CHAPTER 4

RESULTS AND DISCUSSION

The present study is mainly divided into two parts: regeneration and secondary metabolite studies. First objective was to optimize the medium for shoot regeneration utilizing leaf and nodal explants of *Hemidesmus indicus*. The second objective was to carryout secondary metabolite studies using shoots derived from nodal explants. First they were analyzed qualitatively by HPTLC fingerprinting. After that quantification of lupeol and rutin from these shoot cultures was done followed by elicitation studies for the same using three different elicitors. These shoot cultures were treated with elicitors and gene expression analysis for rutin biosynthetic pathway was studied.

4.1 REGENERATION STUDIES

Leaf and node are the two commonly used explants for establishing shoot cultures which were selected for the present regeneration studies and MS media fortified with different combinations and concentrations of PGRs were tried. Organogenesis means formation of shoots or roots from the explants under *in vitro* conditions (Thorpe, 1980). The *in vitro* organogenesis occurs via three steps: (i) the explants acquire competence for organogenesis, (ii) quiescent cells re-enters cell cycle and cell fate is determined to form particular organ primordia and (iii) formation of *in vitro* organs (Christianson and Warnick, 1983, 1984, 1985; Sugiyama, 1999; Zhang and Lemaux, 2004). The results of the regeneration studies are summarized below.

4.1.1 Shoot Regeneration from Leaf Explant

Basal MS medium fortified with sucrose (3%) was maintained as a control for the study and after leaf explant were placed on the medium, swelling was observed during second week, and in the third week callus was induced from the cut end. After four weeks the entire explant with callus was subcultured on fresh medium, it slightly proliferated but it turned brown at the end of sixth week and failed to show further growth till eight weeks. Only 25% cultures induced off-white compact callus in medium without PGRs (Fig. 7). Thus MS basal medium was then fortified with different combinations and concentrations of PGRs to achieve organogenesis from leaf explant.

4.1.1.1 Individual cytokinins (BA and Kn)

Initially two commonly used cytokinins viz. BA and Kn were tried individually at different concentrations. When the explants were placed on medium fortified with BA,

swelling was observed during first week and callus formation started from the lower cut ends within the second week. This callus slightly proliferated along the cut ends and during third and fourth week it started to cover the lower surface. After transfer to fresh medium growth slightly enhanced but ceased in sixth week. In presence of lower concentration of BA (5 µM) a small mass of green compact callus was differentiated in 33.33% cultures. Maximum callus proliferation with 100% response was observed when BA concentration increased to 10 µM. A combination of greenish white compact callus was observed at cut margins whereas brown compact callus formed on the entire lower lamina (Fig. 8a). Further increase in BA concentration to 15 and 20 µM, the response declined to 83.33% and in these combinations the proliferation of callus was comparatively less. Further increasing the BA concentration (25 and 30 µM) failed to evoke a better response and 66.67% and 33.33% response was observed in 25 and 30 µM concentrations respectively (Fig. 7). Hence in all the concentrations of BA, compact callus was formed till eight weeks and in the next experiment Kn was added into the medium instead of BA.

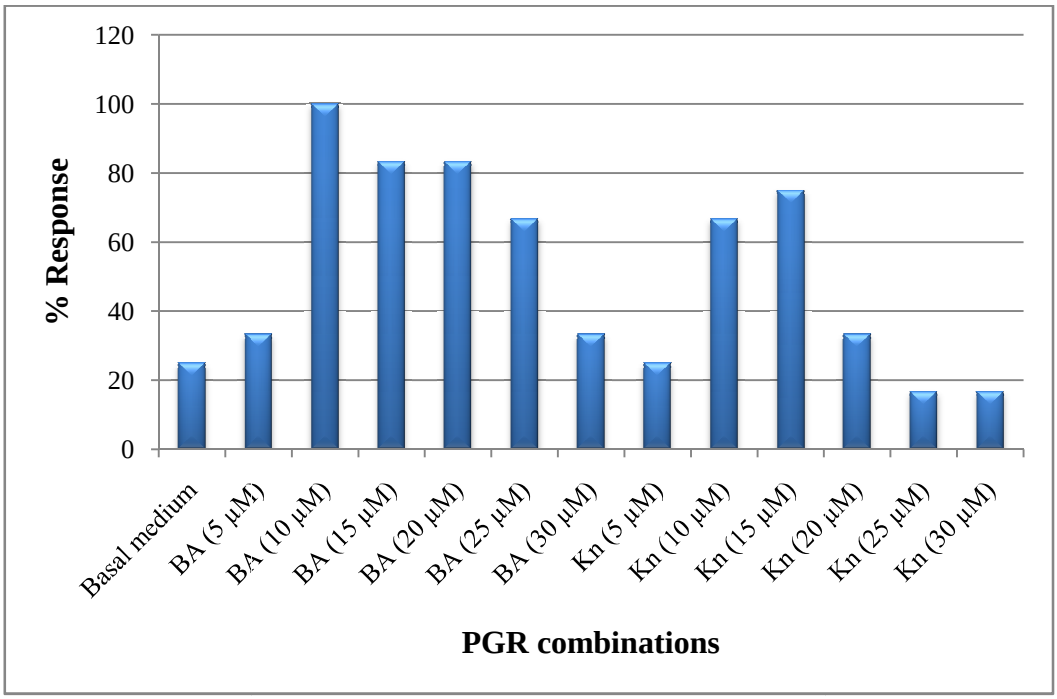

Figure 7: Effect of BA and Kn on callus induction from leaf explant of *H. indicus* after eight weeks.

