from leaf explant in this PGR combination. PGRs are known to control the pathway of embryogenic in several plants like *Scaevola aemula* (Weerakoon, 2010), *Coffea Arabica* (Ibrahim et al., 2013), *Portulaca oleracea* (Sedaghati et al., 2019) and *Scaevola sericea* (Liang et al., 2020).

In the present study, combinations of BA/Kn with NAA induced only SEs which is contrary to earlier study on same plant where only shoot formation was documented in these combinations (Faisal and Anis, 2003; Thomas and Phillip, 2005). Both BA/Kn at high concentrations with IAA/NAA at low concentrations facilitated embryogenesis as this ratio stimulated better embryogenesis as reported in *Eucalyptus camaldulensis* (Prakash and Gurumurthi, 2010) and *Miscanthus giganteus* (Kim et al., 2010). Although BA with IAA/NAA induced SEs, their numbers were less as compared to Kn with IAA/NAA. The observations also revealed that the results were different as the cytokinin was changed from BA to Kn and the later formed only SEs. Whereas in earlier report on same plant in which indirect organogenesis was reported in combinations of Kn-IAA/NAA (Haque and Ghosh, 2013). The combinations of Kn-IAA/NAA also failed to induce shoots in *Digitalis lanata* (Bhusare et al., 2018) which is in line with present study. This might be due to the fact that

the choice of cytokinin affects the pathways of regeneration (Song et al., 2011; Yang et al., 2012).

In earlier study on indirect somatic embryogenesis from leaf derived callus in *T. indica* it was stated that BA at 2 mg/l formed 27.0 SEs (90.3% response) after 150 days (Manjula et al., 2000). Later on it was also observed for the same plant that the use of Kn (0.05 μM) and 2,4-D (9.04 μM) induced embryogenic calli, but SEs differentiated in 2-ip (9.84 μM) with IBA (0.05 μM) and after 10 weeks their number reached to 25.0 SEs with 85% germination (Jayanthi and Mandal, 2001). In another report, MS medium fortified with 0.5 μM TDZ and 1.5 μM 2,4-D proved to be better for differentiation and germination of SEs in the same plant (Chandrasekhar et al., 2006). Whereas Sahai et al. (2010a) developed a medium in which the callus differentiated in presence of either BA (5 μM) or TDZ (2.5 μM) and induced 10.20 ± 0.37 SEs (90% response) after 12 weeks in presence of BA (5 μM). The optimum number of SEs differentiated in present study was higher and rapid as compared to previous reports for somatic embryogenesis from leaf explant of *T. indica* (Manjula et al., 2000; Jayanthi and Mandal, 2001; Chandrasekhar et al., 2006; Sahai et al., 2010a).

In presence of Kn-IAA and Kn-NAA, optimum SEs differentiated in the former combination followed by later, which proved that Kn had profound effect on SE induction in *T. indica*. Beneficial effect of Kn on SEs is well documented in plant species like *Valeriana edulis* (Castillo-España et al., 2000), *Oncidium* sp. (Chen and Chang, 2001), *Cichorium intybus* (Abdin and Ilah, 2007), *Jatropha curcas* (Jha et al., 2007), *Vigna aconitifolia* (Choudhary et al., 2009), *Abelmoschus esculentus* (Daniel et al., 2018) and *Valeriana officinalis* (Abdi et al., 2019). In the same way, beneficial effect of NAA with other cytokinins is reported in *Abutilon indicum* (Seth et al., 2017), *Limonium sinense* (Dam et al., 2017) and *Neolamarckia cadamba* (Li et al., 2019). Maximum embryogenesis was observed in presence of Kn (2.3 μM) and NAA (5.4 μM) in *Mucuna pruriens*, but for maturation $AdSO_4$ (13.6 μM) was also added with Kn-NAA (Vibha et al., 2009). Whereas Daniel et al. (2018) documented that optimum SEs were induced from leaf explants of *Abelmoschus esculentus* in presence of Kn-2,4-D which differs from the present results. Variation in responses to different combination of PGRs may be due to differences in genetic make-up among different plant species (Xie and Hong, 2001).

The present protocol facilitated formation of plantlets from SEs in the same medium within eight weeks and specific treatments for maturation such as transferring of SEs into basal media or dark treatment was not required as well as it took less time for SEs to

germinate into plantlets. This might be due to the profound effect of synergistic combinations of cytokinins and auxins on somatic embryogenesis, and they triggered a signal required for root apical meristem (RAM) and shoot apical meristem (SAM) as well as establishing shoot-root axis during early somatic embryogenesis (Müller and Sheen, 2008; Pinto et al., 2011; Su et al., 2015).

4.3.1.3 Development of Plantlets Derived through Somatic Embryos

In the present study small plantlets developed through SEs were transferred to static and liquid media containing different PGRs combinations for further growth. These plantlets were transferred to liquid and static media fortified with Kn (5 µM) and IAA (0.1 µM), which enhanced to an average length of 3.66 ± 0.34 cm in liquid medium (Fig. 43a) as compare to static medium (2.93 ± 0.17 cm) (Fig. 42a). The same combination showed response for development was high in static medium (80%) compared to liquid (50%). Another combination, Kn (5 µM) and NAA (1.0 µM) when added together, plantlets failed to elongate in both media and were almost similar in length for static (2.71 ± 0.29 cm) (Fig. 42b) as well as liquid (2.86 ± 0.33 cm) media (Fig. 43b). Development response was high in static (70%) in comparison with liquid (50%).

When the combination changed to BA with $AdSO_4$, it showed better result as length increased (7.76 ± 0.19 cm) in liquid medium (Fig. 43c). Though in static medium the length of plantlets was less (3.96 ± 0.31 cm) as compared to liguid medium, but it showed maximum length among all combinations tried for static medium (Fig. 42c). When GA_3 was tried with different concentrations (0.5-2.0 µM), 1 µM significantly gave better response among all the PGRs tried and developed plantlets. Initially small plantlets (Fig. 44a) obtained from SE were transferred to the both static and liquid media, and their length was measured after four weeks. Static medium showed 1.49 ± 0.15 cm length (Fig. 44b) which was comparatively less among all combinations of static media tried. Whereas in liquid medium, all plantlets started to elongate within two weeks (Fig. 44c) and at the end of four weeks there was significant increase in their length (Fig. 44d). Maximum 9.38 ± 0.35 cm length with 90% germination was observed in liquid medium (Fig. 44e; 45). Similarly, CW also demonstrate that liquid medium gave better elongation as length was 4.64 ± 0.22 cm (Fig. 43d) and germination response was also satisfactorily high (80%). Whereas in static medium, plantlets were stunted as length was 1.48 ± 0.2 cm but hyperhydricity was observed in both media (Fig. 42d).

Figure 42. Development and elongation of plantlets in static medium fortified with different PGR combinations at the end of four weeks in *T. indica*: (a) Kn (5 µM) + IAA (0.1 µM), (b) Kn (5 µM) + NAA (1.0 µM), (c) BA (5 µM) + $AdSO_4$ (20 µM) and (d) 4% CW.

Figure 43. Development and elongation of plantlets in liquid medium fortified with different PGRs combinations at the end of four weeks in *T. indica*: (a) Kn (5 µM) + IAA (0.1 µM), (b) Kn (5 µM) + NAA (1.0 µM), (c) BA (5 µM) + $AdSO_4$ (20 µM) and (d) 4% CW.

Figure 44. Development and elongation of plantlets in MS medium fortified with GA_3 (1 µM) in *T. indica*: (a) SEs derived plantlets after eight weeks, (b) further growth of plantlets in static medium after four weeks, (c) rapid growth of plantlets in liquid medium within two weeks, (d) multiplication plantlets by four weeks and (e) healthy elongated plantlets with strong multiple roots.

Therefore all the plantlets derived from SEs were developed within four weeks in liquid medium augmented with GA_3.

- It was concluded that GA_3 proved to be advantageous for the development of SEs derived plantlets.

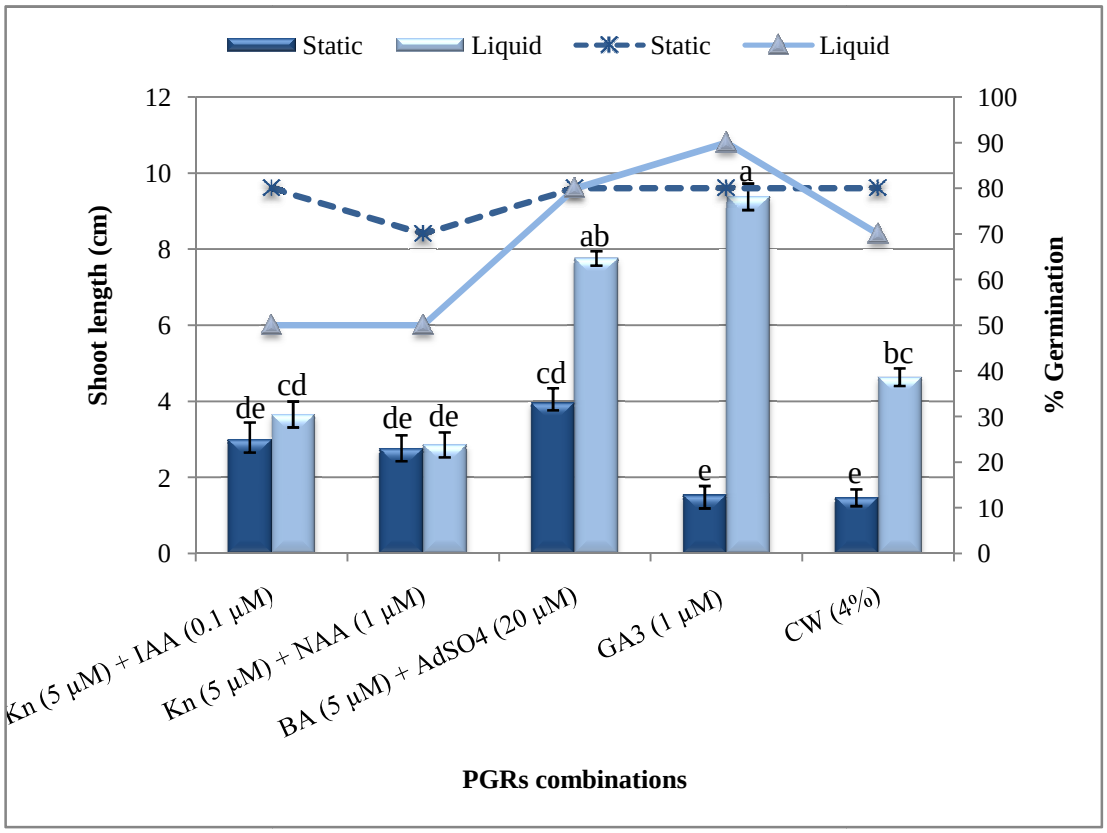

Figure 45. Development of plantlets in different media after four weeks.
Each bar shows the mean values (n = 5), error bar as standard error and the line represents % germination. Bars having same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Mature embryos of *T. indica* were transferred to MS static and liquid media and the results justify that both helped in development of embryos, but liquid medium proved to be superior as compared to static. This is in line with previous study in *Bixa orellana* (Parimalan et al., 2011) and *Ledebouria ovatifolia* (Baskaran et al., 2016) where liquid medium has been documented to have better effect on SE development. Advantage of liquid media for elongation of SE derived plantlets is documented in *Allium sativum* (Kim et al., 2003). Due to

higher response of liquid media, this medium is being used for scale-up cultures using bioreactors (Dave et al., 2003; Kim et al., 2004; Paek et al., 2005; Rizvi et al., 2007).

Among different PGRs tried for plantlet development, GA_3 proved to be better which is in corroboration with results on SEs to plantlet formation from internodal (Thomas, 2006) and root explant (Sahai et al., 2010b) of *T. indica*. But contrasting results has been reported for leaf derived indirect SEs, where the use of static basal medium for SEs conversion into plantlets is suggested (Jayanthi and Mandal, 2001; Chandrasekhar et al., 2006; Sahai et al., 2010a). In line with the present study, development of *Cryptomeria japonica* (Igasaki et al., 2003) and *Panax quubquefolius* (Zhou and Brown, 2006) SEs occurred in presence of GA_3. According to Xiao et al. (2016), gibberellins have profound effect in cell division and elongation of plantlets. The high rate of embryo conversion into plantlets in presence of GA_3 is repeated in *Manihot esculenta* (Syombua et al., 2019). GA_3 was responsible for SEs maturation and plantlet formation in *Catharanthus roseus* (Siddiqui et al., 2011), *Merwilla plumbea* (Baskaran and Van Staden, 2012), *Muscari armeniacum* (Yücesan et al., 2014), *Paris polyphylla* (Raomai et al., 2014) and *Curcuma longa* (Raju et al., 2015). In the present study, it was also noted that in media fortified with GA_3, maximum length of plantlets was achieved and this was because it stimulated shoot elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger, 1998). This is also reported in many earlier studies in which elongation of *in vitro* shoots occurred in presence of this PGR such as in *Viola canescens* (Mokhtari et al., 2016) and many other plants (Siwach and Gill, 2011; Inthima et al., 2017; Manasa et al., 2019; Tata, 2020).

Some reports on embryogenesis suggest the use of GA_3 with other PGRs for SEs development e.g. the combination of GA_3 and phloroglucinol was required for maximum conversion of SEs into plantlets in *Lachenalia viridiflora* (Kumar et al., 2016) and *Ledebouria ovatifolia* (Baskaran et al., 2016). Whereas maximum germination of SEs was achieved in presence of BA and GA_3 in *Acca sellowiana* (Cangahuala-Inocenteet al., 2007), *Vigna aconitifolia* (Choudhary et al., 2009), *Wedelia calendulacea* (Sharmin et al., 2014) and *Passiflora edulis* (Huh et al., 2017). Similarly, GA_3 along with a low dose of cytokinin promoted embryo maturation, germination, and plantlet conversion in many species (Jimenez, 2005; Perera et al., 2009). Moreover formation of secondary somatic embryos were seen directly on primary embryos which is also documented in *Bixa orellana* (Parimalan et al., 2011), *Primulina tabacum* (Yang et al., 2012), *Curcuma longa* (Raju et al., 2015), *Medicago sativa* (Sangra et al., 2019) and *Scaevola sericea* (Liang et al., 2020).

4.3.2 Nodal Explants

Plant twigs containing nodes were collected from botanical garden, washed thoroughly and placed in MS media containing PGRs and MS basal medium served as control.

4.3.2.1 Effect of Individual Cytokinins on Axillary Shoot Regeneration

Nodes of *T. indica* when placed vertically in MS basal medium, axillary buds failed to form shoots. Thus this medium was further supplemented with individual cytokinins (5-20 µM) and their effect on shoot formation was evaluated.

- **Individual cytokinins (BA and Kn)**

Cytokinins are generally known to induce axillary shoot formation. When nodes were inoculated in the medium with various concentration of BA (5-20 µM), the morphogenic response in terms of shoot formation was similar in all the concentrations tried (Table 23) and the average number of shoots per node was only 1.40 ± 0.15. Buds proliferated within a week and elongated into shoots in the further weeks. As each node of *T. indica* has two opposite buds, it was observed that one bud initially started to grow and after two weeks the second bud grew into a shoot. In presence of 5 µM BA, the shoots were able to reach a maximum length of 3.97 ± 0.91 cm by four weeks in 100% cultures. The other concentrations proved to be slightly inferior than the above concentration (Fig. 46a). Gradual increase in BA levels failed to enhance the shoot number and length as well (Table 23).

Replacing BA with Kn in the same concentration range showed similar response like BA. However, there was significant increase in shoot length as observed in all the concentrations of this combination (Fig. 46b). It was recorded that, even the concentration of Kn increased, shoot number remained the same. A total of 1.60 ± 0.15 shoots having 5.92 ± 1.44 cm length was recorded in presence of Kn (15 µM) (Table 23).

- Among BA and Kn, the later proved to be beneficial for regeneration as it induced shoots with broad leaves and increased length.

4.3.2.2 Effect of Synergistic Cytokinins on Axillary Shoot Regeneration

Individual cytokinins mostly stimulated the axillary buds to form one or two shoots, but they failed to form multiples and therefore their combinations were tried.

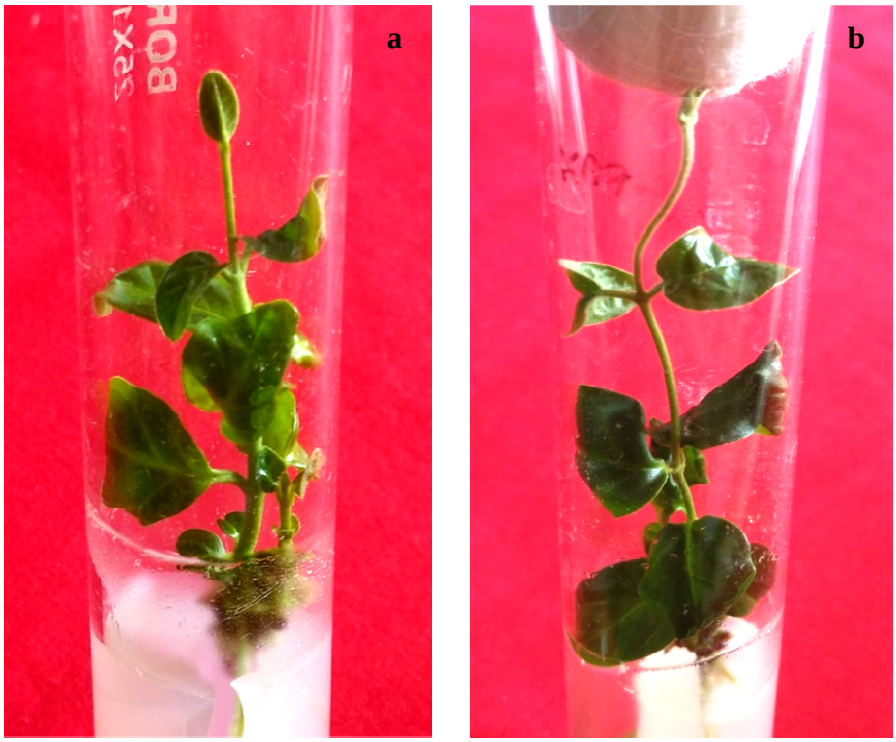

Figure 46. Effect of individual cytokinins on shoot regeneration from nodal explants of *T. indica* at the end of eight weeks: (a) Formation shoots in presence of BA (5 µM) and (b) development of shoot with significant length in Kn (15 µM).

- **BA and Kn**

When medium was fortified with BA and Kn (5-20 µM), lower concentration (5 µM) of BA and Kn formed more than one shoot (1.60 ± 0.21) and the length reached to 3.84 ± 0.64 cm. When BA was kept constant at 5 µM and Kn levels were increased (10-20 µM) it only helped in elongation of shoots as the length reached to 4.50 ± 0.49 cm at 15 µM. Multiples were obtained when BA level increased to 10 µM and it formed maximum 4.10 ± 0.22 shoots with Kn (5 µM) and their length increased to 5.15 ± 0.29 cm by eight weeks. In this combination, the bud proliferated in the first week and immediately formed a shoot within two weeks (Fig. 47a). These shoots elongated within four weeks and another bud emerged from the opposite node (Fig. 47b). Subculture was done after four weeks in the same medium composition and that aided development of multiple shoots within six weeks (Fig. 47c). Shoot proliferation and elongation was observed at the end of eight weeks (Fig. 47d). Further increase in Kn level to 10-20 µM, reduced the number of shoots as well as length. When BA was increased to 15-20 µM, shoot frequency slightly enhanced to 2.40 ± 0.38 in presence of BA (15 µM) with Kn (5 µM) and highest shoot length of 5.34 ± 0.50 cm was achieved as compared to above concentrations. Although maximum number of shoots were recorded at lower concentration of cytokinins, BA (15 µM) and Kn (15 µM) proved to be better for shoot length as it became 6.54 ± 0.86 cm. Increasing the level of Kn failed to improve in number of shoots. At higher concentrations of BA (20 µM) with various levels of Kn, shoots were grown as stunted with hyperhydricity at the end of eight weeks (Table 23).

- **BA and $AdSO_4$**

$AdSO_4$ is a known cytokinin which stimulates multiple shoot formation, thus it was combined with BA and Kn to enhance the shoot number. Initially $AdSO_4$ (5-20 µM) was combined with BA in same concentration range and its effect on nodal explants was assessed. Observation revealed that at low concentration of BA (5 µM) with all concentrations of $AdSO_4$ failed to enhance the number. When BA reached to 10 and 15 µM, the shoot number slightly went up i.e. 2.50 ± 0.25 in presence of BA (15 µM) with $AdSO_4$ (10 µM) in 100% cultures, but this response wasn't significant. Highest length of 7.05 ± 1.16 cm was obtained in presence of 10 µM of BA and $AdSO_4$ (Fig. 48a) among all combinations tried. At high concentration of BA, the shoot number as well as length gradually declined (Table 23).

Figure 47. Synergistic effect of PGRs on optimum shoot regeneration in MS + BA (10 µM) + Kn (15 µM) from nodal explants of *T. indica*: (a) Axillary bud break and its elongation within two weeks, (b) shoot formation and induction of new buds from both sides of node at four weeks, (c) formation of multiple shoots after six weeks and (d) healthy elongated shoots by eight weeks.

Figure 48. Effect of synergistic cytokinins on shoot regeneration from nodal explants of *T. indica* at the end of eight weeks: (a) maximum shoot length in presence of BA (10 µM) + $AdSO_4$ (10 µM) and (b) regeneration of shoot in medium supplemented with Kn (10 µM) + $AdSO_4$ (15 µM).

Table 23. Effect of cytokinins on shoot regeneration from nodal explants of *T. indica* (8 weeks).

PGR (µM)			No. of shoots/explant*	Shoot length (cm)*	Response (%)
BA	Kn	$AdSO_4$			
0	0	0	0.00 ± 0.00 e	0.00 ± 0.00 f	0
5	0	0	1.40 ± 0.15 bcd	3.97 ± 0.91 de	100
10	0	0	1.30 ± 0.14 cd	3.81 ± 0.68 de	100
15	0	0	1.40 ± 0.15 bcd	3.34 ± 0.55 de	100
20	0	0	1.30 ± 0.14 cd	3.54 ± 0.67 de	100
0	5	0	1.20 ± 0.13 d	4.32 ± 1.07 cd	100
0	10	0	1.40 ± 0.15 bcd	4.80 ± 1.19 cd	100
0	15	0	1.60 ± 0.15 bcd	5.92 ± 1.44 bc	100
0	20	0	1.30 ± 0.14 cd	3.80 ± 0.69 de	100
5	5	0	1.60 ± 0.21 bcd	3.84 ± 0.64 de	100
5	10	0	1.20± 0.13 d	3.61 ± 0.57 de	100
5	15	0	1.10 ± 0.09 d	4.50 ± 0.49 cd	100
5	20	0	1.50 ± 0.16 bcd	2.97 ± 0.40 e	100
10	5	0	4.10 ± 0.22 a	5.15 ± 0.29 bcd	100
10	10	0	1.30 ± 0.14 cd	3.02 ± 0.54 e	100
10	15	0	1.50 ± 0.16 bcd	3.54 ± 0.42 de	100
10	20	0	1.40 ± 0.15 bcd	3.99 ± 0.69 de	100
15	5	0	2.40 ± 0.38 b	5.34 ± 0.50 bcd	100
15	10	0	1.80 ± 0.13 bcd	5.75 ± 0.74 bc	100
15	15	0	1.70 ± 0.14 bcd	6.54 ± 0.86 ab	100
15	20	0	1.80 ± 0.13 bcd	3.90 ± 0.50 de	100
20	5	0	2.10 ± 0.22 bcd	2.39 ± 0.29 e	100
20	10	0	1.50 ± 0.16 bcd	4.08 ± 0.44 cde	100
20	15	0	1.80 ± 0.13 bcd	4.61 ± 0.78 cd	100
20	20	0	1.90 ± 0.26 bcd	5.40 ± 0.37 bcd	100
5	0	5	1.30 ± 0.14 cd	4.27 ± 0.79 cd	100
5	0	10	1.70 ± 0.20 bcd	5.67 ± 1.29 bc	100
5	0	15	1.60 ± 0.15 bcd	3.92 ± 0.85 de	100

5	0	20	1.60 ± 0.15 bcd	4.08 ± 1.02 cde	100
10	0	5	1.90 ± 0.22 bcd	4.06 ± 1.00 cde	100
10	0	10	1.40 ± 0.15 bcd	7.05 ± 1.16 a	100
10	0	15	2.10 ± 0.26 bcd	3.11 ± 0.38 de	100
10	0	20	2.00 ± 0.20 bcd	4.07 ± 0.70 cde	100
15	0	5	2.10 ± 0.22 bcd	4.25 ± 0.99 cd	100
15	0	10	2.50 ± 0.25 b	4.15 ± 0.74 cd	100
15	0	15	1.50 ± 0.16 bcd	4.07 ± 0.81 cde	100
15	0	20	1.70 ± 0.20 bcd	4.25 ± 0.89 cd	100
20	0	5	2.30 ± 0.32 bc	3.05 ± 0.39 e	100
20	0	10	1.70 ± 0.20 bcd	3.06 ± 0.75 e	100
20	0	15	1.70 ± 0.14 bcd	2.50 ± 0.73 e	100
20	0	20	1.20 ± 0.13 d	2.87 ± 0.70 e	100
0	5	5	1.40 ± 0.15 bcd	5.58 ± 0.97 bc	100
0	5	10	1.40 ± 0.15 bcd	3.88 ± 0.73 de	100
0	5	15	1.50 ± 0.16 bcd	3.71 ± 0.49 de	100
0	5	20	1.60 ± 0.15 bcd	4.45 ± 0.78 cd	100
0	10	5	1.40 ± 0.15 bcd	5.10 ± 0.92 bcd	100
0	10	10	1.20 ± 0.13 d	4.97 ± 0.52 bcd	100
0	10	15	1.70 ± 0.14 bcd	6.97 ± 0.88 ab	100
0	10	20	1.30 ± 0.14 cd	4.55 ± 0.63 cd	100
0	15	5	1.50 ± 0.16 bcd	5.57 ± 0.81 bc	100
0	15	10	1.40 ± 0.15 bcd	4.37 ± 0.18 cd	100
0	15	15	1.30 ± 0.14 cd	4.06 ± 0.62 cde	100
0	15	20	1.40 ± 0.15 bcd	4.10 ± 0.63 cd	100
0	20	5	1.40 ± 0.15 bcd	4.10 ± 0.67 cd	100
0	20	10	1.50 ± 0.16 bcd	3.38 ± 0.43 de	100
0	20	15	1.40 ± 0.15 bcd	3.26 ± 0.53 de	100
0	20	20	1.30 ± 0.14 cd	2.90 ± 0.51 e	100

*Values represent mean ± SE. Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

- **Kn and $AdSO_4$**

When Kn was added with $AdSO_4$, it hardly improved the shoot number which remained below 1.70 ± 0.14 per node in all concentrations (Table 23). But $AdSO_4$ was able to increase the shoot length as well as leaf broadening when combined with Kn. At lower concentration (5 µM) of both PGRs the shoot length reached to 5.58 ± 0.97 cm which slightly lowered down with increasing concentrations of $AdSO_4$ (10-20 µM). Maximum length 6.97 ± 0.88 cm was observed when Kn at 10 µM and $AdSO_4$ at 15 µM wasadded in the medium (Fig. 48b). Further increasing concentration of Kn (15 and 20 µM) with various concentrations of $AdSO_4$, the length gradually decreased.