When Kn was added to the medium, the morphogenic response was comparatively less as compared to BA, and at all concentrations only green compact callus differentiated. Lower concentration of Kn (5 µM) only 25% cultures responded and it increased to 66.67% when 10 µM Kn was added in the medium. Further increase in Kn concentration to

15 µM enhanced the response to 75% and better proliferation of green compact callus was observed (Fig. 8b). Kn at 20 µM concentration adversely affected the callus formation and only 33.33% cultures induced callus which failed to proliferate. The response further decreased to 16.67% with 25 and 30 µM Kn concentrations (Fig. 7).

Observations revealed that leaf explants showed a better response in presence of individual BA in terms of callus formation as compared to Kn. However both the cytokinins induced callus but failed to form shoots from leaf explant. Presence of different auxins into the medium was also evaluated for callus induction.

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

Auxins like IAA, NAA and 2,4-D were added individually into the MS medium in the range from 0.1-2 µM, and their effect on leaf explant was studied till eight weeks. When the medium was supplemented with IAA, curling and swelling of the explants was observed within a week. Callus differentiated from the upper and lower cut ends of the explant during second week which proliferated towards the periphery in third week. This callus started to cover the lower lamina in fourth week which continued after subculturing during fifth week. Growth of the callus was fast at all the IAA concentrations till eight weeks in 100% cultures. However a variation in the callus type was observed and in 0.1 µM IAA concentration, green compact callus was observed whereas at 0.5 µM, green compact along with brown friable callus was observed. The proliferation of callus was less in both these concentrations which increased at 1 and 2 µM concentration, where a mixture of green compact and brown friable callus was formed (Fig. 9, 10a).

Another auxin NAA when used as a PGR in the medium, profuse callus proliferation was observed as compared to IAA and 100% cultures formed off-white friable callus in all the concentrations (Fig. 9). However the proliferation of callus was less in lower NAA concentrations (0.1 and 0.5 µM) which increased as NAA increased to 1 and 2 µM (Fig. 10b). Incorporation of 2,4-D in the medium induced maximum callusing with 100% response (Fig. 9, 10c). Off-white friable callus was formed in all the combinations and the proliferation increased as the concentration of 2,4-D increased.

Thus all the three auxins formed only callus from leaf explants within eight weeks. This callus varied in texture and colour but none of the concentrations induced morphogenic callus which could differentiate shoot buds. Therefore in the next set experiment, BA and Kn were combined and their effects were recorded.

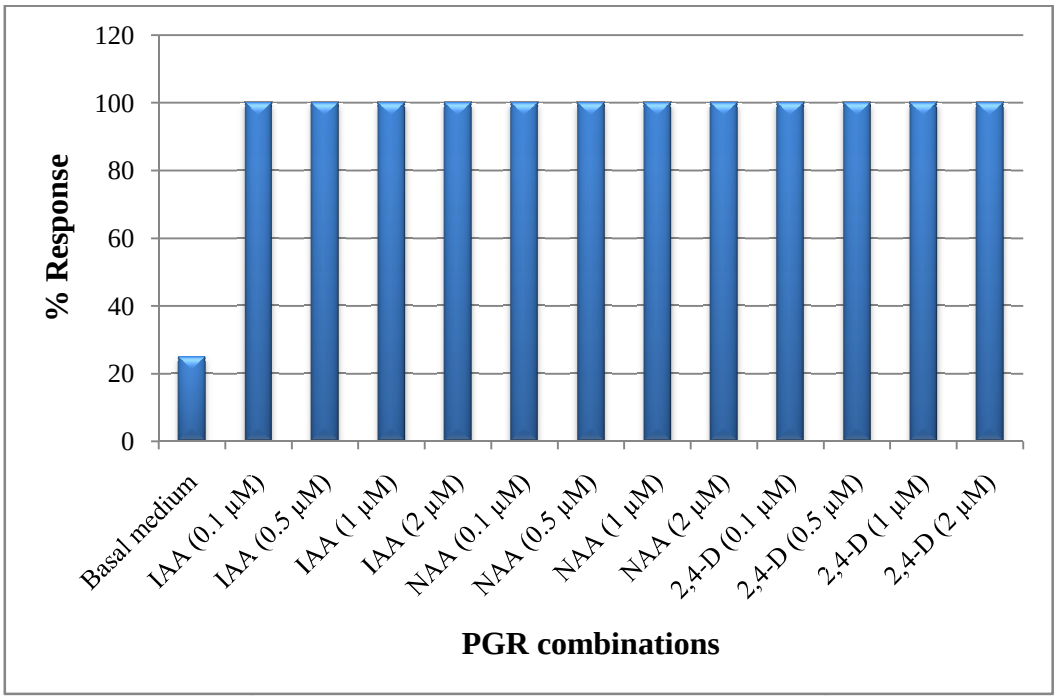

Figure 9: Effect of IAA, NAA and 2,4-D on callus induction from leaf explant of *H. indicus* after eight weeks.

4.1.1.3 Synergism of cytokinins (BA with Kn)

The two cytokinins, BA and Kn when added together from a range of 5-30 µM, but changes were only observed in second week, as leaf started to swell. The explants remained as it is during third week and the callus induction was observed at upper and lower cut ends of the leaf lamina in fourth week. These explants were subcultured on fresh medium at the end of four weeks but the callus failed to proliferate further. The % response for differentcombinations varied (Fig. 11) and in all the combination green compact callus was observed. A maximum of 66.67% cultures responded in medium supplemented with BA (10 µM) and Kn (5 µM) in which green compact callus was observed from cut ends which progressed to upper lamina (Fig. 10d). Medium fortified with BA (10 µM) with Kn (20 µM) and BA (20 µM) with Kn (20 µM) were least responsive and only 16.67% culture responsed. Higher concentrations of both BA and Kn (25 and 30 µM) failed to induce any response and the explants turned brown after three weeks without callus formation.

It was concluded that both the cytokinins individually evoked better response for callus formation, but when the added together in the medium it adversely affected the callus formation and failed toinduce shoots. Therefore BA/Kn were combined with IAA/NAA and their effect on leaf explant was evaluated.

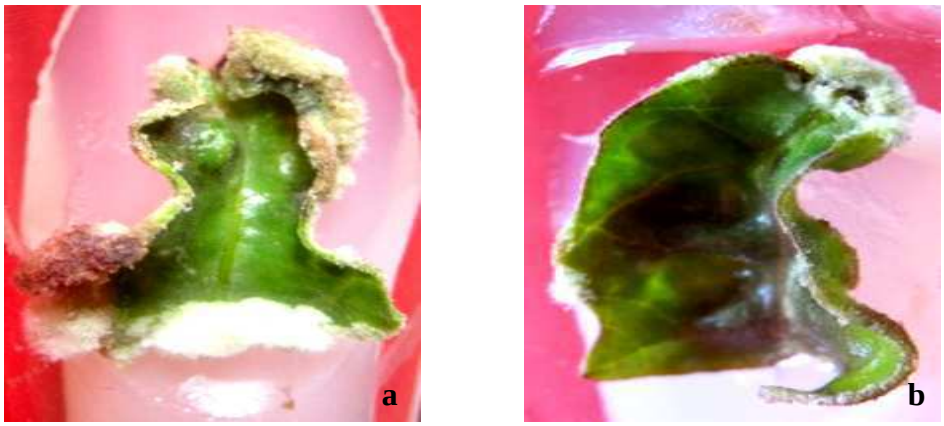

Figure 8: Effect of individual cytokinins on leaf explant after eight weeks- (a) greenish white and brown compact callus differentiated from upper and lower surface in presence of BA (10 µM) and (b) formation of green compact callus in Kn (15 µM).

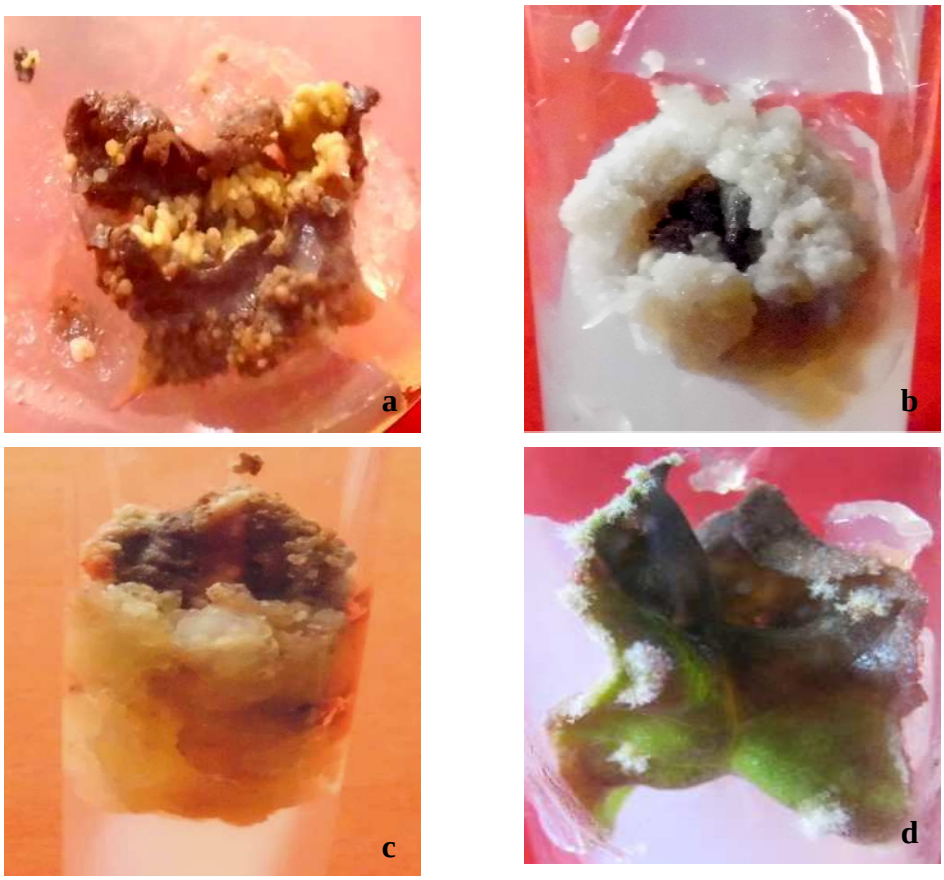

Figure 10: Effect of individual auxins and combinations of cytokinins on leaf explant after eight weeks- (a) a mixture of green compact and brown friable callus in IAA (2 µM), (b) off-white friable callus in NAA (2 µM), (c) profuse off-white friable callus in 2,4-D (2 µM) fortified medium and (d) green compact callus in BA (10 µM) with Kn (5 µM).