- Axillary shoot regeneration experiment facilitated formation of healthy shoots in 100% cultures. BA-Kn proved to be better for regeneration of multiple shoots and BA-$AdSO_4$ helped in elongation of shoots.

Nodal explant of *T. indica* when placed in medium augmented with BA formed only few shoots which is in accordance with previous results for *Hemidesmus indicus* (Siddique and Bari, 2006; Singh et al., 2015), *Desmodium gangeticum* (Vishwakarma et al., 2009) and *Cucumis sativus* (Abu-Romman et al., 2015). Similarly individual Kn also failed to induce multiple shoots and there are similar report for in *Vitex trifolia* (Hiregoudar et al., 2006), *Ceropegia pusilla* (Kondamudi et al., 2010), *Tinospora cordifolia* (Sivakumar et al., 2014) and *T. indica* (Najar et al., 2018). However shoots regenerated in Kn supplemented medium had longer internodes and thus they were long as compared to BA containing medium. This is in accordance with previous report on *T. indica* (Faisal et al., 2007) and other Asclepiadaceae plants like *Gymnema sylvestre* (Komalavalli and Rao, 2000) and *Ceropegia candelabrum* (Beena et al., 2003), as well as for shoot derived from nodal explant of *Solanum nigrum* (Padmapriya et al., 2011) and *C. sativus* (Abu-Romman et al., 2015) where Kn facilitated shoot elongation.

Both the cytokinins individually failed to induce multiples but when added together they induced an optimum response with 4.10 ± 0.22 shoots with 5.15 ± 0.29 cm length in eight weeks. This is may be due to the fact that cytokinins when added in combinations, release bud dormancy and promote axillary bud outgrowth (Shimizu-Sato et al., 2009; Yaish et al., 2010). Similar observations have been reported in nodal explant of *Enicostemma hyssopifolium* (Seetharam et al., 2002) and *Chlorophytum borivilianum* (Ashraf et al., 2014).

In contrary to previous studies on nodal explant of *T. indica* where the use of cytokinin with auxin BA+NAA+ascorbic acid was reported for optimum shoot formation (Faisal et al., 2007). Their report states formation of 8.6 ± 0.71 shoots with 5.2 cm length in 93% response which is slightly higher as compared to the present study. In another report 3.59 shoots are documented after 12 weeks when nodes are grown in presence of Kn (2 mg/l) with IAA (0.5 mg/l) (Soni et al., 2015) and this shoot number is lower as compared to present study. Najar et al. (2018) also used combination of cytokinin and auxin [TDZ (1.5 mg/l) + IBA (0.1 mg/l)] in their study but culture period required for multiple shoot formation was longer (60 days) as compared to 56 days stated in the present result.

In *T. indica*, optimum regeneration was observed when MS medium was fortified with higher BA (10 µM) with lower Kn (5 µM) concentration, similar PGR combination at same level has been documented in *H. indicus* by Pathak et al. (2017). Similarly, in nodal explant of *Artemisia vulgaris*, a combination of higher BA and lower Kn (4.44 and 2.32 µM respectively) evoked optimum regeneration, whereas individual cytokinins differentiated less number of shoots (Sujatha and Kumari, 2007). On the contrary another report on *H. indicus* stated that low level BAP (1.0 mg/l) with high level Kn (2.0 mg/l) showed maximum shoot formation (Rama Devi et al., 2014). The positive effect of BA and Kn is also reported in *Piper longum* (Soniya and Das, 2002), *Crataeva nurvala* (Walia et al., 2007a), *Swertia chirata* (Balaraju et al., 2009), *Andrographis paniculata* (Dandin and Murthy, 2012) and *Catharanthus roseus* (Sain and Sharma, 2013). Moreover, in medicinal plants like *Passiflora edulis* (Shekhawat et al., 2015a), *Morinda citrifolia* (Shekhawat et al., 2015b) and *Farsetia macrantha* (Choudhary et al., 2020) these PGR combinations have been used for efficient multiplication of shoots.

When combinations of BA/Kn with $AdSO_4$ was tried for shoot regeneration from nodes of *T. indica*, it failed to induce multiple shoot, but it enhanced the shoot length in both the combinations. This is in line with previous report of *Bambusa arundinacea* in which maximum shoot length was achieved on MS medium containing BAP/Kn and $AdSO_4$ (Venkatachalam et al., 2015). Similarly, Garima and Amla (2010) have also described the promotive effect of $AdSO_4$ in combination with BAP on shoot elongation of *Thevetia peruviana*. Whereas in *Stevia rebaudiana* significant shoot length has been reported in combination of Kn and $AdSO_4$ (Khan et al., 2014).

Axillary shoot regeneration experiment facilitated simultaneous formation of a morphogenic callus at the base of node. This callus differentiated shoot buds in presence of BA with $AdSO_4$ and it became embryogenic under the influence of Kn and $AdSO_4$ which formed somatic embryos. Thus, the results for these two, different developmental pathways have been given under their respective synergistic combination.

- **Effect of BA and $AdSO_4$ on Indirect Shoot Regeneration**

BA and $AdSO_4$ induced the formation of a morphogenic callus at the base of nodal explant in many combinations. As soon as the axillary bud proliferated into a shoot, simultaneously callus started to differentiate at the base of the nodal explants. There were several combinations in which this response was observed. The callus became nodular in progressive weeks and differentiated shoot buds by end of eight weeks (Fig. 49a). The response varied between 30% to 50% but it reached to 70% in presence of BA (20 µM) with $AdSO_4$ (5 µM) (Fig. 50). The basal portion of nodal explants with the callus was transferred to a fresh medium (Fig. 49b) and immediately the shoot buds elevated into shoots within twelve weeks (Fig. 49c).

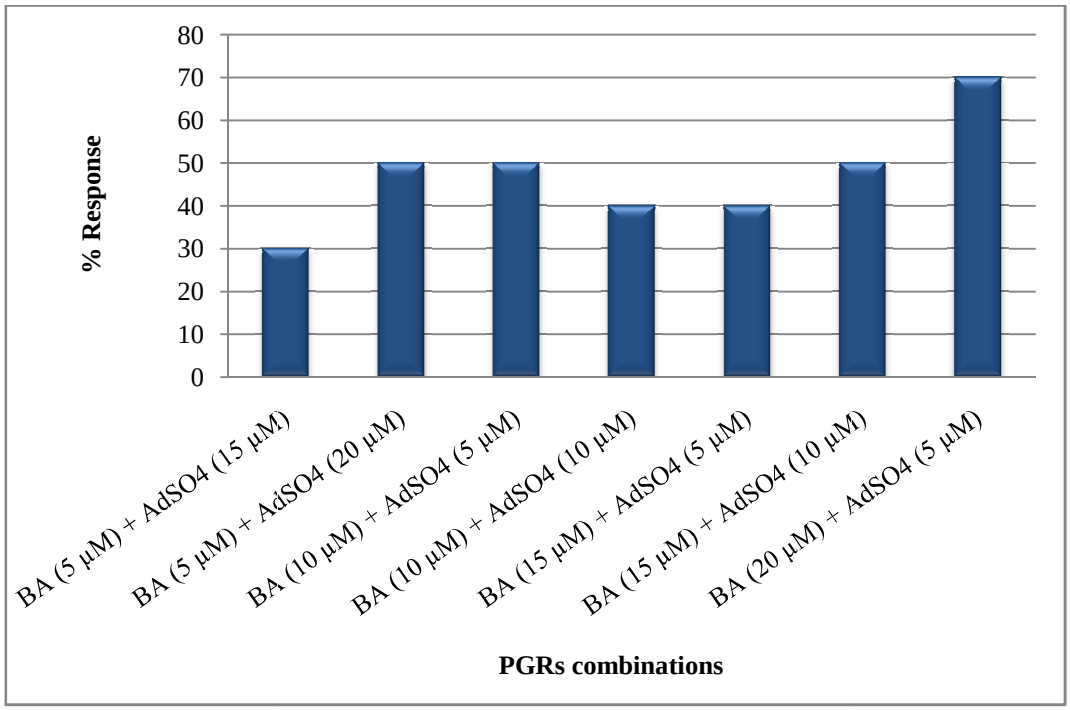

Figure 50. Response of nodal derived callus for shoot regeneration in different concentrations of BA + $AdSO_4$ by the end of eight weeks.

Figure 49. Indirect shoot regeneration from nodal derived callus in presence of BA (20 µM) + $AdSO_4$ (5 µM) in *T. indica*: (a, b) Differentiation of shoot buds and shoots after eight weeks and (c) elongated shoots after twelve weeks.

Generally nodal cultures are aimed to generate shoots via axillary bud proliferation, but sometimes callus formation was observed at the base of node especially when media contains cytokinins which affect callogenesis by decreasing the cell wall lignification, facilitating callus initiation and proliferation (Kumlay and Ercisli, 2015). In present study, *T. indica* nodes when grown in presence of BA-$AdSO_4$, simultaneously formed callus at base which differentiated shoot buds during the culture period. This callus regenerated shoots within four weeks when transferred to fresh medium. This result is in contradiction to previous reports on the same plant (Najar et al., 2018) as they also observed callus at the base but was not morphogenic in nature. Whereas Faisal and Anis (2005) developed shoot regeneration protocol from nodal derived callus in presence of another cytokinin (2,4,5-T) at 10 μM, which differentiated shoots after transferring to Kn (5 μM) fortified medium.

It was observed that higher of BA-$AdSO_4$ induced basal callusing from nodes of *T. indica* which is in line with report of *Ananas comosus* by Ibrahim et al. (2013) but callus was formed in presence of only BA. Similarly in other plants like *Gypsophila paniculata* (Kanchanapoom et al., 2011), *Solanum tuberosum* (Iqbal et al., 2014) and *Lycium barbarum* (Karakas, 2020) callus formed at the base of node differentiated shoots after subculturing. Whereas in *Rauvolfia serpentina* nodal derived callus was transferred to BA containing medium for shoot regeneration (Gupta et al., 2014). Beneficial effect of $AdSO_4$ in presence of BA for indirect shoot regeneration has been documented in *S. tuberosum* (Singh et al., 2017) and *Gerbera jamesonii* (Talla et al., 2019). Previously, nodal explant of *L. reticulata* induce basal callus in presence of Kn with NAA/IBA which formed multiple shoots afterwards (Parabia et al., 2007), similar response was also reported in BA-NAA by Rathore et al. (2013). Whereas Sudipta et al. (2011) documented callus formation in presence of BA and Kn for the same plant. Recently Rohela et al. (2020) depicted optimum regeneration from nodal derived callus of *Morus alba* in combination of BA with another cytokinin (TDZ). Use of nodal derived callus for multiple shoot regeneration is also reported previously in *Aristolochia indica* (Siddique et al., 2002), *Kigelia pinnata* (Thomas and Puthur, 2004), *Cassia alata* (Hasan et al., 2008), *Fragaria* sp. (Biswas et al., 2010), *Vanilla planifolia* (Tan et al., 2011), *Aristolochia indica* (Pattar and Jayaraj, 2012), *Albizia lucida* (Saha et al., 2013), *Withania somnifera* (Udayakumar et al., 2014) and *Solanum tuberosum* (Kumlay and Ercisli, 2015).

- **Effect of Kn and $AdSO_4$ on Somatic Embryogenesis**

Presence of Kn in the medium induced a similar response with above combination as this combination also differentiated callus from the base of node and it was embryogenic. It was noted that in all combinations with different concentrations, the % response for SEs differentiation varied (Fig. 52). Highest number of embryos were in presence of Kn (10 µM) with $AdSO_4$ (15 µM) in 60% cultures. Initially they were in globular stage and after subculture these embryos passed through different stages and germinated into plantlets (Fig. 51a). Few embryos induced later which remained in the globular or heart stages by the end of eight weeks For further development of plantlets/SEs, callus was transferred to static medium with the same combination (Fig. 51b) and also in earlier optimized liquid medium for SE development (1 µM GA_3) generated through leaf expant. After subculture by end of twelve weeks healthy plantlets were obtained from both media, static (Fig. 51c) and liquid medium (Fig. 51d) but later comparatively proved better.

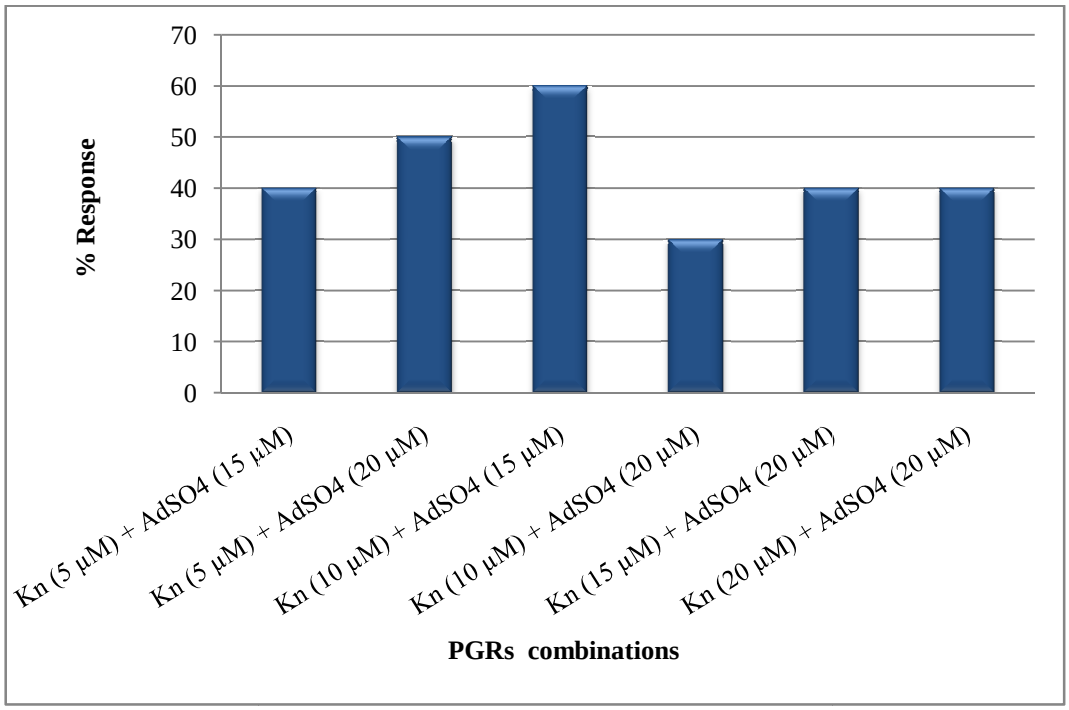

Figure 52. Response of nodal derived callus for differentiation of SEs in different concentrations of Kn + $AdSO_4$ by end of eight weeks.

It was observed that when BA was replaced by Kn and tried with $AdSO_4$ it formed embryogenic callus which simultaneously differentiated SEs. Previously role of Kn in indirect somatic embryogenesis has been well documented in *Gentiana* sp. (Tomiczak et al., 2016). In

Figure 51. Induction of indirect SEs from nodal derived callus in presence of Kn (10 µM) + $AdSO_4$ (15 µM) in *T. indica*: (a) Formation of embryogenic callus at the base of axillary shoot, (b) cluster of differentiating SEs, (c) germination of SEs into plantlets in static medium after twelve weeks and (d) germination of SEs into plantlets in liquid medium containing GA_3 (1 µM) by twelve weeks.

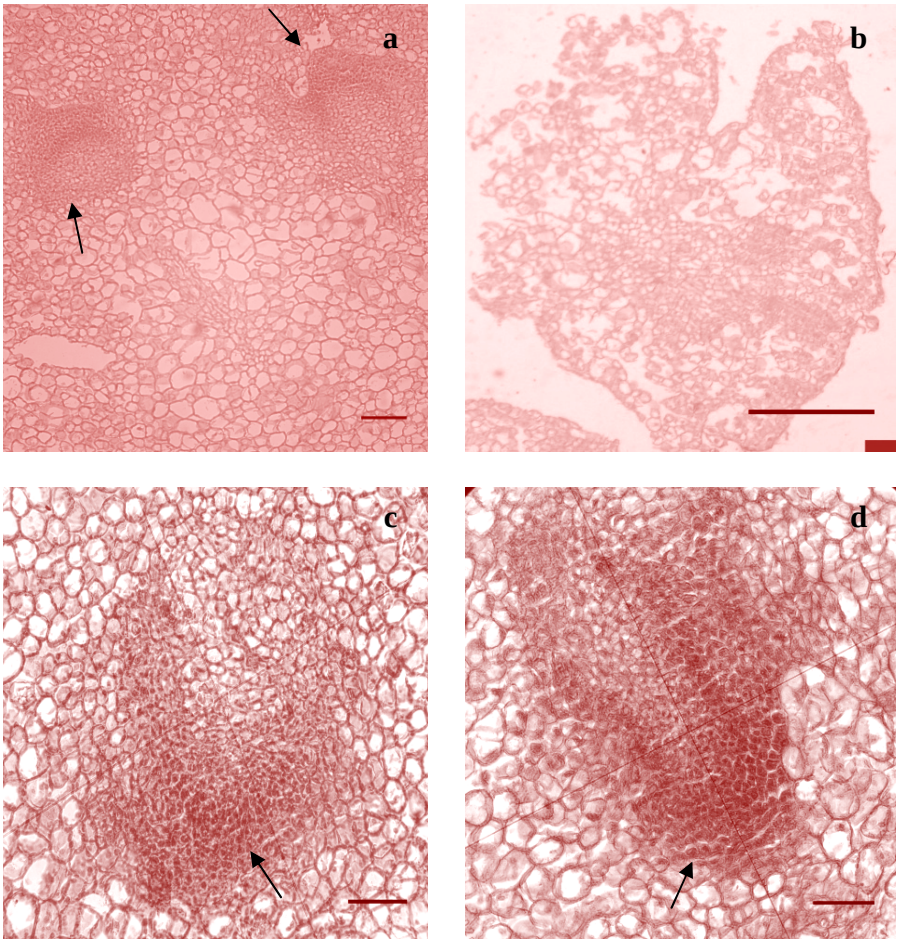

Figure 53. Histology of SEs in *T. indica*: (a) Embryogenic cell clumps, (b) early heart stage embryo, (c) heart and (d) torpedo stage. (Bar = 0.5 mm)

the present study, it was also observed that subculture in the same combination helped in conversion of SEs into plantlets. As the $AdSO_4$ was present in the medium it enhanced the efficiency of SEs maturation which is considered as key step in somatic embryogenesis (Vibha et al., 2009). Earlier reports have suggested that synergism of Kn with $AdSO_4$ facilitated SEs formation in *Gentiana kurroo* (Fiuk and Rybczynski, 2007) and *Gentiana decumbens* (Tomiczak et al., 2015). The embryogenic callus derived from nodal explant has been utilized for plant regeneration through somatic embryogenesis in *Dioscorea alata* (Belarmino and Gonzales, 2008), *H. indicus* (Cheruvathur et al., 2013b), *Dioscorea rotundata* (Manoharan et al., 2016) and *Mirabilis jalapa* (Rohela et al., 2016). Although the combinations of BA/Kn with $AdSO_4$ induced morphogenic callus from nodal explant of *T. indica*, which further differentiated shoots and embryos, the potency of this callus for regenerating shoots and SEs was less as compared to leaf derived callus. This is due to fact that the explant type affects the frequency of organogenesis and embryogenesis (Lincy et al., 2009; Abrha et al., 2013; Kumlay and Ercisli, 2015).

4.3.2.3 Histology of SEs

Histological study of embryogenic calli showed that embryogenic cells were small, closely packed and after four weeks, some darkly stained embryogenic cell masses were observed (Fig. 53a). In the initial weeks early heart and heart stage embryos were visible (Fig. 53b, c) and as culture time increased torpedo stages were observed (Fig. 53d).

4.3.3 Effect of PGRs on Rooting of Shoots

In the present study shoots developed through leaf and nodal explants were placed for rooting in half and quarter strength of MS medium augmented with 1% sucrose and different concentrations (2-8 µM) of IBA and NAA. ¼ and ½ strength MS basal media served as control.

- **IBA**

½MS basal medium formed 1.50 ± 0.25 roots which were smaller in length (0.90 ± 0.10 cm). Supplementing it with different concentrations of IBA induced rooting in all concentrations. Lower concentrations of IBA (2 and 4 µM) formed higher roots compared to basal but bothe concentrations showed almost similar number of roots. Length was less in 2 µM as compared to 4 µM where it reached to 2.03 ± 0.36 cm which was highest in all

concentrations of ½MS. Root number and length decreased at higher concentrations (6 and 8 µM) and response also decreased. Reducing the strength to ¼MS facilitated higher number of roots in basal medium i.e. 3.40 ± 0.54 and at 4 µM an optimum of 9.75 ± 0.74 roots (80% response) were formed with 3.11 ± 0.33 cm length was recorded at the end of four weeks (Fig. 54a). Further raising the level of IBA gradually decreased the number of roots as well as their response (Table 24).

Table 24. Effect of IBA and NAA on rooting in *T. indica* shoots (4 weeks).

PGRs (µM)		No. of roots/shoot*	Root length (cm)*	Response (%)
IBA	NAA			
½MS				
0	0	1.50 ± 0.25 f	0.90 ± 0.10 d	40
2	0	3.71 ± 0.33 de	1.62 ± 0.21 bc	70
4	0	3.80 ± 1.00 de	2.03 ± 0.36 b	50
6	0	1.80 ± 0.33 ef	0.97 ± 0.17 c	40
8	0	1.75 ± 0.41 ef	0.84 ± 0.09 d	40
0	2	3.67 ± 0.77 de	1.07 ± 0.09 bc	50
0	4	6.33 ± 0.51 bc	1.24 ± 0.05 bc	50
0	6	4.60 ± 1.28 cd	0.98 ± 0.12 c	50
0	8	2.00 ± 0.00 ef	0.48 ± 0.11 d	30
¼MS				
0	0	3.40 ± 0.54 de	1.47 ± 0.28 bc	50
2	0	8.33 ± 0.98 bc	1.81 ± 0.28 bc	30
4	0	9.75 ± 0.74 a	3.11 ± 0.33 a	80
6	0	4.20 ± 0.91 cd	1.84 ± 0.14 bc	50
8	0	2.00 ± 0.00 ef	0.73 ± 0.09 d	20
0	2	4.00 ± 0.63 cd	1.89 ± 0.19 b	50
0	4	5.33 ± 1.09 cd	1.59 ± 0.04 bc	30
0	6	3.00 ± 0.47 de	1.39 ± 0.14 bc	30
0	8	3.29 ± 0.80 de	1.64 ± 0.07 bc	70

*Values represent mean ± SE. Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

- **NAA**

Replacing IBA with NAA in the medium also induced rooting in all the concentrations tried. In ½MS it formed 6.33 ± 0.51 roots in 4 µM with 1.24 ± 0.05 cm length in 50% cultures (Fig. 54b). Higher concentrations (6 and 8 µM) reduced the root number and response. The ½MS medium containing NAA was better as compared to IBA containing medium. Reducing the strength to ¼MS and addition of NAA resulted with total 5.33 ± 1.09 roots at 4 µM and percent response reached to 30% which was comparatively lower to half strength. Increased concentration of NAA (6-8 µM), declined the number of roots as well as response (Table 24).