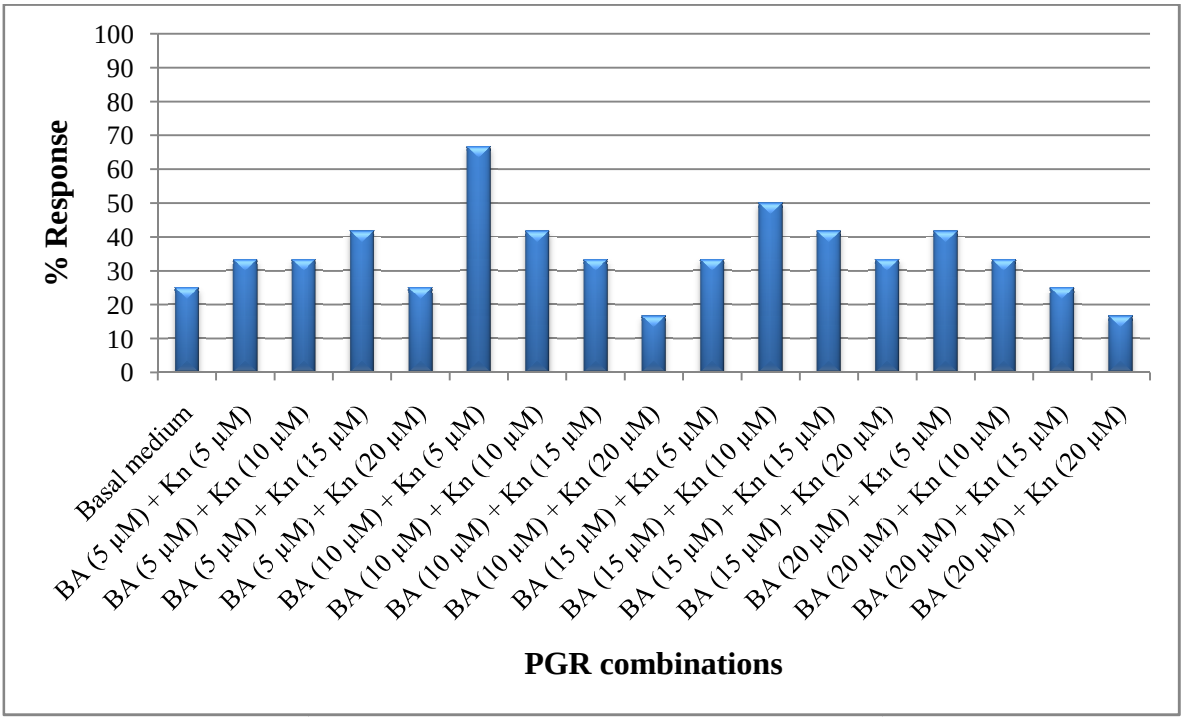

Figure 11: Effect of BA and Kn on callus induction from leaf explant of *H. indicus* after eight weeks.

4.1.1.4 Synergism of BA with IAA/NAA

Leaf explant when placed on medium fortified with BA (5 µM) and IAA (0.1-2 µM) formed green compact callus. The proliferation of callus was less in medium having lower concentrations of IAA (0.1-1 µM) and it increased at 2 µM IAA. Similar response was observed in medium with 10 µM BA with lower concentrations of IAA (0.1 and 0.5 µM) which induced less callus whereas the proliferation increased at 1 and 2 µM IAA. Further increasing the level of BA to 15 µM induced callus which proliferated at a faster rate at all IAA concentrations (0.1-2 µM) but they remained as it is till eight weeks without induction of shoots.

Increase in BA level to 20 µM and combining it with all IAA concentrations (0.1-2 µM) differentiated shoots indirectly. Amongst these combinations, medium fortified IAA (1 µM) evoked optimum regeneration in which the explants curled, swelled and formed callus from cut ends within a week which proliferated, turned nodular and covered the lower midrib of the leaf in the second week (Fig. 12a). In the next week, shoot bud differentiated from the lower surface of the explant which elongated and formed shoots during fourth week (Fig. 12b). This morphogenic callus when subcultured after four weeks further proliferated and simultaneously differentiated shoot buds. These buds elongated

into shoots which branched during sixth week (Fig. 12c) and this continued till eight weeks and healthy shoots were observed at the end of eight weeks (Fig. 12d). At the end of eight weeks, optimum number of 19.67±0.81 shoots with 100% response was observed in this medium. Medium fortified with BA (20 µM) and IAA (0.1 µM) induced 12.64±1.82 shoots with 83.33% response which increased to 17.00±0.62 (100% response) when IAA concentration was increased to 0.5 µM. However highest IAA concentration (2 µM) failed to increase the shoot number and 13.18±1.29 shoots (91.67% response) were formed (Fig. 13).

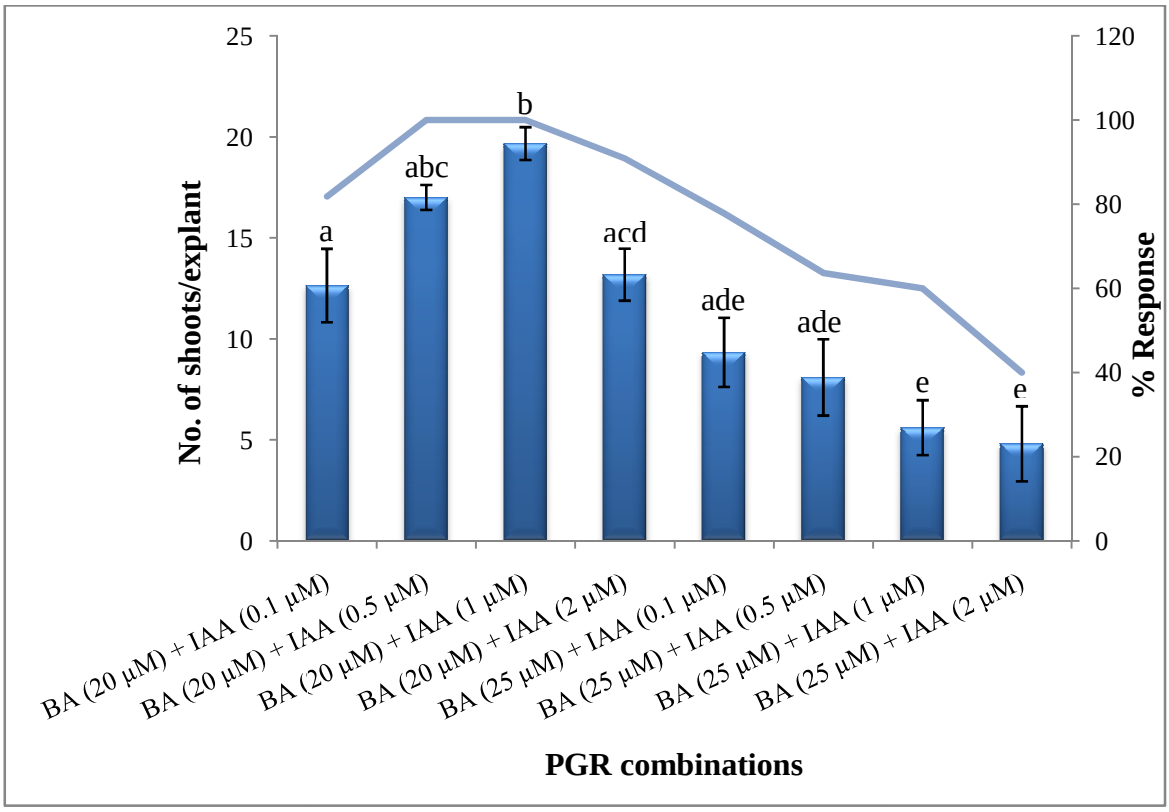

Figure 13: Shoot regeneration from leaf explant of *H. indicus* in different concentrations of BA (20 and 25 µM) with IAA (0.1-2 µM) after eight weeks.
Each bar shows the mean values (n= 12), error bar as standard error and the line represents the % response. Bars having same letters are not significantly different according to Tukey's HSD test ($p \leq 0.05$).

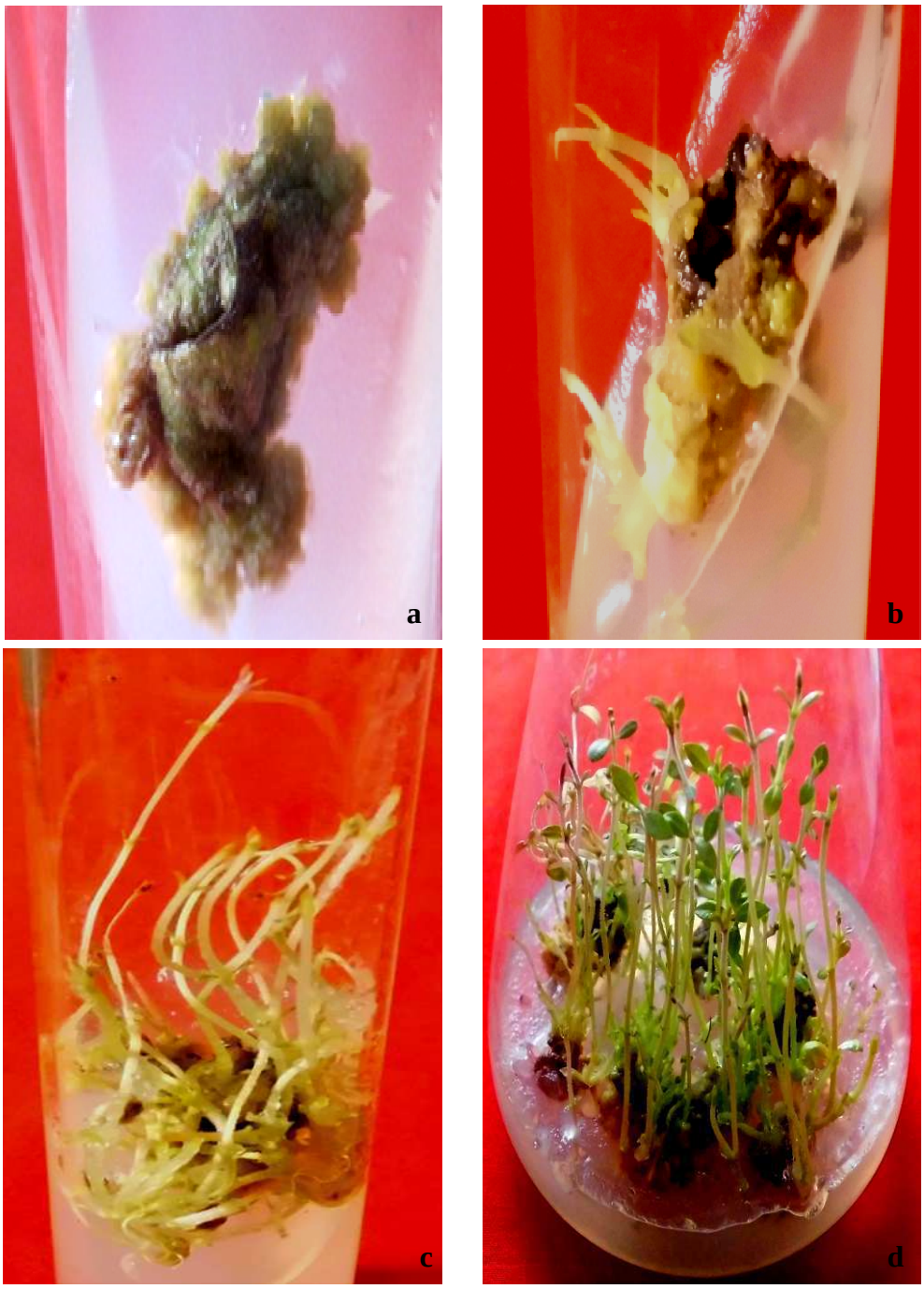

Figure 12: Indirect shoot regeneration from leaf explant in MS medium fortified with sucrose (3%), BA (20 µM) and IAA (1 µM)- (a) induction of greenish brown nodular callus from lower surface after two weeks, (b) elongation of shoot buds in fourth week, (c) further proliferation and elongation of shoots during sixth week and (d) healthy elongated shoots at the end of eight weeks.