- The results for root induction in shoots can be concluded that the strength of MS medium and the type of auxins had a profound effect on the number of roots, their length and the % response. The lower concentration of IBA (2 and 4 µM) in ¼ strength proved beneficial for root induction.

Fortifying the media with different IBA levels (2-8 µM) induced rooting of shoots in all the concentrations and both the strengths. The optimum rooting with 9.75 ± 0.74 roots (80% response) with 3.11 ± 0.33 cm length was observed in ¼MS medium fortified with 4 µM of IBA. Whereas full MS (Soni et al., 2015) and ½MS medium (Faisal and Anis, 2003; Thomas and Phillip, 2005; Faisal et al., 2007; Sahai et al., 2010a; Sadguna et al., 2013; Sharma et al., 2014; Najar et al., 2018) supplemented with IBA has been reported for rooting of *T. indica* shoots, but the percent response achieved in the present study for rooting was higher in comparison to above reports. Similar to present investigation, ¼MS medium fortified with IBA proved to be beneficial for rooting in other medicinal plants such as *Bacopa monnieri* (Mehta et al., 2012a), *Terminalia bellerica* (Mehta et al., 2012b), *Catharanthus roseus* (Sain and Sharma, 2013), *L. reticulata* (Rathore et al., 2013; Patel et al., 2014) and *Hemidesmus indicus* (Shekhawat and Manokari, 2016; Pathak and Joshi, 2017).

When another auxin NAA (2-8 µM) was tried, the root number and length decreased as compared to IBA containing media which is in line with earlier studies on *T. indica* (Thomas and Phillip, 2005; Faisal et al., 2007; Sahai et al., 2010a; Verma et al., 2010; Najar et al., 2018). Martin (2002) suggested that the IBA when added to media showed slow movement as well as degradation which facilitates its localization near the rooting site and thus increases the rooting. Superiority of IBA over NAA has been also observed in *H. indicus* (Sreekumar et al., 2000; Nagahatenna and Peiris, 2007; Sundarmani and Hasina, 2015), *Munronia pinnata*

Figure 54. Rooting of *T. indica* shoots at the end of four weeks: (a) Induction of long multiple roots in ¼MS medium supplemented with IBA (4 µM) and (b) induction of roots in ½MS medium with NAA (4 µM).

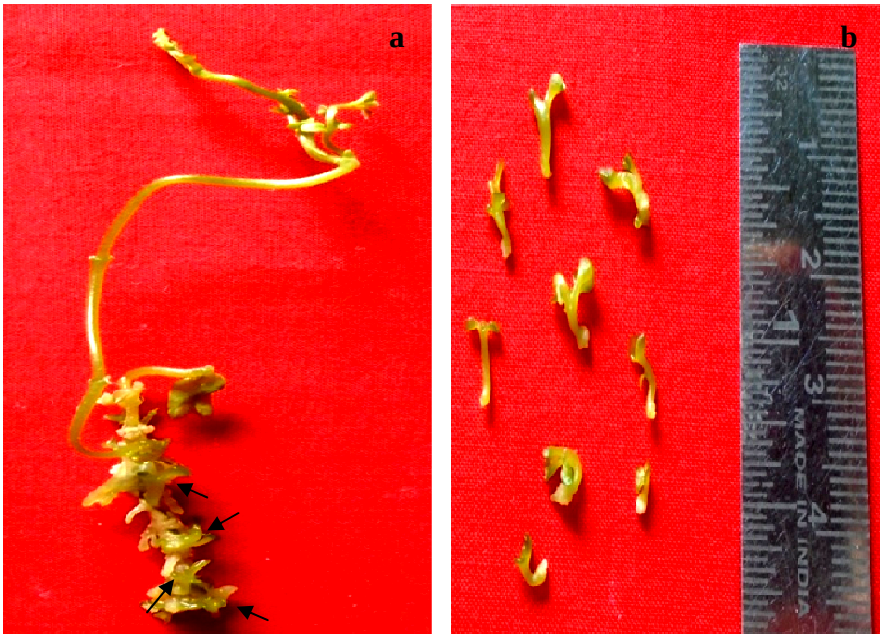

Figure 55. Differentiation of SEs from immersed stem portion in ½MS medium added with NAA at the end of four weeks in *T. indica*: (a) Indirect SEs on stem portion of shoot in NAA (4 µM) and (b) cotyledonary stage embryos separated from callus.

(Gunathilake et al., 2008), *Rubia cordifolia* (Khadke et al., 2013), *Anisochilus carnosus* (Reshi et al., 2017), *Ziziphus jujube* (Hou et al., 2017) and *Brucia mollis* (Das et al., 2018). The adverse effect of NAA may be due to increase in ethylene biosynthesis which affects root formation and its elongation (Riov and Yang, 1989; Taiz and Zeiger, 2003).

Another morphogenic response which accompanied along with rooting of shoots was SEs differentiation from intermodal segment of shoots which were placed in half strength MS medium fortified with different concentrations of NAA. Maximum of 15.4 ± 1.82 SEs were formed at 4 µM in 50% cultures (Fig.55a, 56). SEs differentiated from NAA were developed till cotyledonary stage within four weeks (Fig. 55b). The *in vitro* shoots showed a strong tendency to form SEs in ½ strength MS medium fortified with NAA. This dual organogenic response has been studied for the first time in *T. indica.*

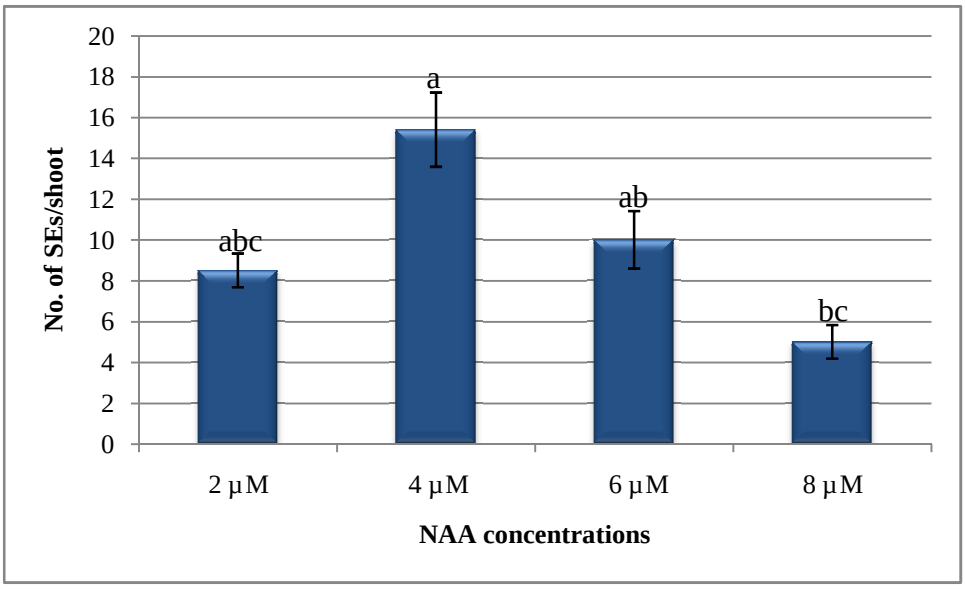

Figure 56. Formation of SEs from stem of shoots in ½MS medium supplemented with NAA.

Each bar shows the mean values (n = 10) and error bar as standard error. Bars having same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

When rooting of shoots was achieved in NAA containing medium, formation of direct SEs occurred on internodal portion of stem. Similarly, the role of NAA on somatic embryogenesis is well documented in *Manihot esculenta* (Taylor et al., 2001) and *Chlorophytum borivilianum* (Akhtar and Choudhary, 2016). Previously in *T. indica*, indirect

somatic embryogenesis from internodal explant is reported in which callus formation occurred in presence of another auxin 2,4-D at 4 μM (Thomas, 2006). Similarly, in *L. reticulata* also the SEs are documented to be induced from stem explant (Sathyanarayana et al., 2008). Nevertheless, the frequency of embryo formation is less in stem explants as compared to leaf explants, which is in corroboration with the results in *Solanum nigrum* (Xu et al., 2014), *Crocus* sps. (Verma et al., 2016) and *Manihot esculenta* (Syombu et al., 2019) and *Portulaca oleracea* (Sedaghati et al., 2019). The differences in the response of different explants to somatic embryogenesis could be attributed to their differences in morphological structure and stages of maturity (Schädel et al., 2010). Leaf explants are with large surface area which is in contact with the medium and thus a better morphogenic response is generated as compared to stem explants which are round in shape. Additionally, the differential response by the two explants could also be due to the fact that leaves are less lignified as compared to stem explants making them more responsive to regulatory hormones and easily programmed to undergo dedifferentiation (Twumasi et al., 2009; San-José et al., 2010; Schädel et al., 2010). Similarly Azad et al. (2009) also documented that SEs differentiated from stem explants is less as compared to other tried explants in *Phellodendron amurense*.

4.3.4 Hardening of Planltlets

Young and healthy plantlets obtained from SEs and rooted shoots derived from 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). Plantlets were covered with polythene bags to maintain humidity and placed in culture room. They were watered every alternate day till four weeks and regularly monitored (Fig. 57a). These healthy plantlets were shifted to greenhouse and transferred in small pots with same composition of substrates for another four weeks. Then they were removed gently from pots without damaging their roots after eight weeks (Fig. 57b) and placed in garden soil. Hardened plants were phenotipically similar to the *in vivo* mother plants and 100% survival of the plants was observed under field conditions (Fig. 57c). These healthy plants formed flowers (Fig. 57d) and fruits (Fig. 57e) after one year. Data for growth of plantlets in terms of shoot and root length was recorded from the day they were transferred to substrate (Day 0) till they were shifted to field (Day 90) (Fig. 58) and these plants were successfully acclimatized.

Figure 57. Hardening of *T. indica* shoots developed from different regenerative pathways: (a) Plantlets transferred in cups filled with cocopeat:sand (1:1) and kept under lab condition after four weeks, (b) plantlets with well developed roots after eight weeks, (c) one year old hardened plants in field and (d, e) flowering and fruit formation in hardened plants.

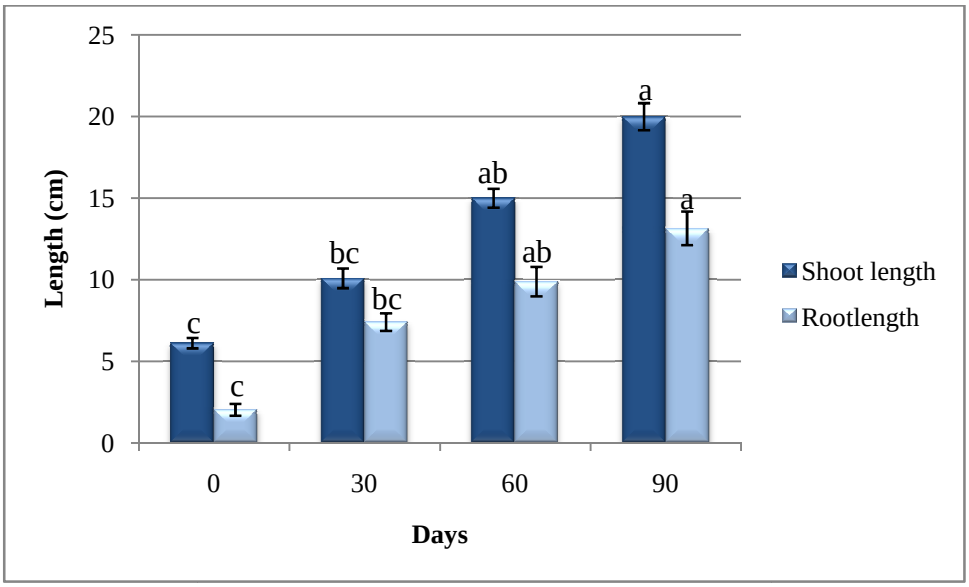

Figure 58. Growth of *T. indica* plantlets during hardening and acclimatization.

Each bar shows the mean values (n = 10) and error bar as standard error. Bars having same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Healthy rooted shoots and SE derived plantlets were successfully hardened and acclimatized within 90 days in cocopeat:sand (1:1). Planting substrate used for hardening of *in vitro* plant is one of the most important factors during acclimatization. In previous studies on *T. indica*, different planting substrates like vermiculite followed by sand:soil (1:1) (Manjula et al., 2000), sand:soil:cow dung (1:1:1) (Jayanthi and Mandal, 2001), vermiculite (Faisal and Anis, 2003; Faisal et al., 2007), soil (Thomas and Phillip, 2005), vermiculite (Chandrashekhar et al., 2006), peat-moss:perlite (3:1) (Sahai et al., 2010a), vermicompost:soil (1:3) (Verma et al., 2010), soilrite (Haque and Ghosh, 2013), soilrite:soil (1:1) (Soni et al., 2015) and sand:soil:manure (1:2:1) (Najar et al., 2018) were uses before final transfer to garden soil. The survival rate of *T. indica* plantlets in present study was 100% throughout hardening and acclimatization, which is higher as compared to earlier reports. This suggests that the mixture of cocopeat:sand (1:1) was suitable for growth of *T. indica*, which is also reported for hardening of *Limonium* hybrid `Misty Blue' (Bose et al., 2017) and *Hibiscus sabdariffa* (Konar et al., 2018).

- Different modes of regenerative pathways were obtained from leaf and nodal explants of *T. indica*, which undergo rooting and successively hardening (Fig. 59).

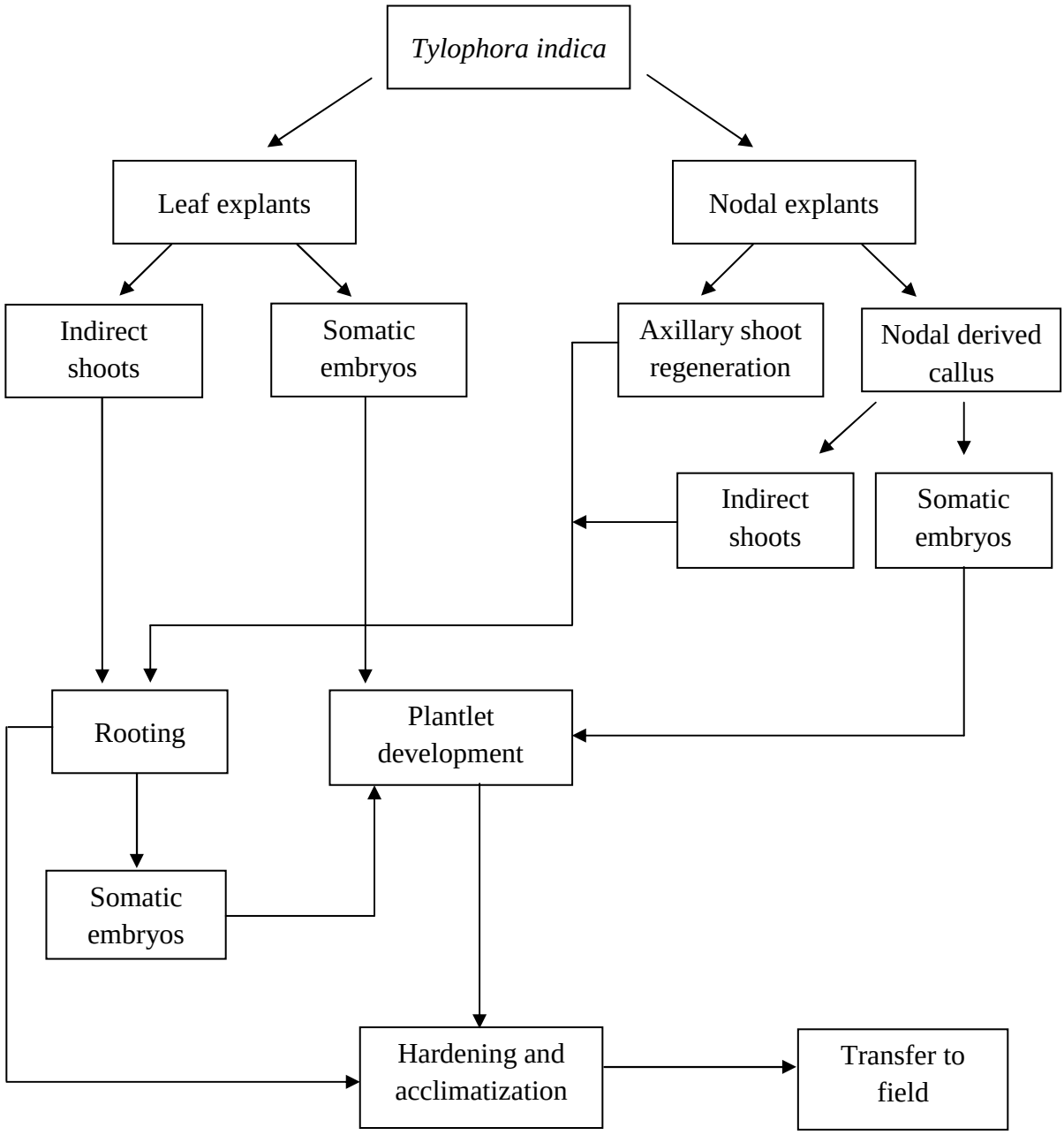

Figure 59. Schematic representation of different regenerative pathways in *T. indica*.

4.4 Phytochemical Analysis

In the present study, *in vivo* shoots served as a control, *in vitro* shoots regenerated from leaf and nodal explants treated with different PGRs and SEs derived hardened plant shoots were harvested (T1-T7) and utilized to analyze qualitatively for their potency to synthesize metabolites using HPTLC fingerprinting. Quantification of lupeol was done using these samples and then elicitation for the targeted metabolite was carried out using different elicitors like yeast extract and salicylic acid.

4.4.1 HPTLC Profiling

Extraction was done using three solvents having different polarities i.e. hexane, ethyl acetate and methanol. All the three extracts were subjected to chromatographic separation on TLC plates in optimized mobile phases followed by derivatization using anisaldehyde-sulphuric acid reagent. Then the plates were scanned at 254, 366 and 525 nm for densitometry analysis and the results are summarized below.

4.4.1.1 Hexane Extract

HPTLC fingerprint experiment was performed using hexane extract for all the samples, were spotted on TLC plates and subjected to chromatographic separation using mobile phase i.e. toluene:methanol (9:1 v/v). Then the plates were observed under 254 and 366 nm and then derivatized to obtain a profile which differed in banding pattern of samples. Scanning was done at 254, 366 and 525 nm which also confirmed the variation in peak numbers and areas.

- **254 nm**

Scanning at 254 nm showed changes in profiling of T1-T7 in which, fingerprint of T2 matched with T6 and T7, was moderately similar with T4 but different from rest of the sample (T1, T3 and T5) (Fig. 60a). T1 sample extract recorded a total of 15 peaks and maximum area was 6570.8 for a peak at Rf (0.02) (Fig. 61a). Samples T2, T3, T5 and T7 recorded almost same peaks 14, 14, 11, 15 and among them highest areas for peak at Rf 0.82 i.e. 22736.9, 10421.1, 13802.0 and 12724.1 (Fig. 61b, c, e, g). The T4 and T6 samples showed similar number of peaks (14) and maximum peak areas were 14282.4 and 16904.8 at Rf 0.13 and 0.39 (Fig. 61d, f).

Table 25. Peak areas of different samples at 254 nm for hexane extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	6570.8	13.64	11248.6	10.78	10051.7	23.12	8981.6	13.93	582.4	1.59	709.6	1.01	275.3	0.51
0.03	-	-	-	-	-	-	-	-	916.0	2.51	-	-	-	-
0.04	-	-	-	-	-	-	-	-	-	-	-	-	489.7	0.91
0.05	3693.8	7.46	-	-	-	-	-	-	-	-	227.6	0.32	-	-
0.07	-	-	-	-	-	-	2657.4	4.12	324.2	0.89	721.3	1.03	-	-
0.08	878.7	1.78	4046.3	3.88	558.4	1.28	-	-	-	-	-	-	1164.5	2.17
0.11	-	-	-	-	498.7	1.15	-	-	-	-	-	-	-	-
0.13	-	-	-	-	-	-	14282.4	22.15	2331.6	6.38	6491.7	9.23	5507.4	10.26
0.14	6245.9	12.62	14589.0	13.99	3672.3	8.45	-	-	-	-	-	-	-	-
0.15	-	-	-	-	-	-	12193.9	18.91	-	-	-	-	-	-
0.16	-	-	-	-	-	-	-	-	1777.5	4.87			4152.6	7.74
0.17	5905.1	11.93	10567.0	10.13	2506.6	5.76	-	-	-	-	5025.9	7.14		
0.18	-	-	-	-	-	-	-	-	677.4	1.85				
0.19	-	-	-	-	-	-	-	-	-	-	1648.4	2.34	1309.1	2.44
0.23	-	-	-	-	-	-	-	-	5313.1	14.54	8632.2	12.27		
0.24	3848.1	7.78	10712.7	10.27	3129.1	7.20	4530.1	7.03	-	-	-	-	7309.7	13.62
0.27	-	-	1658.5	1.59	1111.1	2.56	-	-	532.8	1.46				
0.28	-	-	-	-	-	-	-	-	-	-	1571.6	2.23	1041.0	1.94
0.30	-	-	-	-	-	-	-	-	551.8	1.51	-	-	-	-
0.31	1917.1	3.87	2073.1	1.99	572.5	1.32	-	-	-	-	1248.4	1.77	1258.4	2.34
0.35	2360.7	4.77	-	-	-	-	-	-	-	-	-	-	-	-
0.36	-	-	5410.1	5.19	2399.8	5.52	1050.9	1.63	1660.1	4.54	-	-	-	-
0.39	-	-	9471.6	9.08	1816.1	4.18	-	-	-	-	16904.8	24.02	11319.7	21.09
0.41	662.6	1.34	-	-	-	-	-	-	-	-	-	-	-	-
0.42	-	-	-	-	-	-	-	-	669.0	1.83	-	-	-	-

0.47	-	-	-	-	-	-	-	-	1113.6	3.05	4007.1	5.69	-	-
0.48	2580.4	5.21	-	-	-	-	-	-	-	-	-	-	2237.9	4.17
0.49	-	-	3744.5	3.59	1065.2	2.45			-	-	-	-	-	-
0.50	-	-	-	-	-	-	2198.0	3.41	-	-	-	-	-	-
0.53	-	-	-	-	-	-	-	-	-	-	453.0	0.64	-	-
0.55	-	-	695.1	0.67	-	-	-	-	1448.3	3.96	-	-	192.6	0.36
0.56	679.7	1.37	-	-	-	-	3653.4	5.67	-	-	-	-	-	-
0.57	-	-	-	-	222.5	0.51	-	-	-	-	-	-	-	-
0.58	-	-	216.8	0.21	-	-	-	-	-	-	358.8	0.51	-	-
0.59	-	-	-	-	-	-	-	-	-	-	-	-	274.4	0.51
0.62	-	-	-	-	-	-	189.4	0.29	-	-	-	-	-	-
0.64	-	-	-	-	5457.6	12.55	-	-	4833.1	13.23	-	-	-	-
0.65	-	-	-	-	-	-	-	-	-	-	5383.4	7.65	-	-
0.66	4663.4	9.42	7139.8	6.84	-	-	-	-	-	-	-	-	-	-
0.67	-	-	-	-	-	-	-	-	-	-	-	-	4415.9	8.23
0.70	-	-	-	-	-	-	3766.6	5.84	-	-	-	-	-	-
0.73	1370.6	2.77	-	-	-	-	-	-	-	-	-	-	-	-
0.81	3665.4	7.41	-	-	10421.1	23.97	10966.1	17.01	-	-	-	-	-	-
0.82	-	-	-	-	-	-	-	-	13802.0	37.78	16796.1	23.87	-	-
0.83	-	-	22736.9	21.80	-	-	-	-			-	-	12724.1	23.71
0.85	4265.1	8.62	-	-	-	-	-	-	-	-	-	-	-	-
0.95	-	-	-	-	-	-	-	-	-	-	184.0	0.26	-	-

Chromatogram showed variation in peaks with respect to the samples i.e. *in vivo* and all *in vitro* samples of shoots and callus found unique peaks. Some peaks were common at Rf (0.02, 0.07, 0.13) in all samples (T1-T7) whereas peak at Rf (0.41) was only present in *in vivo* sample (T1). Unique peak was also present in *in vivo* and nodal derived shoots (T6 and T7) at Rf (0.04). Similarly T7 also recorded two unique peaks at Rf (0.59 and 0.67) (Table 25).