Further increasing the BA concentration to 25 µM also induced greenish brown nodular callus along with formation of shoot buds. But these buds failed to elongate into shoots upon subculturing on the same medium and hence rest of the concentrations of BA (25 µM) and IAA (0.1 µM) were transferred to medium fortified with BA (20 µM) and IAA (1 µM) for shoot formation. When callus from medium BA (25 µM) and IAA (0.1 µM) was transferred to BA (20 µM) and IAA (1 µM), it regenerated total 9.33±1.71 shoots (75% response) at the end of eight weeks. The shoots number was decreased to 8.09±1.89 (66.67% response) when callus from medium containing BA (25 µM) and IAA (0.5 µM) was transferred. Further decrease in shoot number was observed (5.60±1.36 and 4.80±1.86) when callus from media supplemented with higher IAA concentrations i.e. 1 and 2 µM were transferred (Fig. 13).

Increasing BA level to 30 µM formed callusing in 100% cultures, but the morphology of callus varied and it was green compact and brown nodular callus in medium having 0.1-1 µM IAA concentrations, whereas in medium with IAA (2 µM) only green compact callus was observed. These calluses proliferated less as compared to earlier BA concentrations (20 and 25 µM). In all combinations shoot buds were formed at the end of four weeks but they failed to elongate into shoots upon subculturing and the explants as well as callus turned brown at the end of eight weeks.

The effect of BA with another auxin NAA in the same concentration range was also evaluated for leaf explant. The explants swelled after first week and remained as it is during second week in presence of 5 µM BA at all NAA concentrations (0.1-2 µM). The callus formation was observed in third week in combinations with lower levels of BA but increasing BA concentrations to 10-20 µM formed callus in second week. However in all the combinations a green compact callus with 100% response was observed, callus proliferation increased as the concentration of NAA increased. Whereas higher concentrations of both BA (25 and 30 µM) failed to induce any response and maximum callus proliferation was observed in medium having BA (5 µM) and NAA (2 µM) in which white compact callus was formed (Fig. 14a, 15).

All the combinations of BA and NAA induced compact callus without formation of nodular callus and shoot buds. Thus another cytokinin Kn was combined with IAA and NAA at same concentrations and their effect was observed.

4.1.1.5 Synergism Kn with IAA/NAA

Combining Kn (5-30 µM) and IAA (0.1-2 µM) induced early response as compared to combinations of BA with IAA and 100% response was observed for all the combinations (Fig. 16). Generally green and white compact callus was formed but at higher concentration of Kn (15-30 µM) brown friable and nodular callus was also observed in some combinations (Fig. 14b). Amongst all the combinations, medium fortified with Kn (20 µM) + IAA (1 µM), Kn (25 µM) + IAA (0.5-2 µM) and Kn (30 µM) + IAA (1-2 µM) induced shoot buds after six weeks; but they failed to elongate further and callus turned brown upon subcultured on same medium or on optimized medium for shoot regeneration i.e. BA (20 µM) and IAA (1 µM). The combinations of Kn with IAA could induced nodular callus and shoot buds in but they failed to elongate into shoots.

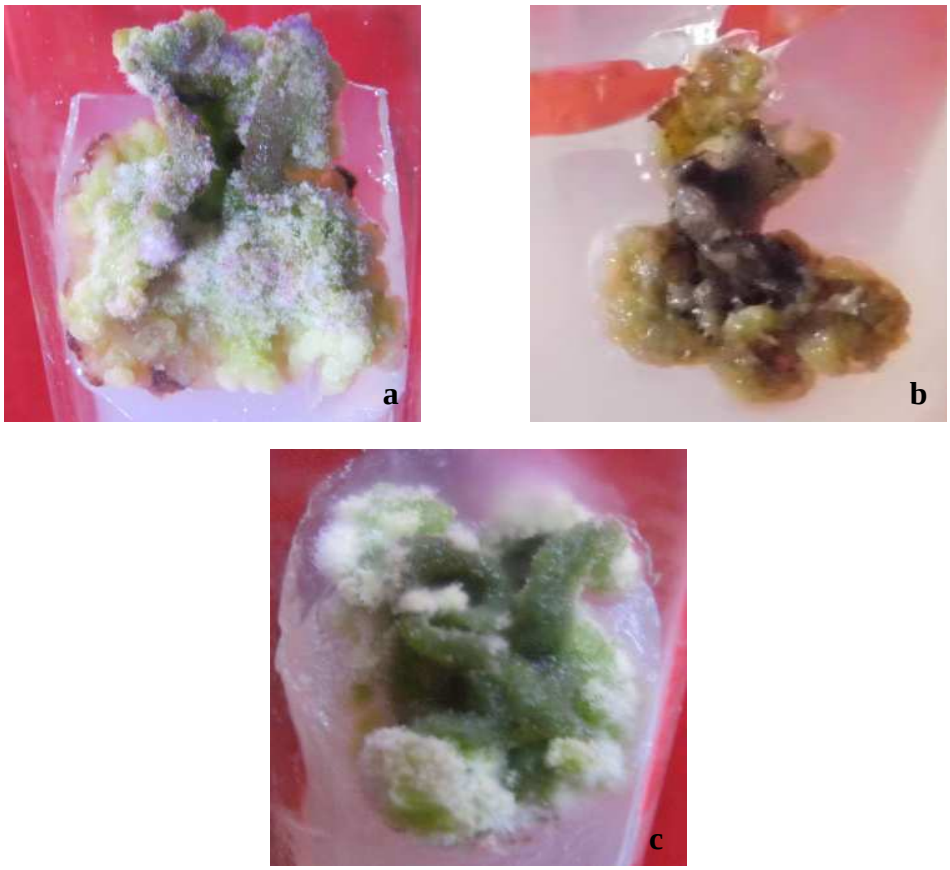

Figure 14: Synergistic effect of cytokinins with auxins on leaf explant after eight weeks- (a) greenish white compact callus in BA (5 µM) with NAA (2 µM), (b) greenish compact and brown nodular callus in Kn (20 µM) with IAA (1 µM) and (c) greenish white compact callus in Kn (5 µM) with NAA (2 µM) fortified medium.

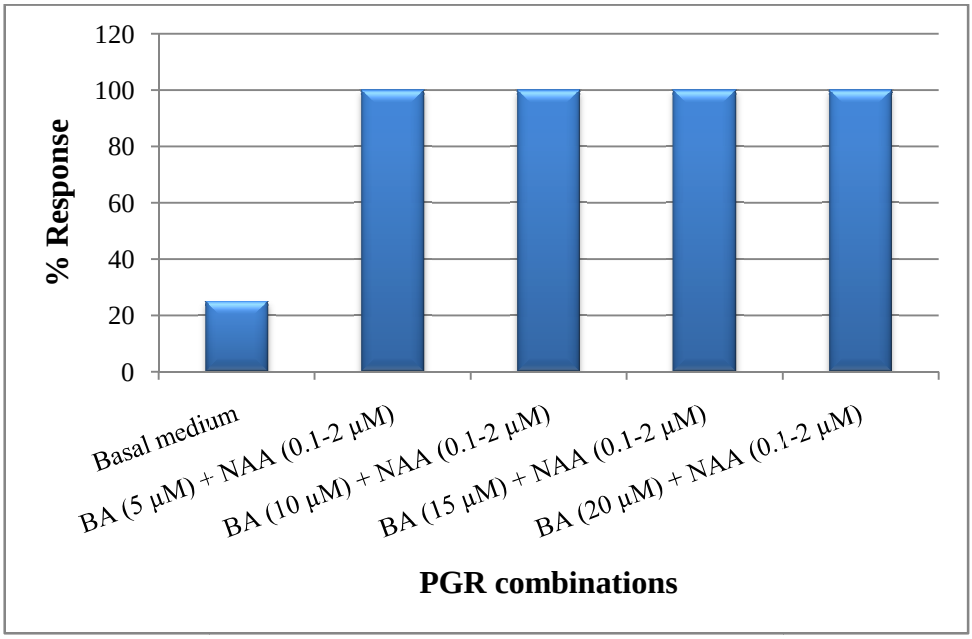

Figure 15: Effect of BA and NAA on callus induction from leaf explant of *H. indicus* after eight weeks.

After IAA, NAA was combined with Kn and observations revealed that it was less responsive as compared to earlier combinations. The medium fortified with Kn 10 µM with differentiated greenish brown nodular callus but it also failed to regenerate shoot buds. In the combinations of Kn (5, 15 and 20 µM) with all NAA concentrations, green and white compact callus was formed and maximum callus proliferation (100% response) was observed in Kn (5 µM) with NAA (2 µM) in 100% cultures (Fig. 14c). When Kn concentration was further increased to 25 and 30 µM, the callus morphology changed to green compact whereas higher Kn concentration (30 µM) with NAA (0.1 and 2 µM) adversely affected the explants and it turned brown without callus formation (Fig. 17).

Hence it can be concluded that the leaves when inoculated on medium containing combination of Kn with NAA induced only callus and failed to regenerate shoot. Thus BA (20 and 25 µM) with IAA (0.1-2 µM) only combinations of induced indirect shoot regeneration from callus whereas other PGRs were formed only callus of different morphology. BA at higher concentrations in combination with IAA had synergistic effect for indirect shoot regeneration and these healthy shoots were with similar morphology to wild shoots was observed.

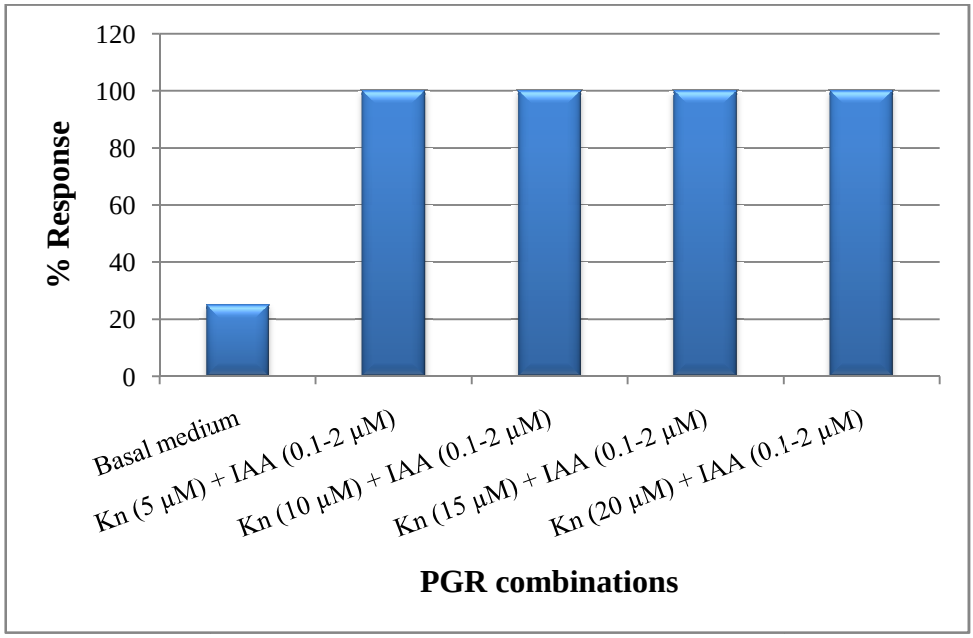

Figure 16: Effect of Kn and IAA on callus induction from leaf explant of *H. indicus* after eight weeks.