- **366 nm**

A fingerprint which was developed on TLC plate was also observed under UV 366 followed by scanning at same wavelength. This resulted with different banding pattern therefore comparison was done between all the samples. *In vivo* sample (T1) had quite similar profile with *in vitro* shoot samples (T3 and T5). Similarly, leaf derived shoot sample (T2) was matched with nodal derived shoots (T6 and T7) but the shoot sample of hardened plant (T4) showed more variation in profile from all the samples (Fig. 60b). T1 and T5 samples reported similar no. of peaks (15), and peak at Rf (0.85) had maximum area of 15159.3 and 23831.5 in chromatogram (Fig. 61h, l). T2 resulted with maximum 17 peaks among all the samples and highest peak area 34853.3 was obtained at Rf 0.89 (Fig. 61i). T3 sample recorded 16 peaks having maximum area of 10819.5 was noted for peak at Rf (0.23) (Fig. 61j). Whereas T4 sample showed less peaks (13) and highest peak area (14577.50) was achieved at Rf 0.56 (Fig. 61k). T6 and T7 sample extract showed similar peaks i.e. 15 and maximum areas (44554.7 and 33207.1) were also recorded at same Rf (0.39) for respective samples (Fig. 61m, n). Chromatogram appeared with different peaks at different Rf and by comparing peaks of all samples, unique peaks were detected. Peak was present in all the samples at Rf (0.02) whereas peaks recorded at Rf (0.07 and 0.11) were only seen in *in vitro* shoot extracts samples (T2, T3, T5-T7). Similarly nodal derived shoot samples (T6 and T7) also had unique peaks at Rf (0.47, 0.54, 0.65) (Table 26).

- **525 nm**

The plate was then derivatized and observed in white light which confirmed that fingerprint was quite similar in all the samples (T1-T7). Although it was observed that, all *in vitro* samples formed higher number of bands compared to *in vivo* sample after derivatization (Fig. 60c). This was further confirmed after scanning at 525 nm which showed change in peak numbers as well as its area in the chromatogram.

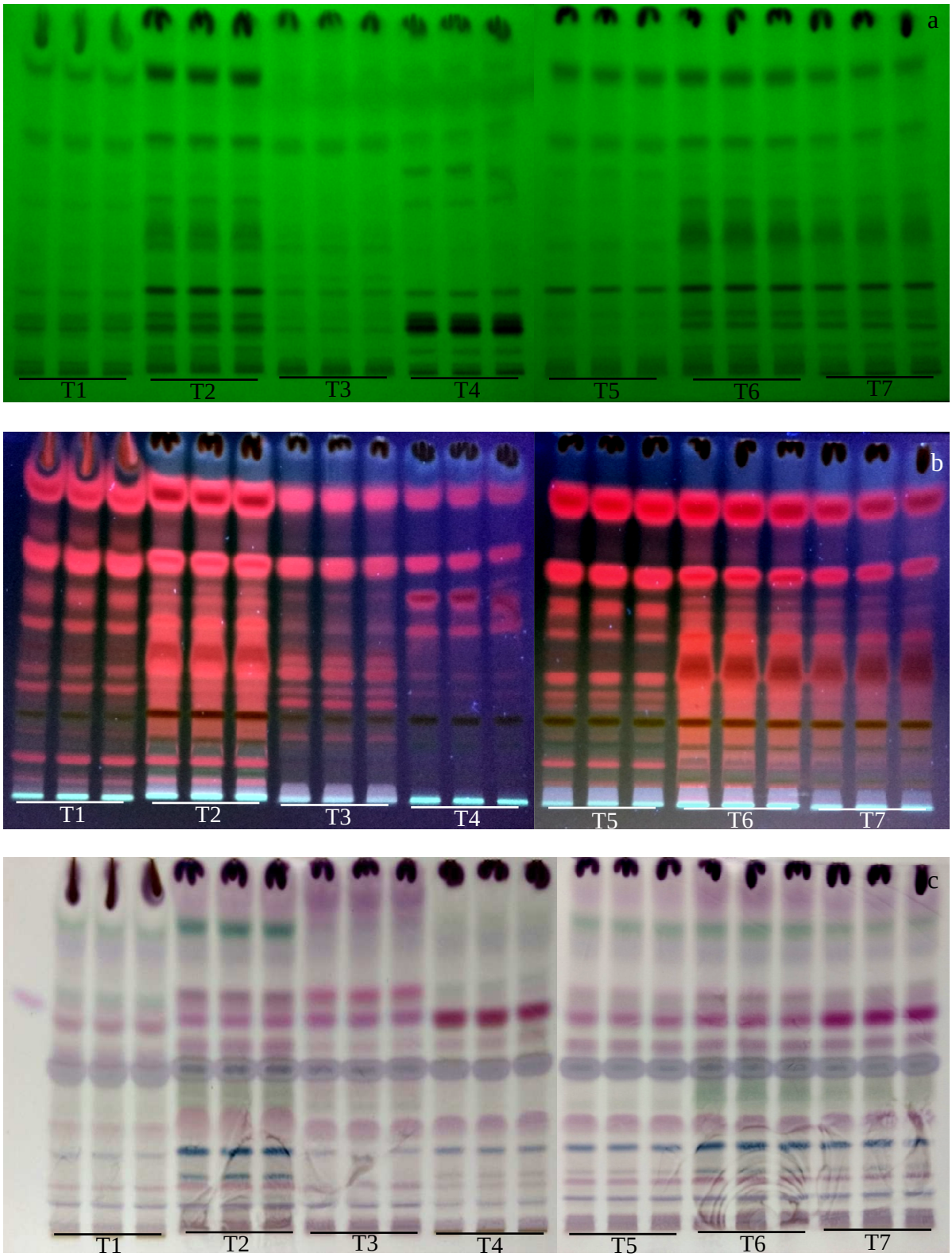

Figure 60. HPTLC fingerprints of hexane extract for different samples of *T. indica*: (a) UV 254 nm, (b) UV 366 nm and (c) white light after derivatization.

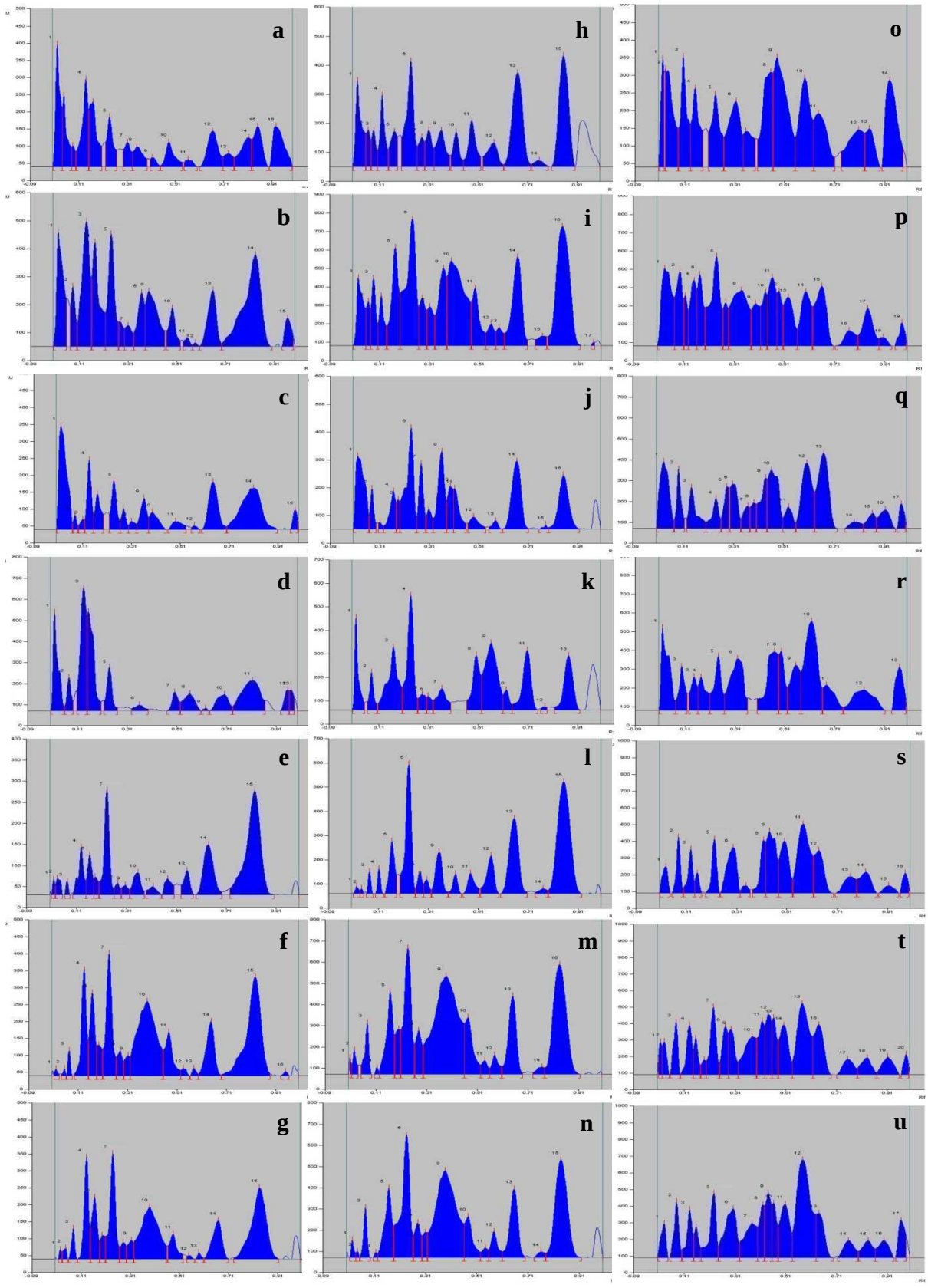

Figure 61. Chromatograms of samples for hexane extract in *T. indica*: (a-g) 254 nm, (h-n) 366 nm and (o-u) 525 nm.

Table 26. Peak areas of different samples at 366 nm for hexane extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	-	-	-	-	-	-	-	-	-	-	437.1	0.31	-	-
0.02	6952.6	8.53	9644.4	5.00	9684.3	14.37	5724.6	7.19	397.8	0.55	1592.2	1.14	865.2	0.75
0.04	-	-	-	-	-	-	-	-	182.7	0.25	-	-	278.5	0.24
0.06	2047.9	2.51	3512.8	1.82	-	-	-	-	-	-	-	-	-	-
0.07	-	-			-	-	-	-	979.3	1.34	3873.7	2.78	3236.5	2.81
0.08	-	-	6471.5	3.35	2081.9	3.09	2689.1	3.38	-	-	-	-	-	-
0.09	2177.9	2.67	-	-	-	-	-	-	-	-	-	-	-	-
0.11	-	-	5355.9	2.77	389.2	0.58	-	-	1163.8	1.60	327.3	0.23	236.8	0.21
0.12	4837.2	5.93	-	-	-	-	-	-	-	-	-	-	-	-
0.16	-	-	-	-	3182.5	4.72	-	-	4693.8	6.44	10205.9	7.31		
0.17	3395.0	4.16	14673.1	7.60	-	-	11031.5	13.86	-	-	-	-	9182.8	7.97
0.18	-	-	-	-	1224.3	1.82	-	-	-	-	-	-	-	-
0.20	-	-	-	-	-	-	-	-	-	-	4097.1	2.94	-	-
0.23	-	-	-	-	10819.5	16.06	-	-	13034.8	17.88	17281.9	12.39	18829.2	16.33
0.24	10724.6	13.15	27091.0	14.03	-	-	12540.0	15.76	-	-	-	-	-	-
0.27	-	-	-	-	4419.5	6.56	-	-	1676.9	2.30	-	-	-	-
0.28	2370.7	2.91	6394.4	3.31	-	-	1855.0	2.33	-	-	5104.5	3.66	3956.5	3.43
0.30	-	-	-	-	-	-	-	-	1098.8	1.51	-	-	-	-
0.31	3122.6	3.83	5412.0	2.80	1436.4	2.13	1182.3	1.49	-	-	-	-	2054.9	1.78
0.35	-	-	-	-	-	-	-	-	4708.4	6.46	-	-	-	-
0.36	4608.0	5.65	-	-	7478.8	11.10	3650.5	4.59	-	-	-	-	-	-
0.37	-	-	13613.2	7.05	-	-	-	-	-	-	-	-	-	-
0.39	-	-	-	-	3469.9	5.15	-	-	-	-	44224.7	31.70	33207.1	28.80
0.40	-	-	30384.4	15.74	-	-	-	-	-	-	-	-	-	-
0.41	-	-	-	-	3221.1	4.78	-	-	-	-	-	-	-	-

0.42	2946.3	3.61	-	-	-	-	-	-	1542.1	2.12	-	-	-	-
0.47	-	-	-	-	-	-	-	-	-	-	8648.8	6.20	5857.3	5.08
0.48	4880.9	5.99	-	-	-	-	-	-	2331.5	3.20	-	-	-	-
0.49	-	-	9201.2	4.77	1684.7	2.50	-	-	-	-	-	-	-	-
0.50	-	-	-	-	-	-	7025.4	8.83	-	-	-	-	-	-
0.54	-	-	-	-	-	-	-	-	-	-	1539.2	1.10	1015.5	0.88
0.56	-	-	3286.0	1.70	-	-	14577.5	18.32	4711.6	6.46	-	-	-	-
0.57	3644.6	4.47	-	-	-	-	-	-	-	-	-	-	-	-
0.58	-	-	-	-	720.7	1.07	-	-	-	-	2375.4	1.70	3154.0	2.74
0.59	-	-	2498.1	1.29	-	-	-	-	-	-	-	-	-	-
0.62	-	-	-	-	-	-	1881.9	2.36	-	-	-	-	-	-
0.65	-	-	-	-	-	-	-	-	11877.8	16.30	11719.0	8.40	10339.2	8.97
0.66	-	-	18598.8	9.63	9356.3	13.89	-	-	-	-	-	-	-	-
0.67	13841.4	16.98	-	-	-	-	-	-	-	-	-	-	-	-
0.71	-	-	-	-	-	-	8812.6	11.07	-	-	-	-	-	-
0.75	822.9	1.01	-	-	-	-	-	-	-	-	-	-	-	-
0.76	-	-	1864.6	0.97	-	-	-	-	-	-	1037.2	0.74	753.8	0.65
0.77	-	-	-	-	-	-	-	-	650.7	0.89	-	-	-	-
0.78	-	-	-	-	235.2	0.35	262.8	0.33	-	-	-	-	-	-
0.83	-	-	-	-	-	-	-	-	-	-	27067.9	19.40	-	-
0.84	-	-	34853.3	18.05	-	-	-	-	-	-	-	-	22322.1	19.36
0.85	15159.3	18.59	-	-	7970.5	11.83	-	-	23831.5	32.70	-	-	-	-
0.87	-	-	-	-	-	-	8359.2	10.50	-	-	-	-	-	-
0.97	-	-	187.5	0.10	-	-	-	-	-	-	-	-	-	-

Table 27. Peak areas of different samples at 525 nm for hexane extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	-	-	-	-	-	-	-	-	-	-	3157.2	2.29	-	-
0.02	4202.6	3.60	-	-	-	-	13222.4	10.13	-	-	-	-	-	-
0.03	8807.8	7.54	17715.8	10.03	12038.1	11.76	-	-	4054.1	3.65	3374.9	2.45	4486.5	3.06
0.08	-	-					-	-	5235.3	4.72	5515.3	4.00	6227.1	4.25
0.09	-	-	10626.4	6.02	4835.1	4.72	5588.7	4.28	-	-	-	-	-	-
0.10	7918.4	6.78	-	-	-	-	-	-	-	-	-	-	-	-
0.11	-	-	3906.9	2.21	-	-	-	-	-	-	-	-	-	-
0.13	-	-	-	-	-	-	-	-	4978.9	4.49	6462.3	4.69	6042.1	4.12
0.14	-	-	-	-	5695.0	5.56	4477.3	3.43	-	-	-	-	-	-
0.15	6483.7	5.55	9003.9	5.10	-	-	-	-	1831.9	1.65	3599.0	2.61	-	-
0.16	-	-	-	-	-	-	-	-	-	-	-	-	3242.3	2.21
0.17	-	-	8637.2	4.89	-	-	5255.1	4.03	-	-	-	-	-	-
0.19	-	-	-	-	-	-	-	-	-	-	1491.1	1.08	-	-
0.22	-	-	-	-	-	-	-	-	6204.0	5.59	9762.7	7.09	-	-
0.23	7272.9	6.22			-	-	-	-	-	-	-	-	9923.9	6.77
0.24	-	-	18416.2	10.43	3808.7	3.72	8067.8	6.18	-	-	-	-	-	-
0.27	-	-	4376.3	2.48	-	-	-	-	-	-	6610.3	4.80	-	-
0.28	-	-	-	-	3915.2	3.82	-	-	-	-	-	-	-	-
0.29	-	-	-	-	-	-	-	-	-	-	6518.1	4.73	-	-
0.30	-	-	-	-	-	-	-	-	10725.6	9.66	-	-	13764.5	9.39
0.31	9180.3	7.86	-	-	6345.7	6.20			-	-	-	-	-	-
0.32	-	-	-	-	-	-	14803.3	11.34	-	-	-	-	-	-
0.34	-	-	20296.4	11.50	-	-	-	-	-	-	-	-	-	-
0.35	3974.5	3.40	-	-	-	-	-	-	1208.5	1.09	-	-	-	-
0.37	-	-	-	-	2700.4	2.64	-	-	-	-	-	-	9928.2	6.77

0.38	-	-	-	-	-	-	-	-	-	-	10838.5	7.87	-	-
0.39	-	-	6987.9	3.96	2530.9	2.47	-	-	-	-	-	-	-	-
0.41	-	-	-	-	-	-	-	-	6704.4	6.04	8001.4	5.81	-	-
0.42	-	-	-	-	-	-	-	-	-	-	-	-	6585.3	4.49
0.43	-	-	6501.9	3.68	-	-	-	-	-	-	-	-	-	-
0.44	-	-	-	-	6643.3	6.49	-	-	14407.9	12.98	8868.5	6.44	9436.1	6.43
0.45	11384.2	9.74	-	-	-	-	-	-	-	-	-	-	-	-
0.46	-	-	11278.3	6.39	11779.1	11.51	-	-	-	-	6025.8	4.38	5774.1	3.94
0.47	-	-	-	-	-	-	14845.7	11.37	-	-	-	-	-	-
0.48	16554.8	14.16	-	-	-	-	-	-	-	-	-	-	-	-
0.49	-	-	5645.2	3.20	-	-	-	-	-	-	-	-	-	-
0.50	-	-	-	-	-	-	7208.8	5.52	11138.2	10.04	10628.0	7.72		
0.51	-	-	-	-	-	-	-	-	-	-	-	-	12174.8	8.30
0.53	-	-	10116.1	5.73	3185.8	3.11	-	-	-	-	-	-	-	-
0.55	-	-	-	-	-	-	9388.5	7.19	-	-	-	-	-	-
0.57	-	-	-	-	-	-	-	-	20600.5	18.56	20691.9	15.02	-	-
0.58	-	-	-	-	-	-	-	-	-	-	-	-	32527.2	22.18
0.59	11388.7	9.74	-	-	-	-	-	-	-	-	-	-	-	-
0.60	-	-	12027.5	6.81	13393.2	13.08	-	-	-	-	-	-	-	-
0.62	-	-	-	-	-	-	24502.0	18.77	-	-	-	-	-	-
0.64	7791.5	6.67	-	-	-	-	-	-	9450.5	8.52	11414.3	8.29	8159.0	5.56
0.66	-	-	14529.9	8.23	-	-	-	-	-	-	-	-	-	-
0.67	-	-	-	-	15566.2	15.21	-	-	-	-	-	-	-	-
0.68	-	-	-	-	-	-	6177.1	4.73	-	-	-	-	-	-
0.76	-	-	-	-	-	-	-	-	4637.1	4.18	4062.7	2.95	4456.3	3.04
0.77	-	-	3699.2	2.10	-	-	-	-	-	-	-	-	-	-
0.79	-	-	-	-	1483.9	1.45	-	-	-	-	-	-	-	-
0.81	6621.7	5.67	-	-	-	-	-	-	-	-	-	-	-	-
0.82	-	-	-	-	-	-	-	-	5834.4	5.26	-	-	-	-
0.83	-	-	-	-	-	-	9535.0	7.30	-	-	4243.9	3.08	4220.0	2.88

0.84	-	-	8537.3	4.84	-	-	-	-	-	-	-	-	-	-
0.85	3803.4	3.25	-	-	-	-	-	-	-	-	-	-	-	-
0.86	-	-	-	-	2326.3	2.27	-	-	-	-	-	-	-	-
0.90	-	-	1404.7	0.80	-	-	-	-	-	-	-	-	4363.6	2.98
0.91	-	-	-	-	3814.5	3.73	-	-	1822.7	1.64	4782.1	3.47	-	-
0.93	11486.5	9.83	-	-	-	-	-	-	-	-	-	-	-	-
0.96	-	-	-	-	-	-	-	-	-	-	-	-	5346.8	3.65
0.97	-	-	-	-	-	-	7476.8	5.73	-	-	-	-	-	-
0.98	-	-	2833.6	1.61	2312.6	2.26	-	-	2151.4	1.94	1674.6	1.22	-	-

The *in vivo* shoots sample (T1) resulted with total 14 peaks which was lowest among all the samples and maximum area of 11486.5 was noted for peak at Rf (0.93) (Fig. 61o). *In vitro* samples i.e. T2, T3 and T4 recorded total 19, 17 and 13 peaks and highest peak areas were also recorded i.e. 18416.2, 12038.1 , 24502.0 for peak of respective samples at different Rf (0.24, 0.03, 0.62) (Fig. 61p-r). Total peaks (16, 20, 17) were noted in nodal derived shoot samples (T5-T7) and their maximum peak areas were 20600.5, 20691.9 and 32527.2 at same Rf (0.57) (Fig. 61s-u). Chromatograms also depiected that there were some common as well as unique peaks present in different samples. A peak at Rf (0.03) which was common in all the samples (T1-T7), whereas peak at Rf (0.08) was only present in *in vitro* sample extracts. Similarly a unique peak was present in all nodal derived shoots (T5-T7) at Rf (0.76) and T7 sample also had unique peaks at Rf (0.51 and 0.96) (Table 27).

4.4.1.2 Ethyl Acetate Extract

Ethyl acetate extract of all the samples were spotted on TLC plates and separated in mobile phase toluene:ethyl acetate:formic acid (6:3:1 v/v).

- **254 nm**

Plate when observed at UV 254 showed variation in banding pattern with respect to number of bands and it was further confirmed through scanning which exhibited the change in peak numbers and areas. It was observed from profile that T3 sample extract was more varied from all other samples. T1 was *in vivo* shoot sample showed similarity with T2 and T4, and it was different from T5-T7 (Fig. 62a). After scanning it was recorded that T1 and T3 sample had a total 16 and 18 peaks and maximum areas were 16513.2 and 25244.9 at same Rf (0.02) (Fig. 63a, c). T4 sample recorded 15 peaks from which peak had maximum area (19074.2) at Rf (0.81) (Fig. 63d). Samples (T2 and T5-T7) recorded almost same peaks 16, 18, 16, 15 and even though the highest peak areas i.e. 25244.9, 22165.9, 33286.0, 25909.0 respectively at Rf 0.83 (Fig. 63b, e, f, g). Similarly by comparing all samples some unique peaks were observed. Unique peak was obtained at 0.68 Rf because it was only present in T1 sample and absent in all the *in vitro* sample. Shoot which were procured from the hardened *in vitro* plants (T4) also recorded a distinct peak at Rf (0.06) and similarly all *in vitro* samples (T2-T7) also had unique peaks at different Rf (0.14, 0.27, 0.34). Leaf derived shoots (T2 and T3) also showed unique peak at Rf (0.48) (Table 28).

Table 28. Peak areas of different samples at 254 nm for ethyl acetate extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	16513.2	23.45	-	-	18271.6	26.42	16823.3	17.19	-	-	-	-	6772.2	7.59
0.03	-	-	19455.5	16.71	-	-	-	-	16526.5	16.43	19367.1	15.14	-	-
0.06	-	-	-	-	-	-	4759.5	4.86	-	-	-	-	-	-
0.09	-	-	-	-	-	-	-	-	1305.5	1.30	1763.7	1.38	245.2	0.27
0.10	8925.4	12.67	-	-	1578.0	2.28	-	-	-	-	-	-	-	-
0.11	-	-	1631.1	1.40	-	-	-	-	-	-	1080.1	0.84	-	-
0.12	-	-	-	-	-	-	12241.4	12.51	-	-	-	-	-	-
0.13	12524.7	17.78	-	-	1588.4	2.30	-	-	-	-	-	-	-	-
0.14	-	-	-	-	-	-	-	-	-	-	5865.9	4.59	2793.5	3.13
0.15	-	-	6151.7	5.28	-	-	9520.0	9.73	4106.5	4.08	-	-	-	-
0.16	7045.7	10.00	-	-	1145.7	1.66	-	-	-	-	-	-	-	-
0.18	-	-	-	-	-	-	-	-	-	-	854.4	0.67	326.0	0.37
0.19	-	-	1597.6	1.37	1303.4	1.88	-	-	688.0	0.68	-	-	-	-
0.20	-	-	-	-	-	-	7613.7	7.78	-	-	-	-	-	-
0.22	-	-	-	-	2003.1	2.90	-	-	-	-	1678.5	1.31	863.9	0.97
0.23	460.1	0.65	4624.9	3.97	-	-	-	-	1175.0	1.17	-	-	-	-
0.26	197.9	0.28	-	-	-	-	-	-	-	-	-	-	-	-
0.27	-	-	-	-	-	-	-	-	-	-	8726.0	6.82	6191.1	6.94
0.28	-	-	8324.9	7.15	5146.6	7.44	6355.3	6.49	5414.6	5.38				
0.31	6549.0	9.30	-	-	-	-	-	-	-	-	-	-	-	-
0.34	-	-	-	-	-	-	4222.4	4.31	-	-	-	-	-	-
0.35	-	-	-	-	2007.5	2.90			3987.1	3.96	8529.0	6.67	5694.6	6.38
0.39	1209.5	1.72	9278.4	7.97	-	-	-	-	-	-	-	-	-	-
0.40	-	-	-	-	-	-	-	-	-	-	-	-	4654.7	5.22
0.41	-	-	-	-	-	-	2106.4	2.15	4632.7	4.60	6641.5	5.19	-	-

0.42	-	-	-	-	2648.8	3.83	-	-	-	-	-	-	-	-
0.43	-	-	8784.5	7.55	-	-	-	-	-	-	9371.7	7.33	4363.6	4.89
0.44	-	-	-	-	-	-	-	-	3458.8	3.44	-	-	-	-
0.46	248.2	0.35	-	-	-	-	-	-	-	-	-	-	-	-
0.47	-	-	-	-	-	-	-	-	-	-	-	-	3774.7	4.23
0.48	-	-	-	-	-	-	472.2	0.48	3618.6	3.60	-	-	-	-
0.49	-	-	3896.9	3.35	1444.7	2.09	-	-	-	-	-	-	-	-
0.55	-	-	-	-	2933.2	4.24	-	-	-	-	-	-	-	-
0.57	-	-	-	-	1927.5	2.79	-	-	-	-	9848.8	7.70	9410.6	10.55
0.58	-	-	9304.1	7.99	-	-	4356.2	4.45	11598.8	11.53	-	-	-	-
0.61	5075.9	7.21	-	-	-	-	-	-	-	-	-	-	-	-
0.64	-	-	-	-	200.8	0.29	435.2	0.44	396.6	0.39	807.3	0.63	-	-
0.66	-	-	400.0	0.34	-	-	-	-	-	-	-	-	-	-
0.68	332.8	0.47	-	-	-	-	-	-	-	-	-	-	-	-
0.70	-	-	-	-	-	-	-	-	-	-	-	-	4074.0	4.57
0.71	-	-	-	-	-	-	3945.2	4.03	-	-	4460.9	3.49	-	-
0.72	-	-			7663.8	11.08	-	-	4474.6	4.45	-	-	-	-
0.73	-	-	6161.8	5.29	-	-	-	-	-	-	-	-	-	-
0.74	-	-	-	-	-	-	-	-	2354.7	2.34	-	-	-	-
0.76	1171.6	1.66	-	-	-	-	-	-	-	-	-	-	-	-
0.81	-	-	-	-	14941.3	21.60	19074.2	19.49	-	-	-	-	-	-
0.83	-	-	-	-	-	-	-	-	22165.9	22.03	-	-	25909.0	29.05
0.84	5207.1	7.39	25244.9	21.68	-	-	-	-	-	-	33286.0	26.02	-	-
0.87	-	-	-	-	-	-	-	-	6567.9	6.53	-	-	-	-
0.88	-	-	-	-	-	-	3125.1	3.19	-	-	9033.7	7.06	7460.2	8.36
0.89	-	-	6346.5	5.45	1197.4	1.73	-	-	-	-	-	-	-	-
0.91	3665.5	5.20	-	-	-	-	2816.6	2.88	3521.6	3.50	6613.0	5.17	6662.9	7.47
0.92	-	-	-	-	2182.7	3.16	-	-	-	-	-	-	-	-
0.93	-	-	4640.6	3.99	-	-	-	-	-	-	-	-	-	-
0.96	-	-	-	-	-	-	-	-	4614.5	4.59	-	-	-	-

- **366 nm**

The TLC plate when observed under UV 366, it confirmed the disparity in banding pattern through the fingerprint seemed roughly similar in all the sample extracts except T3 which was quite different from others (Fig. 62b). Then scanning at 366 nm showed variation with respect to peak number and area by comparing chromatograms of all the samples. T1 sample noted 16 peaks among them maximum area was 18974.8 of peak at Rf (0.31) (Fig. 63h). Rest of all samples contained almost same peaks (14) and maximum areas were 33093.0, 29085.4, 40763.9, 35176.5, 37704.9 and 33642.9 respectively at Rf (0.43 for T2 and 0.82-0.83 for T3-T7) (Fig. 63i-n). Chromatogram showed variation in peaks at different Rf, therefore many unique peaks were identified for all samples i.e. at Rf (0.31, 0.61 and 0.76), peaks were present in only T1 which was *in vivo* shoot sample and absent in all the *in vitro* samples. Whereas peak at Rf (0.72) was present in all *in vitro* samples (T2, T3, T5-T7) therefore it was unique for these samples. Whereas peak present in *in vivo* shoot sample (T1) and nodal derived shoot (T7) at Rf (0.40) and two unique peaks which only present in T7 sample at different Rf (0.47 and 0.88) (Table 29).

- **525 nm**

In white light the fingerprint which developed band pattern was almost similar in all the samples after derivatization. Later TLC plate was scanned at 525 nm and it showed difference in peak numbers and area from the chromatogram in different samples (Fig. 62c). Maximum 16 peaks were reported by both sample T1 and T2 along with maximum area of 18127.5 and 22652.2 on the chromatogram at Rf (0.28 and 0.86) (Fig. 63o, p). SampleT3-T5 recorded similar peaks (14), and their highest peak areas 23081.4, 25595.1, 20731.0 were noted at almost same Rf (0.82-0.84) (Fig. 63q-s). Similarly samples (T6 and T7) recorded with 12 peaks and at Rf (0.85 and 0.83) maximum areas 38088.5 and 30094.6 were noted for peaks with respect to sample (Fig. 63t, u). Similarly the peak variation was observed between samples which found uniqueness between them by comparing peaks at different Rf. Peak at Rf (0.93) was only present in T1-T3 therefore it was unique for these samples. Similarly at Rf (0.55) recorded distinct peak which was only present in *in vivo* shoot sample (T1). Nodal derived shoots (T6 and T7) also identified distinct peaks at different Rf (0.23 and 0.35) and hardened plant shoots (T4) also had a unique peak at Rf (0.34) (Table 30).

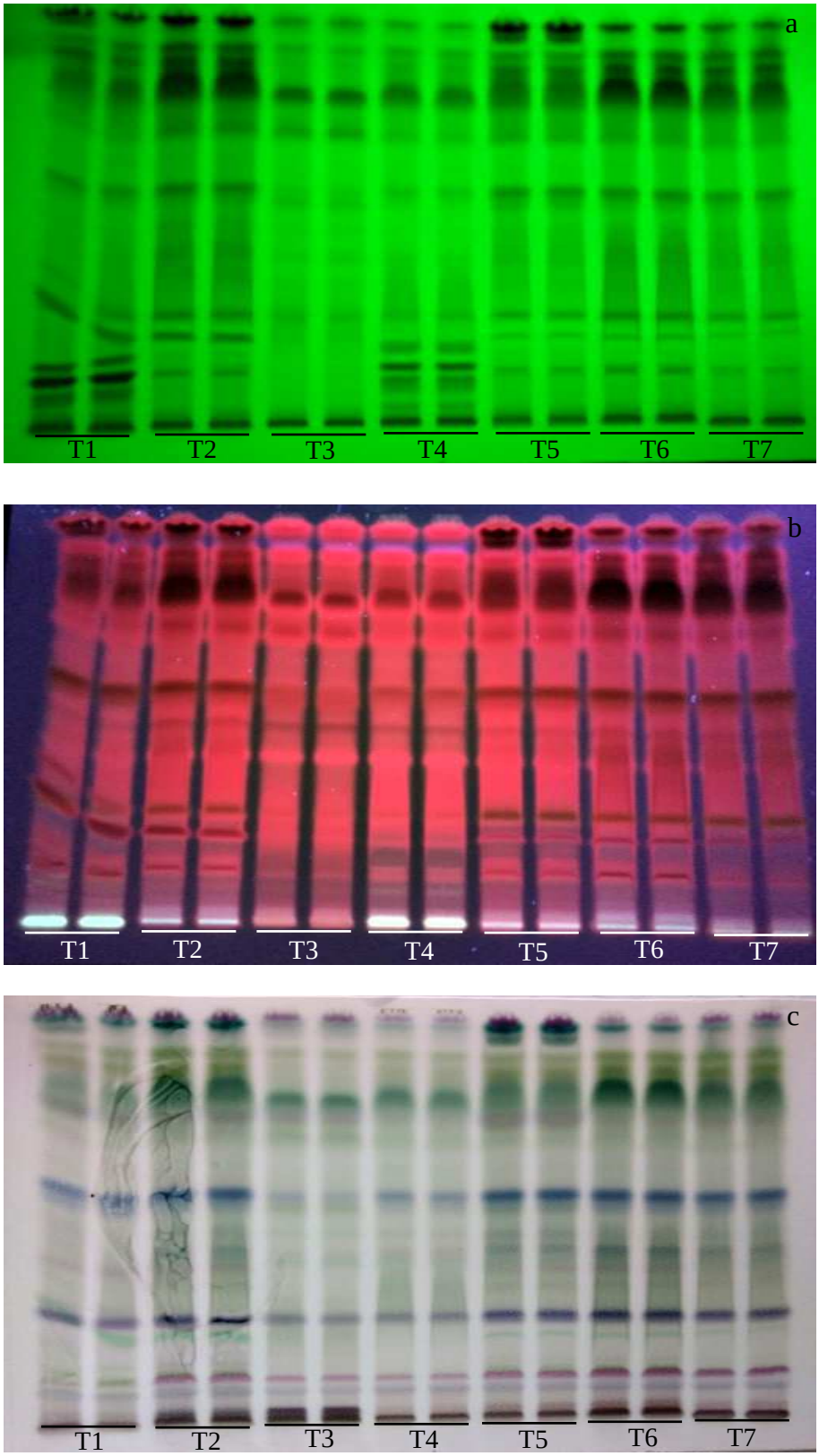

Figure 62. HPTLC fingerprints of ethyl acetate extract for different samples of *T. indica*: (a) UV 254 nm, (b) UV 366 nm and (c) white light after derivatization.

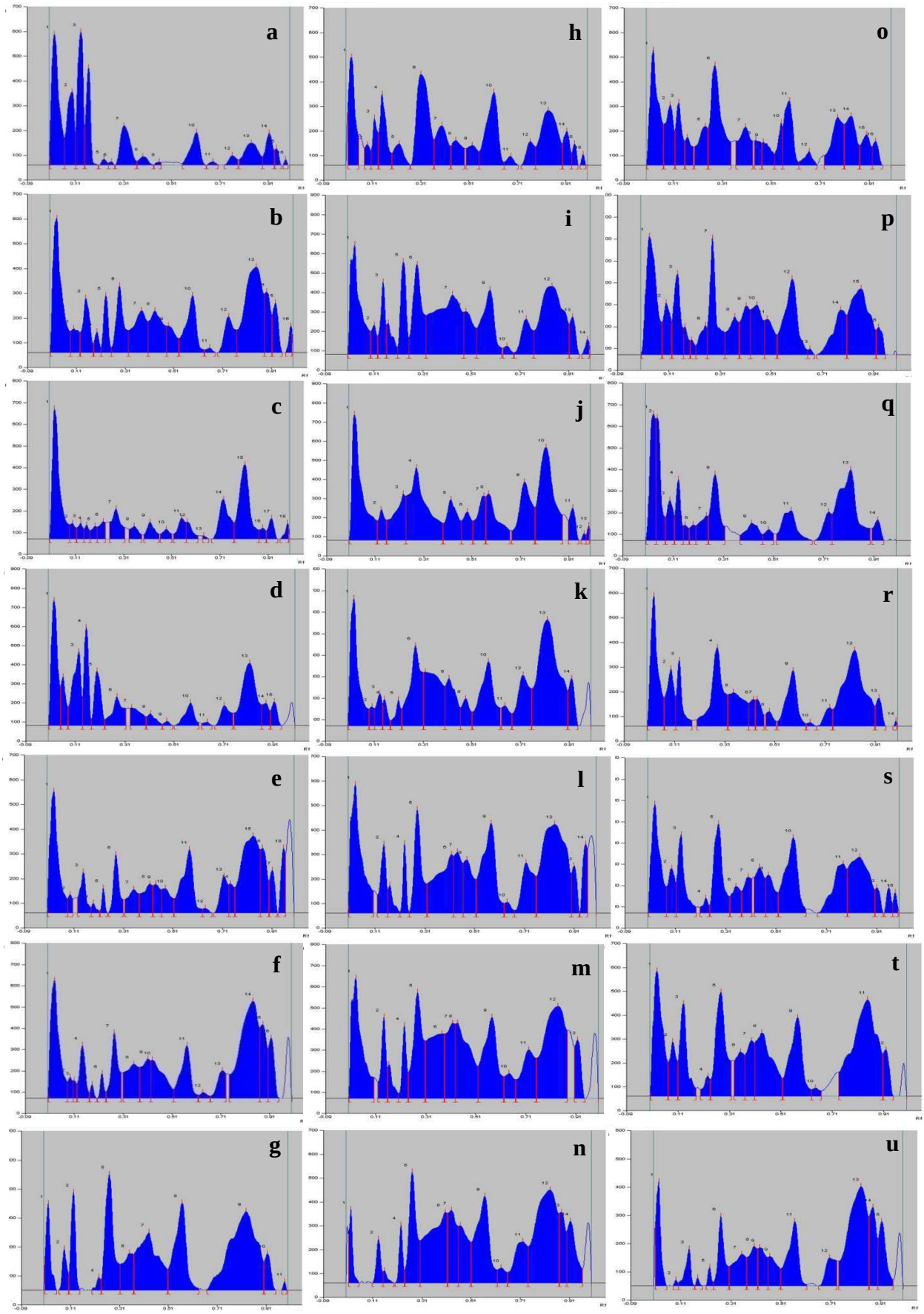

Figure 63. Chromatograms of samples for ethyl acetate extract in *T. indica*: (a-g) 254 nm, (h-n) 366 nm and (o-u) 525 nm.

Table 29. Peak areas of different samples at 366 nm for ethyl acetate extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	11541.2	11.36	-	-	26605.0	17.39	-	-	-	-	-	-	4449.0	2.97
0.03	-	-	22777.7	13.39	-	-	19482.1	12.61	22227.9	14.41	23848.6	12.29	-	-
0.09	1868.5	1.84	-	-	-	-	-	-	-	-	-	-	-	-
0.10	-	-	-	-	-	-	1607.1	1.04	-	-	-	-	-	-
0.11	-	-	3157.2	1.86	-	-	-	-	-	-	-	-	-	-
0.12	3692.2	3.64	-	-	-	-	-	-	-	-	-	-	-	-
0.13	-	-	-	-	4178.3	2.73	3409.8	2.21	-	-	-	-	3280.9	2.19
0.14	-	-	-	-	-	-	-	-	6253.4	4.05	7300.6	3.76	-	-
0.15	7443.2	7.33	7008.6	4.12	-	-	2194.0	1.42	-	-	-	-	-	-
0.16	-	-	-	-	-	-	-	-	-	-	3132.5	1.61	871.3	0.58
0.17	-	-	3839.6	2.26	-	-	-	-	2195.2	1.42	-	-	-	-
0.21	-	-	-	-	-	-	2621.6	1.70	-	-	-	-	-	-
0.22	3518.1	3.46	-	-	-	-	-	-	-	-	5907.1	3.04	3523.9	2.36
0.23	-	-	10995.2	6.46	11132.9	7.28	-	-	3641.9	2.36	-	-	-	-
0.27	-	-	-	-	-	-	-	-	-	-	-	-	13970.0	9.34
0.28	-	-	-	-	27086.0	17.70	17366.5	11.24	12406.2	8.04	18566.2	9.57	-	-
0.29	-	-	17175.2	10.10	-	-	-	-	-	-	-	-	-	-
0.31	18974.8	18.68	-	-	-	-	-	-	-	-	-	-	-	-
0.32	-	-	-	-	-	-	17415.1	11.27	-	-	-	-	-	-
0.37	-	-	-	-	-	-	-	-	-	-	19129.8	9.86	-	-
0.40	7635.5	7.52	-	-	-	-	-	-	-	-	-	-	22079.7	14.76
0.41	-	-	-	-	-	-	9490.8	6.14	15851.0	10.28	-	-	-	-
0.42	-	-	-	-	9856.9	6.44	-	-	-	-	12631.2	6.51	-	-
0.43	-	-	33093.0	19.45	-	-	-	-	-	-	21214.1	10.93	11138.9	7.45
0.44	-	-	-	-	-	-	-	-	8141.1	5.28	-	-	-	-

0.46	4428.2	4.36	-	-	-	-	-	-	-	-	-	-	-	-
0.47	-	-	-	-	-	-	-	-	-	-	-	-	9420.9	6.30
0.48	-	-	-	-	-	-	-	-	8244.1	5.34	-	-	-	-
0.49			8497.1	4.99	5181.7	3.39	4104.3	2.66	-	-	-	-	-	-
0.52	3379.1	3.33	-	-	-	-	-	-	-	-	-	-	-	-
0.56	-	-	-	-	7790.5	5.09	-	-	-	-	-	-	-	-
0.57	-	-	-	-	-	-	-	-			21285.2	10.97	19483.7	13.03
0.58	-	-	-	-	11248.0	7.35	16258.7	10.52	19650.1	12.74	-	-	-	-
0.59	-	-	16548.2	9.73	-	-	-	-	-	-	-	-	-	-
0.61	13979.2	13.76	-	-	-	-	-	-	-	-	-	-	-	-
0.63	-	-	-	-	-	-	-	-	-	-	-	-	2003.0	1.34
0.64	-	-	-	-	-	-	-	-	1161.7	0.75	4269.7	2.20	-	-
0.65	-	-	-	-	-	-	3079.5	1.99	-	-	-	-	-	-
0.66	-	-	1166.5	0.69	-	-	-	-	-	-	-	-	-	-
0.68	1052.7	1.04	-	-	-	-	-	-	-	-	-	-	-	-
0.72	-	-	-	-	-	-	11478.9	7.43	9935.6	6.44	12273.0	6.32	9294.0	6.21
0.73	-	-	-	-	15605.7	10.20	-	-	-	-	-	-	-	-
0.74	-	-	7754.9	4.56	-	-	-	-	-	-	-	-	-	-
0.76	3143.3	3.09	-	-	-	-	-	-	-	-	-	-	-	-
0.81	-	-	-	-	29085.4	19.01	-	-	-	-	-	-	-	-
0.82	-	-	-	-	-	-	40763.9	26.38	-	-	-	-	-	-
0.83	-	-	-	-	-	-	-	-	35176.5	22.80	-	-	33642.9	22.49
0.84	15499.9	15.26	32547.1	19.13	-	-	-	-	-	-	37704.9	19.43	-	-
0.88	-	-	-	-	-	-	-	-	-	-	-	-	7245.9	4.84
0.91	3499.7	3.45	-	-	-	-	-	-	3839.9	2.49	-	-	9169.6	6.13
0.92	-	-	-	-	-	-	5245.6	3.39	-	-	6810.1	3.51	-	-
0.93	-	-	4430.6	2.60	4149.9	2.71	-	-	-	-	-	-	-	-
0.95	1416.8	1.39	-	-	-	-	-	-	-	-	-	-	-	-
0.96	-	-	-	-	-	-	-	-	5534.3	3.59	-	-	-	-
0.97	-	-	-	-	431.7	0.28	-	-	-	-	-	-	-	-

Table 30. Peak areas of different samples at 525 nm for ethyl acetate extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	-	-	-	-	-	-	-	-	-	-	-	-	5412.8	5.51
0.03	16049.1	14.65	21676.4	15.24	13538.9	14.23	15224.6	15.50	17598.4	13.56	18832.8	12.98	-	-
0.05	-	-	-	-	10385.7	10.92	-	-	-	-	-	-	-	-
0.08	-	-	-	-	-	-	-	-	-	-	-	-	2651.6	2.70
0.09	-	-	-	-	-	-	-	-	5651.1	4.35	5906.8	4.07	-	-
0.10	7823.7	7.14	6352.7	4.47	4769.2	5.01	6732.1	6.85	-	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	-	-	-	-	6173.3	6.28
0.13	6140.2	5.61	-	-	5353.9	5.63	5673.8	5.77	10213.3	7.87	10933.8	7.54	-	-
0.14	-	-	9746.1	6.85	-	-	-	-	-	-	-	-	-	-
0.16	-	-	-	-	1374.2	1.44	-	-	-	-	-	-	-	-
0.17	3004.2	2.74	2224.8	1.56	-	-	-	-	-	-	-	-	-	-
0.19	-	-	-	-	1537.2	1.62	-	-	-	-	-	-	-	-
0.20	-	-	1081.8	0.76	-	-	-	-	-	-	-	-	-	-
0.23	-	-	-	-	-	-	-	-	1521.8	1.17	1932.4	1.33	787.9	0.80
0.24	5999.0	5.48	-	-	4027.6	4.23	-	-	-	-	-	-	-	-
0.25	-	-	3691.3	2.59	-	-	-	-	-	-	-	-	-	-
0.27	-	-	-	-	-	-	-	-	-	-	-	-	13739.1	13.98
0.28	18127.5	16.55	13852.8	9.74	10465.9	11.00	14682.5	14.94	14752.0	11.37	17531.4	12.09	-	-
0.34	-	-	-	-	-	-	8567.1	8.72	-	-	-	-	-	-
0.35	-	-	-	-	-	-	-	-	4376.3	3.37	6350.8	4.38	5403.4	5.50
0.37	-	-	6832.7	4.80	-	-	-	-	-	-	-	-	-	-
0.39	-	-	-	-	-	-	-	-	-	-	6953.9	4.79	-	-
0.40	7532.6	6.88	-	-	-	-	-	-	5298.7	4.08	-	-	-	-
0.41	-	-	7238.4	5.09	-	-	-	-	-	-	-	-	-	-
0.42	-	-	-	-	4185.1	4.40	2374.9	2.42	-	-				

0.43	-	-	-	-	-	-	-	-	-	-	17139.1	11.82	16528.2	16.81
0.44	-	-	-	-	-	-	2636.3	2.68	7534.0	5.80	-	-	-	-
0.45	2713.6	2.48	9648.1	6.78	-	-	-	-	-	-	-	-	-	-
0.48	3356.6	3.06	-	-	-	-	-	-	6053.6	4.66	-	-	-	-
0.49	-	-	5791.4	4.07	1603.8	1.69	1681.7	1.71	-	-	-	-	-	-
0.55	3173.0	2.90	-	-	-	-	-	-	-	-	-	-	-	-
0.57	-	-	-	-	-	-	-	-	-	-	15918.8	10.98	14036.7	14.28
0.58	9125.2	8.33	-	-	7967.1	8.38	9635.4	9.81	17704.6	13.64	-	-	-	-
0.59	-	-	17513.4	12.31	-	-	-	-	-	-	-	-	-	-
0.64	-	-	-	-	-	-	-	-	-	-	984.8	0.68	-	-
0.65	-	-	-	-	-	-	333.0	0.34	-	-	-	-	-	-
0.66	1880.9	1.72	410.2	0.29	-	-	-	-	-	-	-	-	-	-
0.73	-	-	-	-	4225.0	4.44	2304.5	2.35	-	-	-	-	-	-
0.78	9101.0	8.31	10542.7	7.41	-	-	-	-	12664.7	9.76	-	-	-	-
0.81	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.82	-	-	-	-	23081.4	24.26			-	-	-	-	-	-
0.83	-	-	-	-	-	-	25595.1	26.05	-	-	-	-	30094.6	30.62
0.84	8773.2	8.01	-	-	-	-	-	-	20731.0	15.97	-	-	-	-
0.85	-	-	-	-	-	-	-	-	-	-	38088.5	26.26	-	-
0.86	-	-	22652.2	15.92	-	-	-	-	-	-	-	-	-	-
0.90	4533.8	4.14	-	-	-	-	-	-	-	-	-	-	-	-
0.91	-	-	-	-	-	-	-	-	2275.9	1.75	-	-	3165.2	3.22
0.92	-	-	-	-	-	-	2669.7	2.72	-	-	4463.0	3.08	-	-
0.93	-	-	3003.9	2.11	2609.4	2.74	-	-	-	-	-	-	-	-
0.94	2208.7	2.02	-	-	-	-	-	-	-	-	-	-	-	-
0.96	-	-	-	-	-	-	-	-	2123.9	1.64	-	-	-	-
0.98	-	-	-	-	-	-	-	-	1295.1	1.00	-	-	-	-
0.99	-	-	-	-	-	-	144.0	0.15	-	-	-	-	302.3	0.31

4.4.1.3 Methanol Extract

In sequential extraction, methanol was the last solvent used for preparation of extracts. Extracts of all the samples were separated using mobile phase i.e. toluene:ethyl acetate:formic acid (6.5:3.0:0.5 v/v) for the qualitative analysis by HPTLC.

- **254 nm**

The TLC plate was observed under UV 254 which showed variation in banding pattern (Fig. 64a). Then plate was scanned which generated chromatograms with number of peaks and areas. T1 sample recorded highest peaks (17) among all samples analyzed and maximum area 6570.8 was obtained at Rf (0.02) (Fig. 65a). Samples (T2, T3, T5 and T7) had total 14 peaks, and it was observed that the highest areas were for same peak at Rf (0.83) and there is significant difference in their values (22736.9, 10421.1, 13802.0 and 12724.1) (Fig. 65b, c, e, g). T4 sample obtained 12 peaks of which the highest area was 14282.4 at Rf (0.13) (Fig. 65d). Total peaks (10) were recorded in T6 sample and maximum area 16904.8 was observed for a peak at Rf (0.39) (Fig. 65f). Chromatogram resulted with different peaks which were present at respective Rf. By comparing all the samples including *in vivo* and *in vitro*, identified unique peaks present in them. As peak at Rf 0.02 and 0.24 was common in all the samples (T1-T7) while at Rf 0.41 and 0.85 peaks were present in only T1 sample therefore peaks were unique for this sample. Similarly at Rf (0.59) recorded distinct peak which was present in only T7 sample. The unique peak was identified in nodal derived shoots (T5-T7) at Rf (0.82) (Table 31).

- **366 nm**

The fingerprint profile was also observed in UV chamber at 366 nm and then scanned at the same wavelength. Observation showed change in banding pattern (Fig. 64b) and scanning depicted the total peaks present with their areas. T1 and T2 sample reported with total 14 and 10 peaks, and among them maximum peak areas 15159 and 34853.3 were recorded at same Rf (0.85) (Fig. 65h, i). Similarly for T3 and T5 both samples resulted with total 7 and 11 peaks respectively their highest areas were 10819.5 and 13034.8 recorded for peak at Rf 0.23 (Fig. 65j, l). Whereas T4 sample showed total 10 peaks from which maximum area 14577.5 for peak at Rf 0.56 (Fig. 65k). T6 and T7 samples reported same peaks (10) and highest area 44224.7 and 33207.1 was noted at Rf (0.39) (Fig. 65m, n).

Table 31. Peak areas of different samples at 254 nm for methanol extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.03	-	-	-	-	-	-	-	-	-	-	4643.0	27.11	4944.1	34.08
0.04	6900.3	25.64	7124.8	43.91	5371.0	21.16	5755.5	26.85	4285.8	15.50	-	-	-	-
0.09	-	-	-	-	327.1	1.29	-	-	-	-	-	-	-	-
0.10	-	-	-	-	-	-	-	-	3194.4	11.55	-	-	1513.1	10.43
0.11	3029.2	11.26	1607.6	9.91	-	-	-	-	-	-	-	-	-	-
0.12	-	-	-	-	135.4	0.53	5568.0	25.98	-	-	3288.0	19.20	-	-
0.13	-	-	-	-	-	-	-	-	-	-	-	-	623.2	4.30
0.14	-	-	-	-	-	-	-	-	1242.5	4.49	-	-	-	-
0.15	1232.6	4.58	967.9	5.96	-	-	-	-	-	-	-	-	-	-
0.16	-	-	-	-	-	-	1884.7	8.79	323.8	1.17	2046.2	11.95	-	-
0.17	-	-	110.1	0.68	-	-	-	-	-	-	-	-	-	-
0.18	533.7	1.98	-	-	-	-	-	-	-	-	423.4	2.47	-	-
0.19	-	-	-	-	-	-	-	-	120.5	0.44	-	-	-	-
0.20	245.2	0.91	-	-	-	-	254.1	1.19	-	-	-	-	-	-
0.21	-	-	112.2	0.69	-	-	-	-	-	-	-	-	-	-
0.22	-	-	-	-	-	-	-	-	-	-	-	-	423.9	2.92
0.23	-	-	-	-	-	-	-	-	597.0	2.16	-	-	-	-
0.24	712.0	2.65	200.6	1.24	-	-	-	-	-	-	-	-	-	-
0.25	-	-	-	-	-	-	-	-	-	-	566.7	3.31	-	-
0.26	-	-	-	-	-	-	1142.1	5.33	-	-	-	-	-	-
0.27	-	-	-	-	232.0	0.91	-	-	-	-	-	-	528.9	3.65
0.28	-	-	-	-	-	-	-	-	1606.3	5.81	-	-	-	-
0.29	-	-	597.0	3.68	-	-	-	-	-	-	-	-	-	-
0.30	1390.1	5.17	-	-	-	-	-	-	-	-	-	-	-	-
0.31	-	-	-	-	-	-	-	-	-	-	618.8	3.61	-	-

0.33	-	-	-	-	909.0	3.58	577.8	2.70	-	-	-	-	-	-
0.35	-	-	658.3	4.06	-	-	-	-	2404.7	8.70	-	-	383.3	2.64
0.36	1889.3	7.02	-	-	-	-	86.9	0.41	-	-	637.2	3.72	-	-
0.38	-	-	-	-	722.0	2.84	517.9	2.42	-	-	-	-	-	-
0.41	-	-	-	-	444.9	1.75	-	-	2395.2	8.66	-	-	1712.6	11.81
0.42	-	-	1761.3	10.85	-	-	-	-	-	-	1327.2	7.75	-	-
0.43	3756.8	13.96	-	-	-	-	-	-	-	-	-	-	-	-
0.44	-	-	-	-	-	-	1395.3	6.51	-	-	-	-	-	-
0.47	-	-	-	-	106.0	0.42	-	-	324.3	1.17	-	-	-	-
0.52	237.0	0.88	-	-	-	-	-	-	-	-	-	-	-	-
0.54	152.5	0.57	-	-	-	-	-	-	-	-	-	-	-	-
0.56	-	-	-	-	201.9	0.80	-	-	-	-	-	-	-	-
0.57	-	-	-	-	-	-	350.2	1.63	-	-	-	-	-	-
0.58	166.4	0.62	-	-	-	-	-	-	430.6	1.56	-	-	-	-
0.61	-	-	195.0	1.20	-	-	-	-	142.2	0.51	-	-	-	-
0.63	269.0	1.00	-	-	408.6	1.61	-	-	-	-	-	-	-	-
0.64	-	-	134.4	0.83	-	-	-	-	-	-	-	-	-	-
0.65	106.3	0.40	-	-	-	-	-	-	-	-	-	-	-	-
0.68	-	-	189.3	1.17	-	-	-	-	-	-	-	-	-	-
0.78	-	-	-	-	296.7	1.17	-	-	-	-	-	-	-	-
0.82	1092.0	4.06	-	-	-	-	-	-	-	-	-	-	-	-
0.90	-	-	-	-	-	-	2332.1	10.88	-	-	-	-	-	-
0.91	-	-	-	-	9316.1	36.70	-	-	6004.7	21.72	1974.8	11.53	2410.8	16.62
0.92	3807.5	14.15	941.2	5.80	-	-	-	-	-	-	-	-	-	-
0.96	-	-	-	-	6916.7	27.24	1567.8	7.32	4578.4	16.56	-	-	-	-
0.97	1387.9	5.16	1626.8	10.03	-	-	-	-	-	-	1601.5	9.35	1965.9	13.55

Table 32. Peak areas of different samples at 366 nm for methanol extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.03	5157.9	17.91	-	-	-	-	4858.0	23.40	-	-	3794.1	22.39	4145.0	31.92
0.04	-	-	5796.5	40.17	7520.7	40.64	-	-	3809.1	12.64	-	-	-	-
0.08	1560.1	5.42	-	-	-	-	-	-	-	-	-	-	-	-
0.10	-	-	-	-	-	-	-	-	4790.9	15.90	-	-	2391.6	18.42
0.11	5231.9	18.17	2263.6	15.69	-	-	-	-	-	-	5267.5	31.08	-	-
0.12	-	-	-	-	-	-	8454.8	40.72	-	-	-	-	-	-
0.14	-	-	-	-	-	-	-	-	1287.4	4.27	-	-	658.2	5.07
0.15	-	-	1361.0	9.43	-	-	-	-	-	-	2820.1	16.64	-	-
0.16	1599.3	5.55	-	-	-	-	-	-	335.8	1.11	-	-	-	-
0.17	-	-	-	-	-	-	1820.5	8.77	-	-	-	-	-	-
0.19	215.3	0.75	-	-	-	-	-	-	-	-	-	-	-	-
0.20	-	-	-	-	-	-	185.5	0.89	-	-	-	-	-	-
0.22	-	-	-	-	340.3	1.84	-	-	-	-	-	-	-	-
0.23	-	-	191.5	1.33	-	-	-	-	-	-	-	-	354.0	2.73
0.24	-	-	-	-	-	-	-	-	-	-	154.4	0.91	-	-
0.25	2127.1	7.39	-	-	-	-	-	-	-	-	-	-	-	-
0.26	-	-	-	-	-	-	297.6	1.43	-	-	-	-	-	-
0.27	-	-	-	-	-	-	-	-	-	-	-	-	951.0	7.32
0.28	1423.8	4.94	-	-	-	-	-	-	3752.4	12.46	-	-	-	-
0.29	-	-	952.3	6.60	-	-	1098.9	5.29	-	-	1086.9	6.41	-	-
0.31	1728.6	6.00	-	-	-	-	-	-	-	-	-	-	-	-
0.32	-	-	-	-	3971.1	21.46	550.6	2.65	-	-	-	-	-	-
0.34	-	-	-	-	-	-	-	-	-	-	-	-	787.1	6.06
0.35	-	-	1041.4	7.22	-	-	-	-	7142.9	23.71	-	-	-	-
0.36	-	-	-	-	-	-	-	-	-	-	1038.7	6.13	-	-

0.38	5373.9	18.66	-	-	-	-	742.2	3.57	1432.1	4.75	-	-	-	-
0.40	-	-	-	-	399.9	2.16	-	-	-	-	-	-	769.0	5.92
0.41	-	-	782.0	5.42	-	-	-	-	-	-	692.0	4.08	-	-
0.42	-	-	-	-	-	-	-	-	3287.4	10.91	-	-	-	-
0.43	-	-	-	-	-	-	533.1	2.57	-	-	-	-	-	-
0.44	1401.1	4.86	-	-	-	-	-	-	-	-	-	-	-	-
0.54	-	-	-	-	329.5	1.78	-	-	-	-	-	-	-	-
0.55	505.2	1.75	-	-	-	-	-	-	-	-	-	-	-	-
0.57	-	-	-	-	-	-	-	-	128.9	0.43	-	-	-	-
0.60	-	-	-	-	197.9	1.07	-	-	-	-	-	-	-	-
0.61	-	-	-	-	-	-	-	-	424.5	1.41	-	-	-	-
0.64	-	-	188.9	1.31	-	-	-	-	-	-	-	-	-	-
0.68	-	-	181.4	1.26	-	-	-	-	-	-	-	-	-	-
0.73	148.9	0.52	-	-	-	-	-	-	-	-	-	-	-	-
0.79	-	-	-	-	-	-	-	-	-	-	207.6	1.22	-	-
0.86	-	-	-	-	-	-	-	-	-	-	-	-	398.0	3.06
0.90	-	-	-	-	5744.5	31.04	2220.7	10.70	3735.7	12.40	-	-	-	-
0.91	-	-	1671.6	11.58	-	-	-	-	-	-	1559.7	9.20	2285.9	17.60
0.93	1843.3	6.40	-	-	-	-	-	-	-	-	-	-	-	-
0.96	485.5	1.69	-	-	-	-	-	-	-	-	324.7	1.92	-	-
0.97	-	-	-	-	-	-	-	-	-	-	-	-	245.3	1.89

The chromatograms were also evaluated for unique peaks present in particular sample. Peak at Rf (0.02) was present in all the samples (T1-T7), whereas peaks identified at Rf (0.09, 0.57, 0.67 and 0.75) were unique because they were only present in T1 sample. T6 sample also obtained one distinct peak at Rf (0.20), similarly T5-T7 sample also had unique peaks at Rf (0.47 and 0.65) (Table 32).

- **525 nm**

Fingerprint formed after derivatization also confirmed variation among the sample when observed under white light (Fig. 64c). When TLC plate was scanned at 525 nm, different samples showed number of peaks and their areas. T1-T4 samples showed almost similar peaks (10, 8, 10 and 9) and among them areas for peak at different Rf (0.48, 0.34, 0.67 and 0.62) gave maximum area i.e. 16554.8, 20296.4, 15566.2 and 24502.0 at respective Rf. (Fig. 65o-r). Nodal derived shoot samples T5-T7 showed total peaks 14, 7 and 7 and they had maximum areas (20600.5, 20691.9 and 32527.2) at the same Rf 0.57 (Fig. 65s-u). Chromatogram showed varied peaks at different Rf and it confirmed presence of distinct peaks by comparing all the samples. At Rf (0.85 and 0.93) peaks present only in *in vivo* (T1) whereas at Rf (0.98) showed peak only present in *in vitro* samples. Unique Peak was also identified in T2 and T3 samples at Rf (0.46). Similarly in nodal shoot samples like T5-T7 identified unique peaks at Rf (0.13 and 0.76). Moreover T7 sample also had unique peaks at Rf (0.16, 051 and 0.58) (Table 33).

- It was revealed from fingerprint analysis that all the selected *in vitro* samples have potency to synthesize metabolites. The biosynthetic potential of *in vitro* shoot cultures derived through nodal explants was high as they have synthesize more number of metabolites as compared to *in vivo* shoots as well as *in vitro* shoots of leaf explants derived.

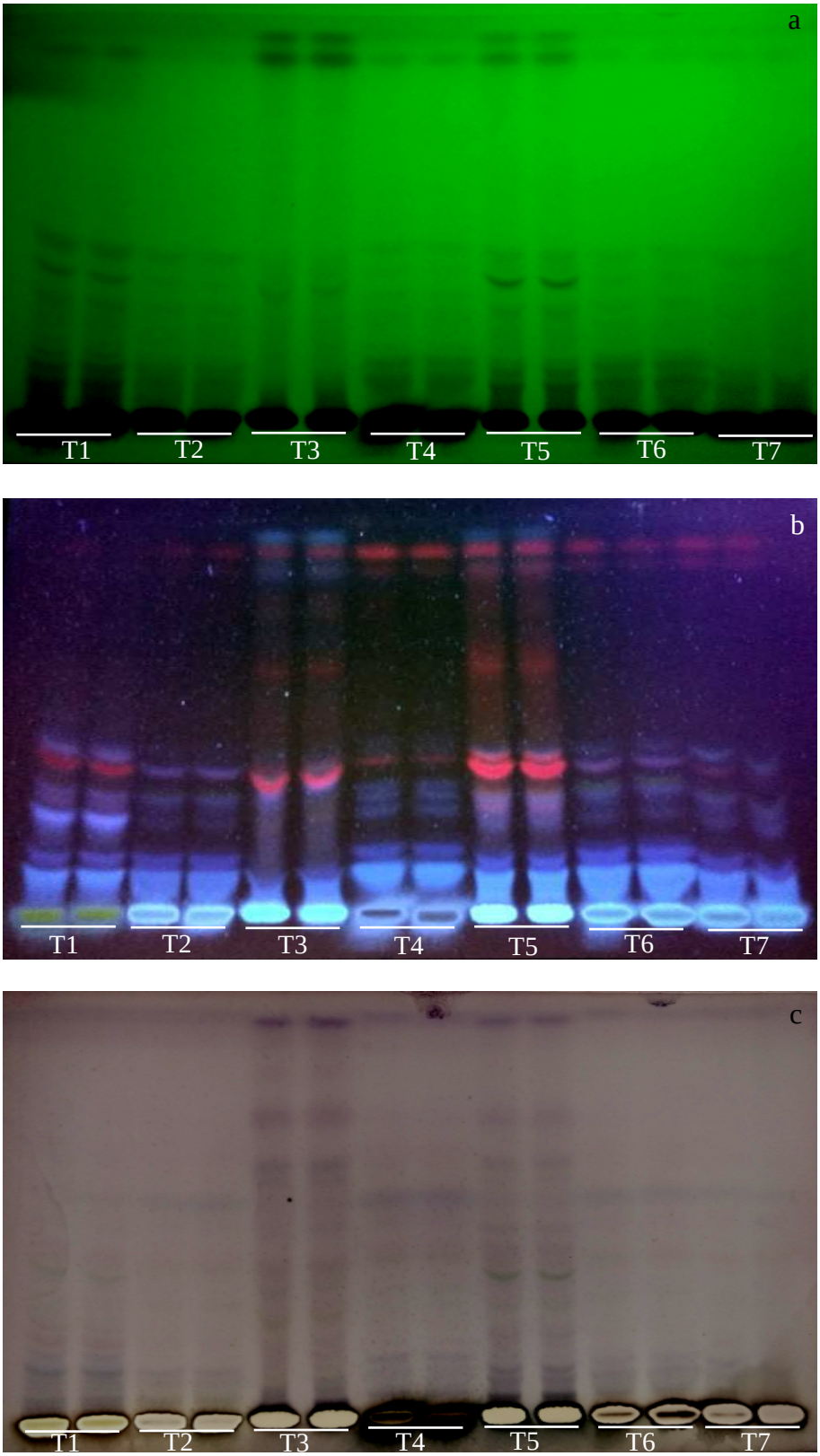

Figure 64. HPTLC fingerprints of methanol extract for different samples of *T. indica*: (a) UV 254 nm, (b) UV 366 nm and (c) white light after derivatization.

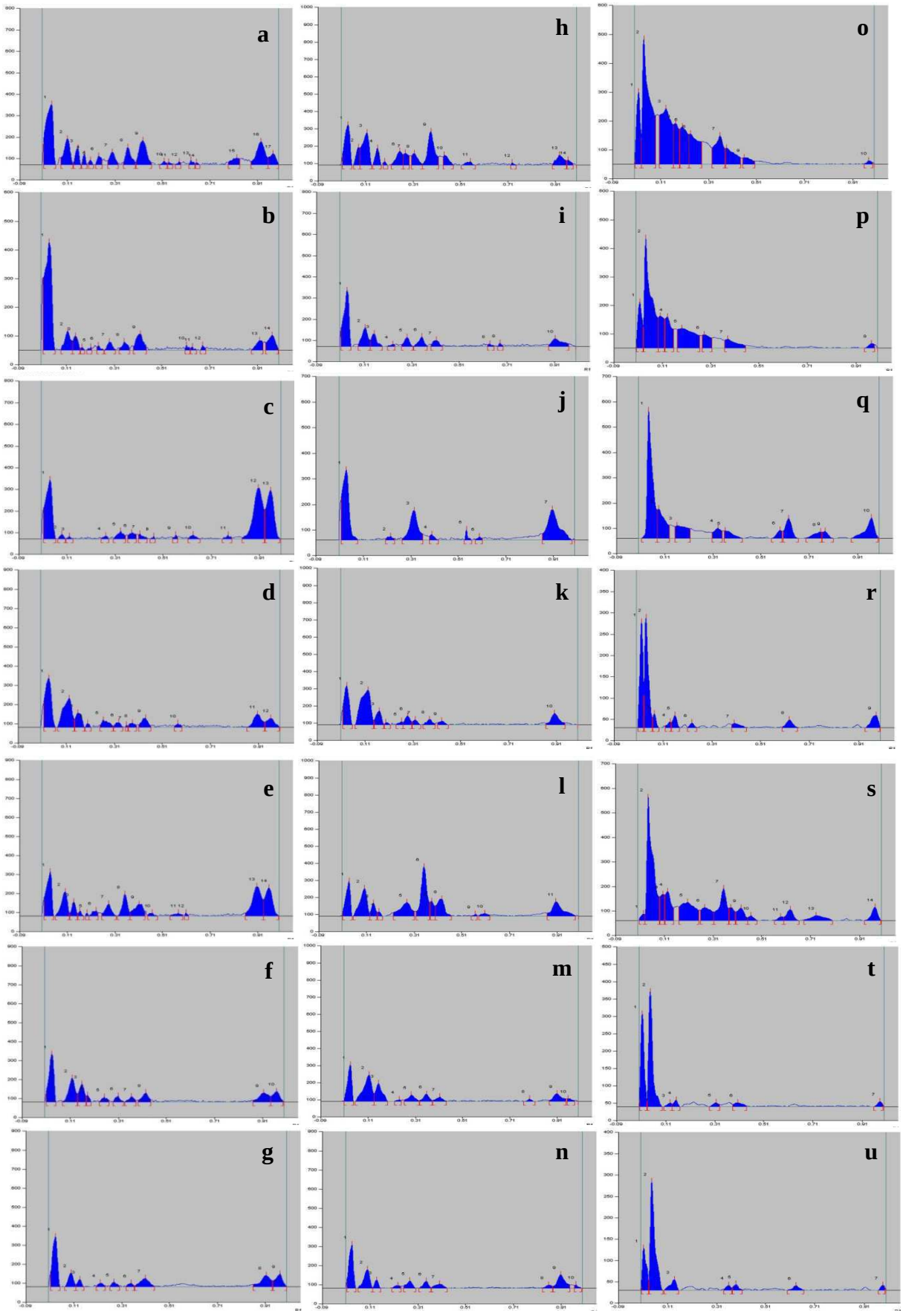

Figure 65. Chromatograms of samples for methanol extract in *T. indica*: (a-g) 254 nm, (h-n) 366 nm and (o-u) 525 nm.

Table 33. Peak areas of different samples at 525 nm for methanol extracts.

Rf value	T1		T2		T3		T4		T5		T6		T7	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	-	-	-	-	-	-	-	-	-	-	3820.1	35.62	1636.9	20.45
0.02	3912.0	8.97	2759.8	10.34	-	-	3112.2	30.97	413.4	1.19	-	-	-	-
0.04	12740.5	29.21	10352.0	38.78	10789.0	41.10	3949.9	39.30	11897.4	34.13	-	-	4768.0	59.56
0.05	-	-	-	-	-	-	-	-	-	-	5498.1	51.27	-	-
0.07	-	-	-	-	-	-	411.8	4.10	-	-	-	-	-	-
0.08	-	-	-	-	3670.4	13.98	-	-	-	-	-	-	-	-
0.10	-	-	2476.9	9.28	-	-	-	-	1970.5	5.65	-	-	-	-
0.12	-	-	-	-	-	-	-	-	2885.1	8.28	-	-	-	-
0.13	8785.6	20.14	2927.3	10.97	-	-	-	-	-	-	189.0	1.76	-	-
0.14	-	-	-	-	-	-	204.3	2.03	-	-	-	-	629.5	7.86
0.15	-	-	-	-	-	-	-	-	-	-	300.9	2.81	-	-
0.16	-	-	-	-	2465.6	9.39	495.6	4.93	-	-	-	-	-	-
0.17	3072.7	7.04	-	-	-	-	-	-	-	-	-	-	-	-
0.19	-	-	4790.4	17.94	-	-	-	-	-	-	-	-	-	-
0.20	4021.9	9.22	-	-	-	-	-	-	4456.1	12.78	-	-	-	-
0.23	4091.6	9.38	-	-	-	-	193.1	1.92	-	-	-	-	-	-
0.27	-	-	-	-	-	-	-	-	2196.7	6.30	-	-	-	-
0.28	-	-	1529.1	5.73	-	-	-	-	-	-	-	-	-	-
0.31	-	-	-	-	-	-	-	-	-	-	251.3	2.34	-	-
0.33	-	-	-	-	1192.8	4.54	-	-	-	-	-	-	-	-
0.35	-	-	-	-	-	-	-	-	3851.2	11.05	-	-	-	-
0.36	3591.1	8.23	-	-	1057.3	4.03	-	-	-	-	-	-	229.3	2.86
0.38	2313.8	5.30	1517.1	5.68					1123.8	3.22				
0.39	-	-	-	-	-	-	-	-	-	-	-	-	281.0	3.51
0.40	-	-	-	-	-	-	343.8	3.42	-	-	400.9	3.74	-	-

0.42	-	-	-	-	-	-	-	-	1578.5	4.53	-	-	-	-
0.46	852.7	1.95	-	-	-	-	-	-	-	-	-	-	-	-
0.47	-	-	-	-	-	-	-	-	410.8	1.18	-	-	-	-
0.59	-	-	-	-	626.9	2.39	-	-	326.9	0.94	-	-	-	-
0.63	-	-	-	-	2125.0	8.10	497.2	4.95	1253.3	3.59	-	-	283.5	3.54
0.73	-	-	-	-	-	-	-	-	1178.4	3.38	-	-	-	-
0.76	-	-	-	-	851.9	3.25	-	-	-	-	-	-	-	-
0.78	-	-	-	-	631.0	2.40	-	-	-	-	-	-	-	-
0.97	-	-	344.3	1.29	2840.5	10.82	-	-	1321.7	3.79	-	-	-	-
0.98	242.0	0.55	-	-	-	-	841.8	8.38	-	-	264.2	2.46	-	-
0.99	-	-	-	-	-	-	-	-	-	-	-	-	176.9	2.21

Fingerprint analysis revealed that there was variation between the *in vitro* samples regenerated via different pathways, the same observations have been reported in *Curculigo orchioides* where the shoots regenerated via different pathways showed change in HPTLC fingerprint (Alagar et al., 2014). This is also previously documented in medicinal plant *Arnicae folium* and *A. caulis* (Stefanache et al., 2014). Moreover, shoots regenerated via nodal explant (in presence of cytokinins) were more similar to *in vivo* plants as compared to leaf and SE derived shoots. This might be due to beneficial effect of cytokinins on synthesis of metabolites and the same phenomenon was recorded in *Eclipta alba* (Pathak et al., 2021) as well as in other studies (Sangwan et al., 2001; Arikat et al., 2004). The chromatograms also showed that profile of shoots from hardened plant was almost similar to *in vivo* plants as compared to shoots under *in vitro* conditions. This is in corroboration with report in *Erigeron breviscapus* in which the profile changed when the cultures are under *in vitro* conditions but after hardening it showed the similar profile in comparison with mother plants (Liu et al., 2008). De Silva and Senarath, 2009) documented that hardened plants and seed raised plants of *Withania somnifera* showed similar HPTLC profile. Similarly, chromatograms of the conventionally propagated plant of *Curcuma angustifolia* had similar fingerprint pattern to that of micropropagated plants (Jena et al., 2018). The uniformity of chemical components are reported between tissue culture derived plants and source plant in *Munronia pinnata* (Gunathilake et al., 2008), *Passiflora caerulea* (Busilacchi et al., 2008), *P. caerulea* and *P. incarnata* (Ozarowski et al., 2013), *Curcuma longa* (Nayak et al., 2011; Singh et al., 2011b), *Kaempferia galanga* (Mohanty et al., 2011a, b) and *Sarcandra glabra* (Zhu et al., 2011).

It was also noted that the variation in number of bands differed with solvents used for extraction, this is because of the affinity of solvent for particular type of metabolites and the same is reported in *Tabernaemontana catharinensis* (Boligon et al., 2013). HPTLC fingerprint is advantageous to assess the metabolite biosynthesis in *in vitro* cultures, it has been reported in important medicinal plants like *Primula veris* (Morozowska and Wesołowska, 2004), *Celastrus paniculatus* (Martin et al., 2006), *Bacopa monnieri* (Patni et al., 2010), *Piper nigrum* (Ahmad et al. 2013), *Withania coagulans* and *W. somnifera* (Preethi et al., 2014) and *Nothapodytes nimmoniana* (Prakash et al., 2016).

4.4.2 Quantification of Lupeol

Hexane extract of different samples when separated on TLC plate, the presence of lupeol was confirmed in all the samples as a band corresponding to the standard.

Chromatogram showed the peak of standard lupeol and the sample at same Rf (Fig. 66a, b). The content of lupeol varied in the samples and it was 24.43 ± 1.28 µg/gm *in vivo* shoot sample (T1) whereas the lupeol quantity decreased in leaf derived shoots (T2) i.e. 19.12 ± 0.26 µg/gm which was least among all the samples. T3 and T4 samples were from leaf derived somatic embryos and shoots from hardened plants respectively. When they were analyzed for lupeol content, it was observed that in T3 sample the quantity slightly increased (29.92 ± 1.12 µg/gm) as compared to *in vivo* sample. But the content was 3.01 fold higher in T4 sample i.e. 73.77 ± 4.14 µg/gm in comparison to *in vivo* shoot (T1). Nodal derived shoots sample (T5 and T6) synthesized almost similar quantity of lupeol i.e. 42.53 ± 1.48 µg/gm and 47.45 ± 0.78 µg/gm respectively. One of the sample from nodal derived shoots (T7) showed an elevated content of lupeol i.e. 254.16 ± 6.90 µg/gm among all samples analyzed, which was 10.40 fold higher than *in vivo* (Table 34).

Table 34. Quantification of lupeol in *in vivo* and *in vitro* samples of *T. indica* using HPTLC.

Sample	Source	Medium	Lupeol quantity (µg/gm)*
T1	*In vivo* shoots	-	24.43 ± 1.28 cd
T2	Leaf derived shoots	BA (10 µM) + Kn (15 µM)	19.12 ± 0.26 d
T3	SEs derived from leaf	GA_3 (1.0 µM)	29.92 ± 1.12 cd
T4	Shoots of hardened plants	-	73.77 ± 4.14 b
T5	Nodal derived shoots	BA (10 µM) + Kn (5 µM)	42.53 ± 1.48 bc
T6	Nodal derived shoots	BA (15 µM) + $AdSO_4$ (10 µM)	47.45 ± 0.78 bc
T7	Nodal derived shoots	Kn (10 µM) + $AdSO_4$ (15 µM)	254.16 ± 6.90 a

*Values represent mean ± SE. Means (n = 3) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

- It was concluded from above study of quantification that *in vitro* sample especially nodal derived shoots (T7) high potency to synthesize lupeol compared to *in vivo* shoots (T1). Moreover one year old hardened plant shoots (T4) also showed significant content then T1.

Shoot cultures are known to synthesize high amount of secondary metabolites (Lorence and Nessler, 2004; Liu et al., 2006) and thus in the present study shoot samples from different media were assessed. HPTLC analysis revealed that lupeol quantity varied among different

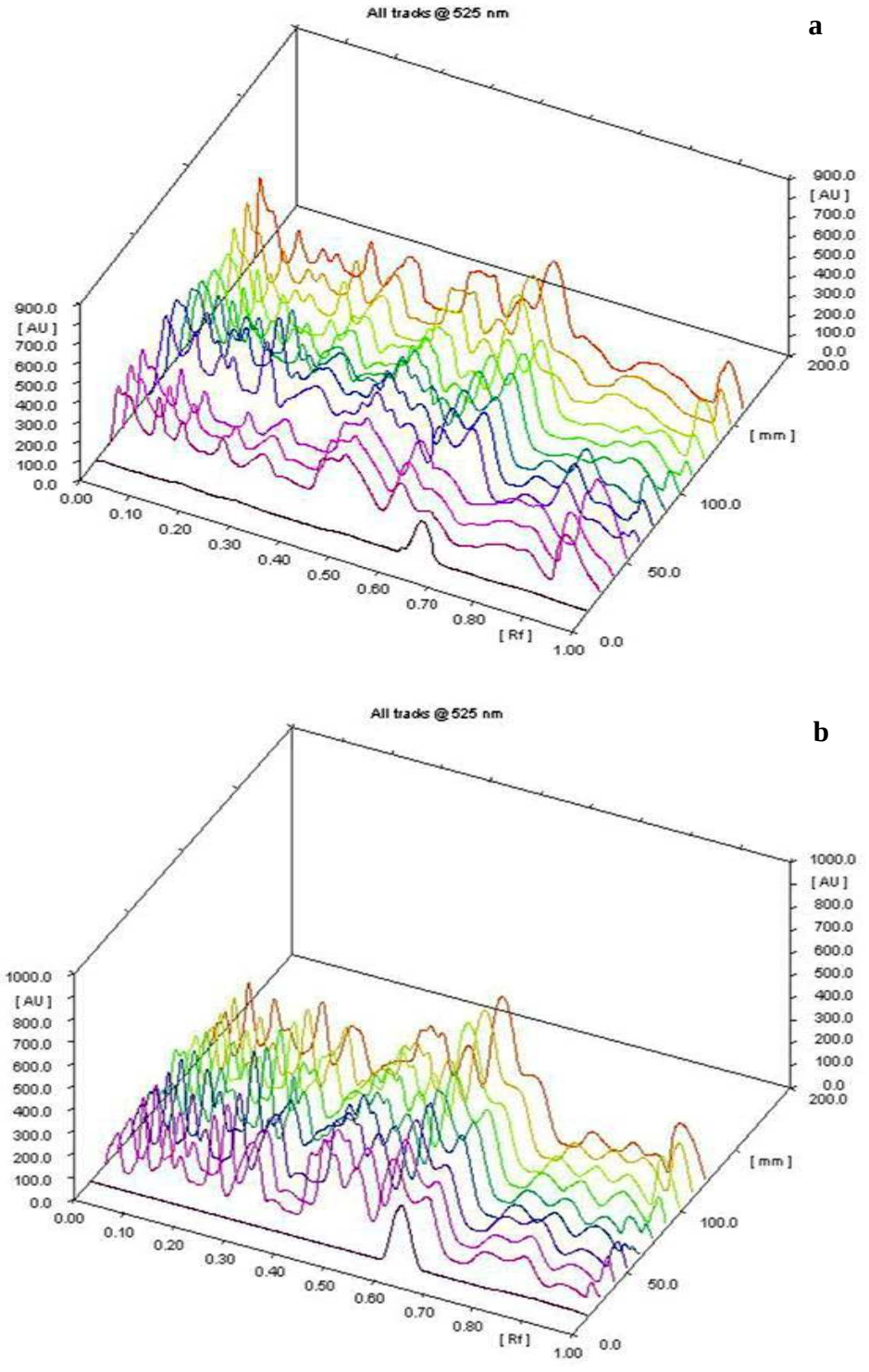

Figure 66. Quantification of lupeol in different samples of *T. indica*: (a) T1-T4 and (b) T5-T7.

samples which may be due to the presence of PGRs in the medium (Dornenburg and Knorr, 1995; Lee et al., 2011). This result is in accordance with earlier report for lupeol accumulation in *Hemidesmus indicus* in which its amount differed according to combinations and concentrations of PGRs (Misra et al., 2005). Effect of hormones has been also reported to affect bacoside A and coronarin D in *Bacopa monnieri* and *Hedychium coronarium* respectively (Parale and Nikam, 2009; Parida, 2018).

Amongst all the media tested, shoots from cytokinin based medium (Kn+AdSO_4) had significantly high content of lupeol as compared to *in vivo* shoots of *T. indica*, this is in line with report of *Cichorium intybus* in which more esculin content has been observed in shoots grown in medium fortified with Kn (Rehman et al., 2003). Previously many reports also documented beneficial effect of Kn on metabolite synthesis in *Nicotiana tabacum* (Angelova et al., 2001), *Artemisia annua* (Baldi and Dixit, 2008), *Genista tinctoria* (Luczkiewicz et al., 2014), *Scutellaria alpina* (Grzegorczyk-Karolak et al., 2015) and *Coffea arabica* (Acidri et al., 2020). In line with present results, *in vitro* cultures of *H. indicus* (Misra et al., 2003; Purohit et al., 2015; Pathak et al., 2017) and *Cnidoscolus chayamansa* (Pérez-González et al., 2019) also accumulated more lupeol as compared to their source plants when developed in media fortified with cytokinins. Similarly, presence of cytokinins enhanced the content of hypericin and pseudohypericin in *Hypericum hirsutum* and *H. maculatum* (Coste et al., 2011), esculin in *Cichorium intybus* (Rafsanjani et al., 2011) and andrographolide in *Andrographis paniculata* (Dandin and Murthy, 2012). The stimulatory effect of cytokinins on secondary metabolite synthesis has also been well documented in other medicinal plants like *Huernia hystrix* (Amoo and Van Staden, 2013), *Stevia rebaudiana* (Aman et al., 2013), *Withania somnifera* (Sabir et al., 2013) and *Scutellaria alpina* (Grzegorczyk-Karolak et al., 2015).

As nodal derived shoot cultures synthesized significantly high content of lupeol, it can be considered as potential alternative to *in vivo T. indica* plants, this is in accordance with other studies in which *in vitro* cultures has been source for better production of metabolite such as withanolide A in *Withania coagulans* and *W. somnifera* (Preethi et al., 2014), barbaloin in *Aloe vera* (Pandey et al., 2016), vasicinone in *Adhatoda vasica* (Panigrahi et al., 2017) and diosgenin in *Dioscorea deltoidea* (Nazir et al., 2020). It was also depicted that the lupeol accumulation was more in nodal derived as compared to leaf derived shoots, this has been also documented in recent report of Mukherjee et al. (2020) on *Rauvolfia serpentina* in which the reserpine content is more in direct shoots as compared to indirect regeneration. Similarly, variation in metabolite with change in culture type has been reported in many

plants (Singh and Chaturvedi, 2013; Ng et al., 2016). Another observation recorded was that the shoots derived from SE have lower content as compared to micropropagated plants which is also reported in *Rosa rugosa* (Jang et al., 2016). It was also noted that hardened plants of *T. indica* synthesized more lupeol as compared to mother plant, which is in contradiction with earlier report on *H. indicus* (Misra et al., 2003) and *Curcuma angustifolia* (Jena et al., 2018) where lupeol and curcumin content is almost similar in wild and tissue culture derived plant. The reason for more metabolite content in hardened plant as compared to *in vitro* culture is because they are exposed to stressful conditions which activate defence mechanisms as well as due to different light conditions which may increase the compounds (Jang et al., 2016). The same results are also reported in other species where the micropropagated plants have similar metabolite content as *in vivo* plants (Sasidharan et al., 2017; Behera et al., 2018) whereas in many studies it has been reported that metabolites content in micropropagated plant material is higher than *in vivo* plants (Arikat et al., 2004; Guo et al., 2007; Ncube et al., 2011; Rather et al., 2012; Chen et al., 2014; Hayta et al., 2017).

4.4.3 Elicitation of Lupeol using Elicitors

In vitro shoots developed in the medium supplemented with Kn (10 µM) + $AdSO_4$ (15 µM) within four weeks and they were transferred to fresh medium fortified with same PGRs concentrations for further growth. Shoots were harvested at weekly interval from the fourth week to sixth week and they were served as control because medium devoid of elicitor. Shoot growth was also monitored weekly and the observation showed that biomass increased with the time interval. Shoots were developed and their FW (0.468 ± 0.05 gm) and DW (0.108 ± 0.01 gm) was recorded at four weeks and it increased to 0.704 ± 0.09 gm (FW) and 0.129 ± 0.01 gm (DW) at six weeks. HPTLC analysis detects the presence of lupeol after derivatization in all the samples (Fig. 67a) which was also confirmed after densitometric scanning at 525 nm that the peak of the sample corresponds to standard (Fig. 67b). However, the AUC of lupeol peak were different in all the samples which depicted that there was variation in lupeol content. The content 254.16 ± 6.90 µg/gm was obtained after four weeks, that reached to 446.13 ± 8.69 µg/gm at five weeks and slightly increased to 457.08 ± 2.79 µg/gm in six weeks which was almost similar with five weeks but higher compared to *in vivo* shoots (Table 35).

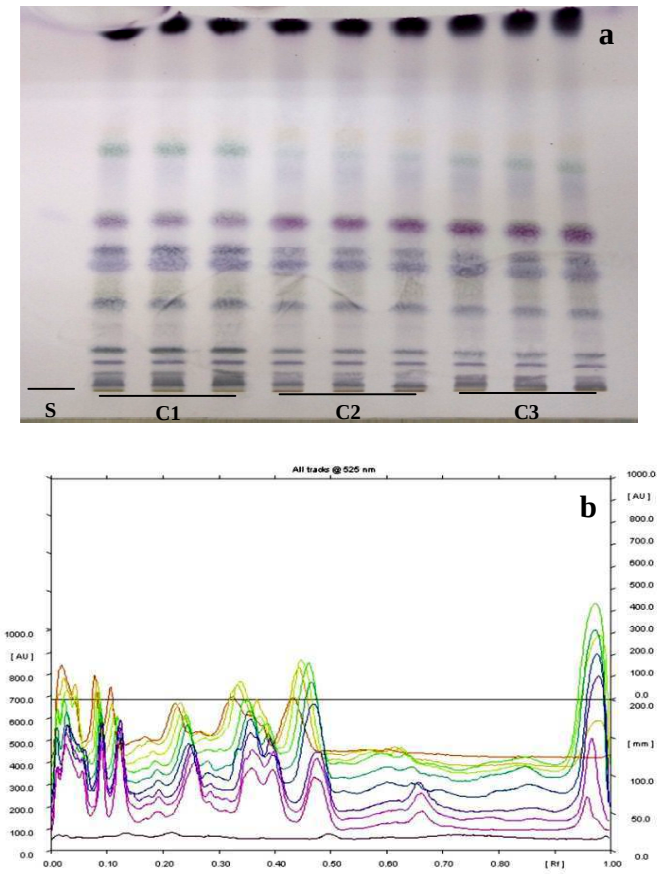

Figure 67. TLC plate and respective chromatogram for hexane extract of control shoot samples: (a) C1- four weeks, C2- five weeks, C3- six weeks and (b) spectra for control samples of all weeks. S- Standard of lupeol. Scanning of plate was done at 525 nm.

4.4.3.1 Effect of Yeast Extract on Shoot Biomass and Lupeol Content

In vitro cultures were then treated with different concentrations of yeast extract (25-200 mg/l) which facilitated better shoot growth as compared to control. Biomass increased gradually with the increasing concentration of YE as well as time interval. Further analyzing the samples by densitometric scanning confirmed the presence of lupeol in all the concentrations of YE after derivatization. Analysis showed variation in content of lupeol among all the concentrations and treatment time.

- **25 mg/l**

When 25 mg/l of YE added to the medium, shoot growth increased moderately with the time interval and that was observed from FW and DW. When shoots were harvested at four weeks, FW (0.511 ± 0.08 gm) and DW (0.0958 ± 0.01 gm) was recorded slightly increased in fifth week i.e. FW 0.529 ± 0.11 gm and DW 0.116 ± 0.02 gm. Further enhancement in the biomass [FW (0.590 ± 0.06 gm) and DW (0.122 ± 0.02 gm)] was in six weeks which was close to control for the same weeks (Fig. 68). Lupeol quantity was also analyzed by scanning at 525 nm and it was 420.33 ± 4.75 µg/gm in four weeks, which was 1.65 fold higher than control for the same weeks. Content slight increased to 448.86 ± 5.09 µg/gm in fifth week and it was similar with control for the same weeks. In sixth week lupeol content (445.70 ± 7.40 µg/gm) remained similar with five weeks and had slight decreased in comparison with control six weeks (Fig. 69a-f; Table 35).

- **50 mg/l**

YE concentration when increased to 50 mg/l, shoot growth and biomass was rapidly enhanced. FW (0.706 ± 0.07 gm) and DW (0.127 ± 0.01 gm) was recorded in four weeks which were higher than control and 25 mg/l. Shoot biomass slightly increased when it was harvested at five weeks and it reached to 1.019 ± 0.06 gm (FW) and 0.221 ± 0.02 gm (DW) in six weeks which was higher than above concentration as well as control (Fig. 68). Lupeol content was also analyzed after scanning the TLC plate at 525nm. The content obtained for four weeks was 400.78 ± 5.82 µg/gm, which was about to similar with 25 mg/l for the same weeks. Yield of lupeol decreased in five and six weeks i.e. 184.17 ± 4.54 µg/gm and 239.95 ± 4.60 µg/gm respectively (Fig. 69a-f; Table 35).

- **100 mg/l**

Variation was not observed in biomass when shoots were treated with 100 mg/l of YE except four weeks showed FW 0.505 ± 0.07 gm and DW 0.098 ± 0.01 gm respectively and it was lower than 50 mg/l and similarity observed with control and 25 mg/l. When shoots were harvested in five and six weeks, biomass obtained was FW (0.852 ± 0.05 gm and 0.948 ± 0.06 gm) and DW (0.153 ± 0.01 gm and 0.233 ± 0.01 gm) which were similar to above concentration (Fig. 68). The densitomatric scanning showed that the lupeol yield (382.92 ± 7.61 µg/gm) was recorded nearer with both above mentioned concentrations for four weeks. The yield was reduced (162.44 ± 3.32 µg/gm and 222.08 ± 3.67 µg/gm) in five and six weeks likewise 50 mg/l (Fig. 69a-f; Table 35).

- **200 mg/l**

When 200 mg/l of YE treatment was given to the *T. indica* shoots, biomass level recorded higher compared to all the above concentrations as well as control. Shoots when harvested at four weeks, its FW and DW was i.e. 0.664 ± 0.07 gm and 0.128 ± 0.02 gm which enhanced at five weeks as FW (0.821 ± 0.06 gm) and DW (0.136 ± 0.01 gm) was recorded. Moreover when FW (1.081 ± 0.05 gm) and DW (0.233 ± 0.01 gm) was noted for shoots harvested at six weeks, observations revealed that it was highest biomass among all YE concentrations (Fig. 68). The lupeol yield obtained at four weeks was 400.94 ± 4.89 µg/gm and was similar with all above concentrations for the same weeks. But it was slightly decreased (386.19 ± 5.61 µg/gm) in five weeks. Gradual declined in the lupeol yield from four to six weeks was recorded as in six weeks it showed 180.22 ± 3.35 µg/gm content (Fig. 69a-f; Table 35).

- It was observed that YE (200 mg/l) gave highest biomass in six weeks among all concentrations. Lupeol yield was higher in 25 mg/l, as it gave 1.65 fold higher yield than control in four weeks.

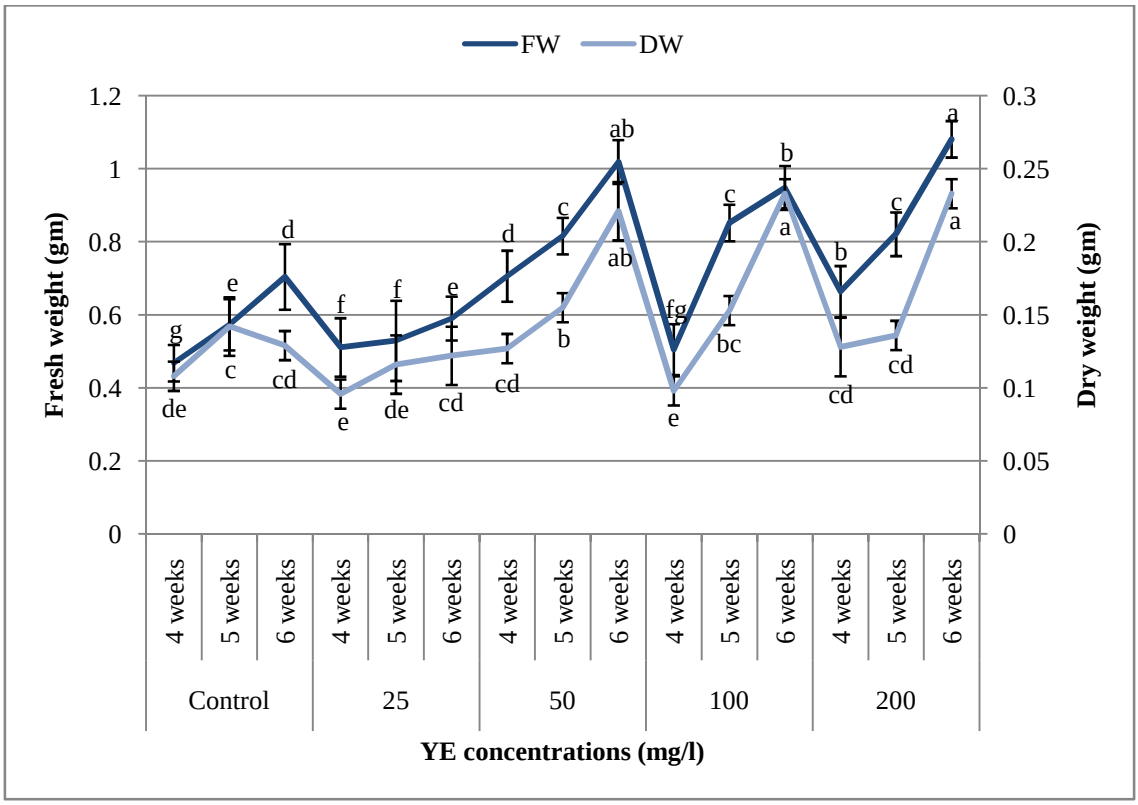

Figure 68. Effect of yeast extract on shoot biomass derived through nodal explants of *T. indica.*

Each line shows the mean values (n = 5) and error bar as standard error. Means followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Table 35. Quantification of lupeol in presence of YE at different time intervals.

YE concentrations (mg/l)	Lupeol (µg/gm)*		
	4 weeks	5 weeks	6 weeks
Control	254.16 ± 6.90 d	446.13 ± 8.69 ab	457.08 ± 2.79 a
25	420.33 ± 4.75 bc	448.86 ± 5.09 ab	445.70 ± 7.40 ab
50	400.78 ± 5.82 bc	184.17 ± 4.54 e	239.95 ± 4.60 d
100	382.92 ± 7.61 c	162.44 ± 3.32 e	222.08 ± 3.67 de
200	400.94 ± 4.89 bc	386.19 ± 5.61 c	180.22 ± 3.35 e

*Values represent mean ± SE. Means (n = 3) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

Although lupeol is important metabolites, scanty reports are available for its enhancement like in callus culture of *Hemidesmus indicus* using γ-rays (Misra and Mehrotra, 2006), using SA in *Cryptostegia grandiflora* (Singh et al., 2011a), UV-B exposure in *Vitis*

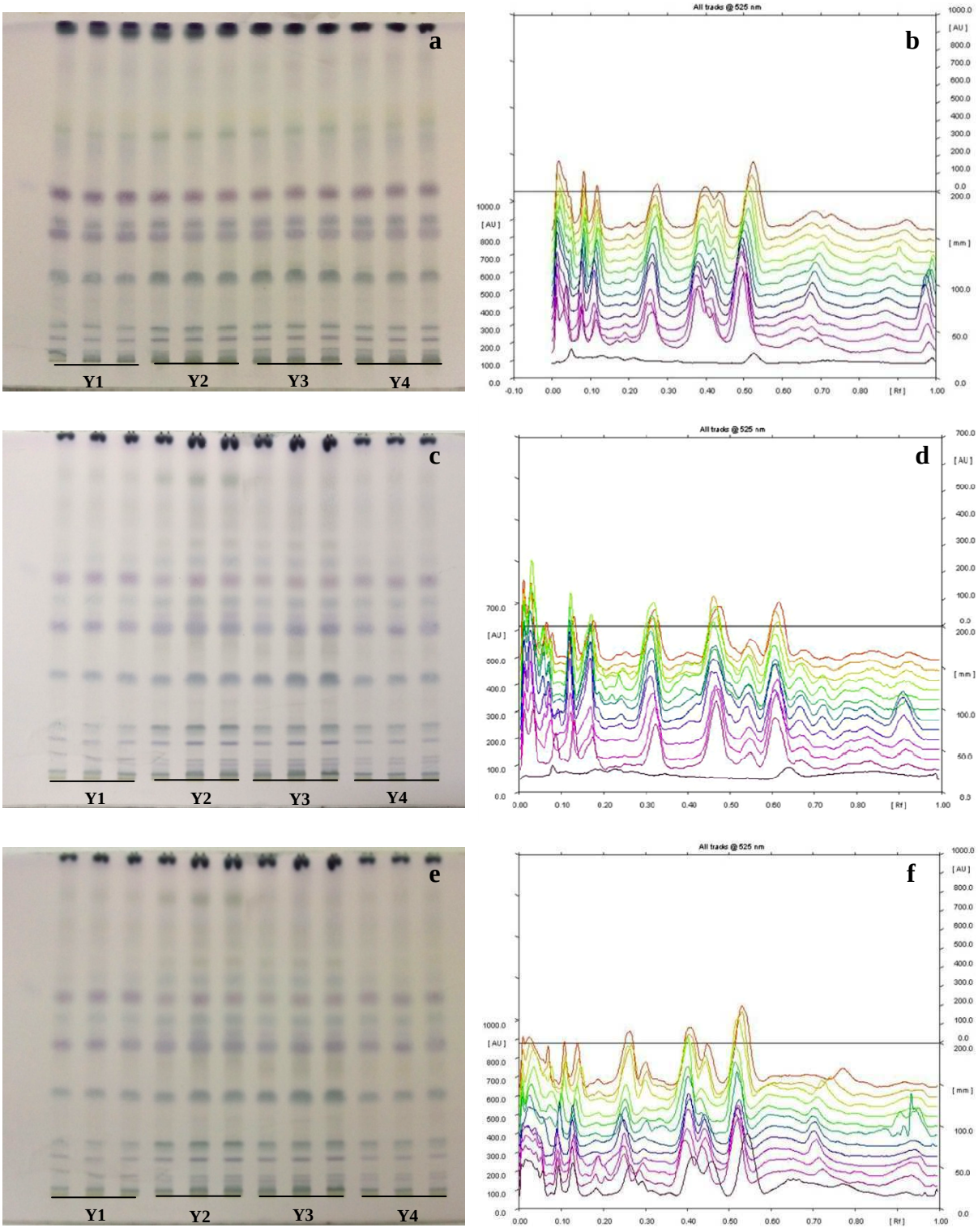

Figure 69. TLC plates and respective chromatograms for hexane extract of shoot samples after YE treatment: (a, b) Four weeks, (c, d) five weeks and (e, f) six weeks. Y1- 25 mg/l, Y2- 50 mg/l, Y3- 100 mg/l and Y4- 200 mg/l. Scanning of plates was done at 525 nm.

vinifera (Gil et al., 2012) and jasmonic acid in *Jatropha curcas* (Zaragoza-Martinez et al., 2016). Hence the effect of YE and SA needs to be explored on its production.

Shoots of *T. indica* when placed in medium fortified with different concentrations of YE, enhanced shoot growth was observed. Most of the combinations evoked better shoot regeneration but maximum biomass was achieved at 25 mg/l and it is in accordance with the fact that lower concentrations of YE is beneficial for growth of shoots (Vasil and Hilderbrandt, 1966). Similarly, low concentration of YE is reported to be effective for growth as compared to higher concentration in *Curcuma mangga* (Abraham et al., 2011). The additive effect is because it provides amino acid, chitin, N-acetyl-glucosamine oligomers, β-glucan, glycopeptides, ergosterol and vitamin B-complex which is beneficial for growth of plant (George et al., 2008). YE is also reported to be a good source of nitrogen which plays pivotal role in growth of plants under *in vitro* conditions (Vasconsuelo and Boland, 2007) and the same has been observed in *Salvia miltiorrhiza* (Shi et al., 2007) and *Zingiber officinale* (El-Nabarawy et al., 2015). Radman et al. (2003) suggested elicitor-receptor interaction is the basis for rapid array of biochemical responses in plants. The variation in lupeol accumulation was also recorded according to concentration and the same effect of YE concentration has been studied on production of different pharmaceutically important metabolites like pseudojujubogenin in *Bacopa monnieri* (Kamonwannasit et al., 2008), plumbagin in *Drosera indica* (Thaweesak et al., 2011), flavonoid in *Merwilla plumbea* (Baskaran et al., 2012) vinblastine and vincristine in *Catharanthus roseus* (Maqsood and Abdul, 2017) and phenolics production in *Impatiens balsamina* (Kasem, 2018).

In the present study, optimum lupeol was observed in shoots treated with 25 mg/l and increased concentrations failed to enhance lupeol synthesis. This is in accordance with earlier findings for ginsenoside production in *Panax quinquefolium* (Kochan et al., 2017) in which lower YE concentration has been proved to be beneficial. Similarly optimum synthesis of plumbagin and rosmarinic acid in *Drosera burmanii* (Putalun et al., 2010) and *Melissa officinalis* (Nasiri-Bezenjani et al., 2014) respectively is reported at lower levels of YE. The elicitation of lupeol might be due to increased expression of genes related to terpenoid biosynthesis as documented by Rahimi et al. (2014) in which the accumulation of ginsenoside in *P. ginseng* is increased due to up-regulation of farnesyl diphosphate synthase (*FPS*) and isopentenyl pyrophosphate isomerase (*IPPI*) genes. Similarly, Li et al. (2016) depicted that enhancement of terpenoid tanshinone in *S. miltiorrhiza* has been correlated with increased expression of terprnoid metabolism genes viz. 3-hydroxy-3-methylglutaryl-CoA reductase

(*HMGR*), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*), isopentenyl diphosphate isomerise (*IPPI*) and geranylgeranyl diphosphate synthase (*GGPPS*) after YE treatment. In line with these results, addition of YE enhanced production of terpenoids by increasing the expression of genes related to terpenoid biosynthesis is well documented in *Scutellaria baicalensis* (Yoon et al., 2000), *Medicago truncatula* (Suzuki et al., 2002) and *S. miltiorrhiza* (Zhao et al. 2010).

Nevertheless the exact mechanism of secondary metabolites enhancement is unclear as it is diverse in different plant groups, but it could be attributed due to peptide and polysaccharide moieties of YE which stimulate metabolite synthesis (Boller, 1995; Menke et al., 1999). Another hypothesis depicted that metal ions like Zn, Ca and Co act as abiotic stress to plants and thus in turn increased production can be achieved (Suzuki et al., 1985). Blume et al. (2000) reported that it activates different signalling pathways which involve G-proteins, protein kinases and ion channels. They further transmit signals for reactive oxygen species (ROS) production and jasmonate pathway which in turn induce secondary metabolite synthesis. Later on Sanchez-Sampedro et al. (2005) also suggested that YE trigger the production of endogenous jasmonic acid/methyl jasmonate which in turn influence the synthesis of secondary metabolites.

4.4.3.2 Effect of Salicylic Acid on Shoot Biomass and Lupeol Content

Shoot cultures of *T. indica* when treated with SA (25-200 µM), the shoot growth was less as compared to control as well as YE treated shoots. The observations also revealed that different SA concentrations and treatment time affected the lupeol content. But in contrary to YE concentrations, the amount of lupeol increased as the treatment time increased in all SA concentrations.

- **25 µM**

When 25 µM of SA was added in the medium shoot growth gradually increased and at four weeks the FW was 0.08 ± 0.00 gm and DW was 0.016 ± 0.00 gm and it increased to 0.502 ± 0.01 gm (FW) and 0.09 ± 0.01 gm (DW) in six weeks (Fig. 70). But the biomass recorded was less for all weeks compared to control medium. Lupeol production was calculated after densitomatric scanning at 525 nm. Yield (176.70 ± 6.24 µg/gm) was recorded in four weeks and it increased to 365.87 ± 9.10 µg/gm in five weeks but content of both weeks were less compared to control and 25 mg/l of YE. In six weeks its level reached to

584.26 ± 8.14 µg/gm (Fig. 71a-f; Table 36) which was recorded as optimum yield among all the concentrations of SA as well as YE, and it was 1.28 fold higher than control for the same weekso.

- **50 µM**

Medium added with 50 µM of SA to treat the shoots, biomass was enhanced i.e. FW (0.281 ± 0.02 gm) and DW (0.056 ± 0.00 gm) in four weeks as compared to previous concentration. In five weeks biomass was reduced [FW (0.227 ± 0.01 gm) and DW (0.058 ± 0.00 gm)] and again enhanced [FW (0.293 ± 0.04 gm) and DW (0.077 ± 0.01 gm)] when shoots were harvested at six weeks (Fig. 70). The yield of lupeol was higher (246.37 ± 6.51 µg/gm) in four weeks compared to above treatment level. Further it slightly enhanced to 305.24 ± 7.18 µg/gm with the prolonged time (five weeks). Six weeks helped in increasing lupeol production (465.55 ± 9.55 µg/gm) which was nearer to control but lower than recorded in 25 µM (Fig. 71a-f; Table 36).

- **100 µM**

Similarly when SA level was increased to 100 µM the biomass level gradually increased from four to six weeks. Shoots when harvested at four weeks had FW (0.224 ± 0.01 gm) and DW (0.048 ± 0.00 gm) respectively which slightly enhanced in fifth week i.e. FW (0.249 ± 0.02 gm) and DW (0.050 ± 0.00 gm). The FW (0.355 ± 0.05 gm) and DW (0.067 ± 0.01 gm) was recorded for six weeks was lower than control and 25 µM of SA (Fig. 70). Slight variation was observed in content of lupeol at four and five weeks i.e. 221.43 ± 2.12 µg/gm and 244.84 ± 8.19 µg/gm. Yield increased to 465.55 ± 9.55 µg/gm in six weeks of SA treatment (Fig. 71a-f; Table 36) which was similar with above SA level for the same weeks.

- **200 µM**

The highest level of SA when added to the medium, significantly enhanced the growth of shoots compared to other concentrations of SA. The growth observed after four weeks was almost similar with above all levels i.e. FW (0.238 ± 0.010 gm) and DW (0.059 ± 0.00 gm) but it increased in five weeks i.e. FW (0.359 ± 0.02 gm) and DW (0.088 ± 0.00 gm). Maximum biomass i.e. FW 0.527 ± 0.05 gm and DW 0.112 ± 0.01 gm was recorded in six weeks among all SA concentrations tried (Fig. 70). In this concentration, lupeol production was less as compared to all weeks of control i.e. 236.66 ± 4.29 µg/gm, 380.86 ± 9.83 µg/gm

and 442.65 ± 9.84 µg/gm were noted for four, five and six weeks respectively (Fig. 71a-f; Table 36).

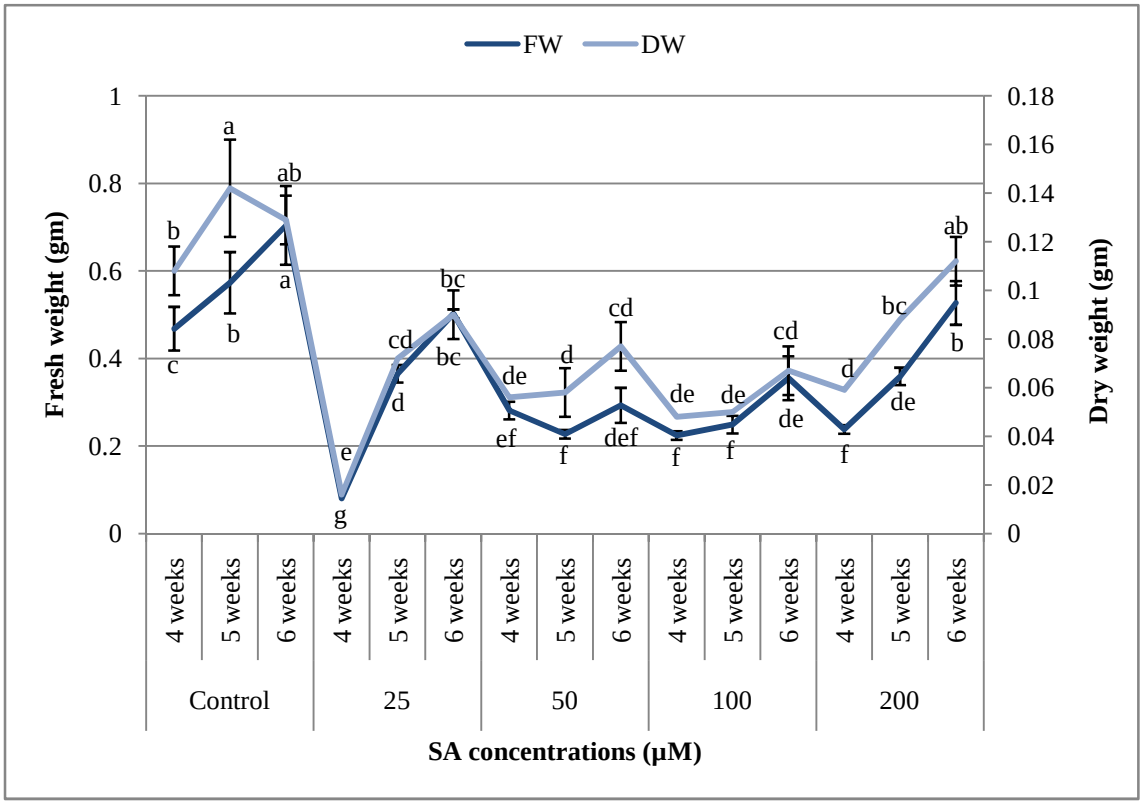

Figure 70. Effect of salicylic acid on shoot biomass derived through nodal explants of *T. indica*.

Each line shows the mean values (n = 5) and error bar as standard error. Means followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Table 36. Quantification of lupeol in presence of SA at different time intervals.

SA concentrations (µM)	Lupeol (µg/gm)*		
	4 weeks	5 weeks	6 weeks
Control	254.16 ± 6.90 de	446.13 ± 8.69 bc	457.08 ± 2.79 b
25	176.70 ± 6.24 f	365.87 ± 9.10 c	584.26 ± 8.14 a
50	246.37 ± 6.51 de	305.24 ± 7.18 d	465.55 ± 9.55 b
100	221.43 ± 2.12 ef	244.84 ± 8.19 de	465.55 ± 9.55 b
200	236.66 ± 4.29 def	380.86 ± 9.83 c	442.65 ± 9.84 bc

*Values represent mean ± SE. Means (n = 3) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test.

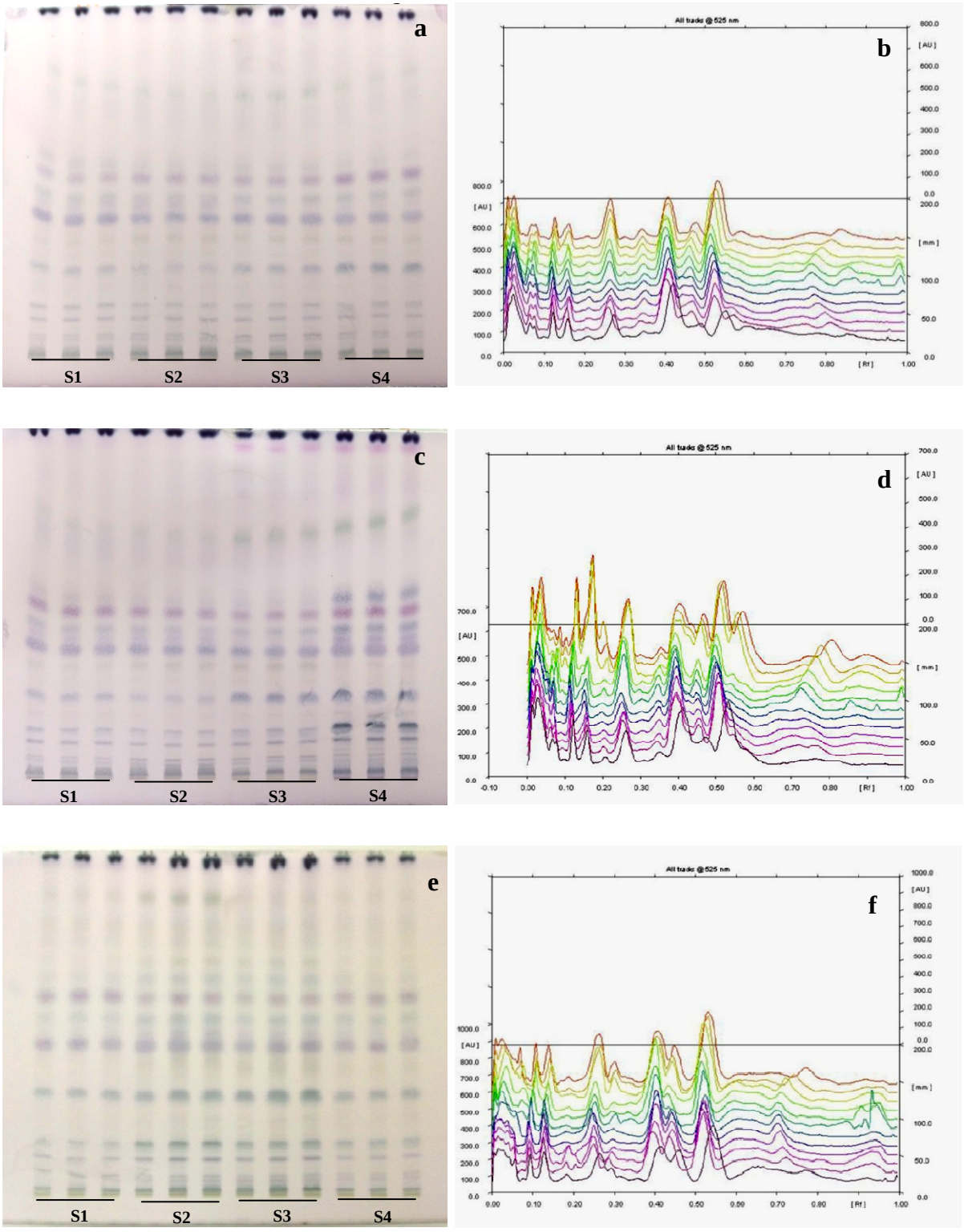

Figure 71. TLC plates and respective chromatograms for hexane extract of shoot samples after SA treatment: (a, b) Four weeks, (c, d) five weeks and (e, f) six weeks. S1- 25 µM, S2- 50 µM, S3- 100 µM and S4- 200 µM. Scanning of plates was done at 525 nm.

- It was observed that, six weeks cultures treated with YE (200 mg/l) showed maximum biomass in all concentrations of YE and SA as well as control. Optimum yield of lupeol was recorded in six weeks of SA (25 µM) which was 1.28 fold higher than control. Additionally, 25 mg/l of YE at four week showed 1.65 fold higher content of lupeol than control for the same weeks. Therefore, these two concentrations of SA and YE proved to be beneficial for lupeol production in *T. indica*.

Addition of SA at different concentrations also increased the biomass of *T. indica* shoots in comparison to control as it is known to have beneficial effect on the growth of plants (Vicente and Plasencia, 2011). However, maximum biomass was achieved at higher concentrations of SA i.e. 200 µM which is in contradiction to earlier reports in *Andrographis paniculata* and *Matricaria chamomilla* shoot cultures (Kovácik et al., 2009; Zaheer and Giri, 2015) where lower concentration is proved to be better. Recently, Sliwinska et al. (2021) also documented that lower concentration of SA is beneficial for *Polyscias filicifolia* shoots. Similarly positive effect of SA on plant growth under *in vitro* conditions is well reported in many previous studies (Luo et al., 2001; Quiroz-Figueroa et al., 2001; Hao et al., 2006). Upon binding with plant receptors, SA is also known to induce systemic acquired resistance (SAR) synthesize pathogenesis related (PR) proteins and hypersensitive responses which subsequently activates defence mechanism (Durrant and Dong, 2004; Chen et al., 2006; Horvath et al., 2007).

Previous reports suggested that application of SA enhanced different metabolites e.g. caffeic acid and rosmarinic acid in *Thymus membranaceus* (Perez-Tortosa et al., 2012), hypericin and pseudohypericin in *Hypericum perforatum* (Gadzovska et al., 2013), withanolides in *Withania somnifera* (Sivanandhan et al., 2013), phenol, flavanols, proanthocyanidins, lignin, gallic acid and hydroxycinnamic acid derivatives in *Cistus heterophyllus* (Lopez-Orenes et al., 2013), carotenoid in *Ruta angustifolia* (Othman et al., 2015), essential oils and total phenolics in *Achillea millefolium* (Gorni and Pacheco, 2016), psoralen in *Psoralea corylifolia* (Hari et al., 2018), phenolic compounds in *Ajuga integrifolia* (Abbasi et al., 2020), reserpine and ajmalicine in *Rauvolfia serpentina* (Dey et al., 2020) and amarogentin, swertiamarin and mangiferin in *Swertia paniculata* (Kaur et al., 2020). Thus in the present study, effect of different concentrations of SA on lupeol biosynthesis was also evaluated. Results revealed that similar to YE elicitation, different concentrations and time intervals of SA treatment affected the lupeol quantity. This is in corroboration with previous

report in lupeol elicitation using SA in *Cryptostegia grandiflora* (Singh et al., 2011). Increased lupeol synthesis after elicitation with SA is reported to be due to its positive effect on terpenoid biosynthesis in many plants (Gorelick and Bernstein, 2014). This could be due to over-expression of branch point genes of terpenoid biosynthesis pathway i.e. 3-hydroxy-3-methylglutaryl-CoA synthase (*HMGS*) and HMG CoA reductase (*HMGR*) as reported in *Brassica juncea* (Alex et al., 2000). Whereas Hayashi et al. (2004) depicted that SA treatment accumulated precursors of terpenoids biosynthesis in *Glycyrrhiza glabra*. In line with present study, enhanced terpenoid biosynthesis has been achieved using SA as an elicitor in *Taxus chinensis* (Wang et al., 2004), *Panax ginseng* (Ali et al., 2006), *T. baccata* (Khosroushahi et al., 2006) and *T. chinensis* var. *Mairei* (Fan et al., 2006).

The results also revealed that maximum lupeol production was achieved at 25 μM concentration and further increase in its level adversely affected the synthesis. This is in accordance with report of *Matricaria chamomilla* in which higher SA level adversely affected phenolic acids synthesis (Kovacik et al., 2009). In *Ginkgo biloba* the accumulation of ginkgolides A and B was higher as compared to control only when SA was applied at lower concentrations (Kang et al., 2009). In line with present study, lower concentrations of SA are beneficial for enhanced production of secondary metabolites in *Orostachys cartilaginous* (Wen et al., 2019) and *Anethum graveolens* (Bulchandani and Shekhawat, 2020). Similarly, maximum production of different metabolites in shoot cultures have been documented at lower levels of SA such as hypericin and pseudohypericin in *H. hirsutum* (Coste et al., 2011), rosmarinic acid in *Solenostemon scutellarioides* (Sahu et al., 2013), digitoxin in *Digitalis purpurea* (Patil et al., 2013) and bacoside A in *Bacopa monnieri* (Largia et al., 2015; Sharma et al., 2015). Whereas recently Nazir et al. (2021) observed that higher concentration of SA (200 μM) showed highest diosgenin production in shoot cultures of *Dioscorea deltoidea*. It is believed that SA interferes the activity of antioxidant enzymes and inhibit catalase enzyme, which in turn increase secondary metabolites production (Askari and Ehsanzadeh, 2015).

The elicitation experiment confirmed that both YE and SA significantly increased lupeol accumulation but elicitor type, its concentration and duration of treatment affected the content, which is in accordance with earlier reports in *Salvia miltiorrhiza* (Zhao et al., 2010) and *Corylus avellana* (Salehi et al., 2019). In the present observation SA was better in terms of lupeol synthesis as compared to YE. Similarly, Singh et al. (2019) also documented that both YE and SA increased synthesis of uscharidin, uzarigenin, calotropin and uscharin but the later proved to be better. In line with this, beneficial role of SA over YE in enhancement of

secondary metabolites is well documented in medicinal plants like *Digitalis purpurea* (Patil et al., 2013), *Swertia chirata* (Kumar et al., 2013), *Caesalpinia pulcherrima* (Aswathy et al., 2017) and *Trifolium pratense* (Reis et al., 2019).

- A part of the last objective was gene expression analysis using molecular technique for elicited samples. The content of both targated metabolites increased after elicitation which can be interpreted that the elicitor(s) up-regulated the genes of respective pathways. Thus carrying out gene expression analysis was an additional study to confirm the same. This study required the gene sequences for metabolic pathways of the selected plants, and after a thorough search it was known that this was not available in KEGG or NCBI database. In the mean time due to the pandemic out break the cultures were lost and this further experimental work which was planned in collaboration failed to materialize. Therefore this study was omitted from present research.