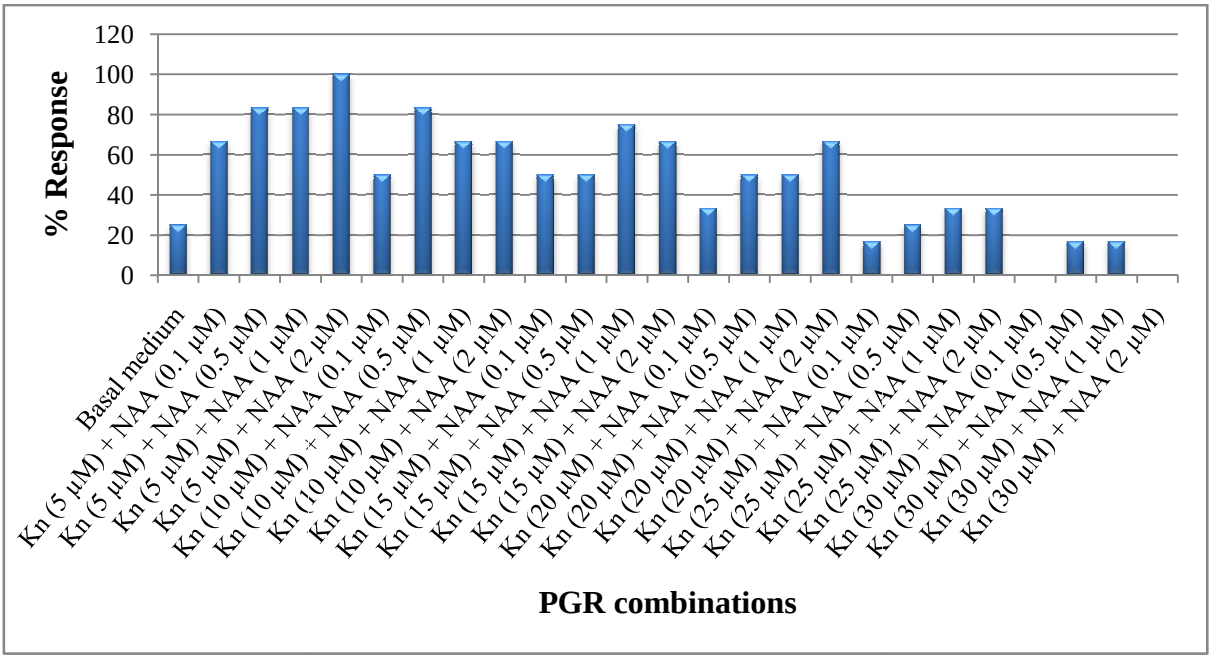

Figure 17: Effect of Kn and NAA on callus induction from leaf explant of *H. indicus* after eight weeks.

The square piece of leaf lamina containing midrib was used as an explant as the efficient absorption of nutrients and growth regulators is better through cut edges of leaves (Sarwar and Skirvin, 1997). Only 25% cultures formed callus on basal medium which failed to differentiate shoots. Similarly leaves of *Plumbago rosea* and *P. zeylanica* failed to

induce shoots from callus on basal MS medium (Das and Rout, 2002). Incorporating BA in the medium formed green and brown compact callus till eight weeks, and similar results are documented for the same plant by Shanmugapriya and Shivakumar (2011). But they have reported greenish friable callus which is contrary to the present findings. In leaves of *Biophytum sensitivum* and *Stevia rebaudiana* also, only green compact callus was induced but it failed to induce shoots (Janarthanam et al., 2009; Kala et al., 2014). Leaves of *Justicia gendarussa* also formed only callus in BAP fortified medium (Agastian et al., 2006).

When leaf explants were inoculated on Kn (5-30 µM) fortified medium, moderate growth of compact callus was observed. This is in line with report of Patel and Patel (2013) for leaf explant of *Tecomella undulata* in which Kn (1.5-3 mg/l) induced compact callus with moderate proliferation. Observations also revealed that the callus forming capacity of the explant varied as the concentrations of Kn changed in the medium without differentiating shoots, which is also reported for the same plant by Purohit et al. (2015). Similarly leaves of *Feronia limonia* failed to induce shoots in medium supplemented with Kn (Hiregoudar et al., 2003). It was also observed that the callus proliferation as well as response of leaves in Kn fortified medium was less as compared to BA containing medium, and these findings are similar to earlier reports on *Gmelia arborea* and *Biophytum sensitivum* (Ujjwala et al., 2013; Kala et al., 2014). Both BA and Kn failed to induce shoots when tried individually, which is also reported in *Withania somnifera* (Joshi and Padhya, 2010).

Inoculating leaves on IAA containing medium evoked callus formation in 100% cultures. In this callus morphology varied and green compact callus was formed at lower IAA concentration (0.1 µM), whereas brown friable callus was observed at 0.5-2 µM concentration. Shanmugapriya and Shivakumar (2011) also reported friable callus with 70% response in IAA (1 mg/l) for the same plant. Similar to present findings, Ujjwala et al. (2013) have reported formation of compact as well as friable callus from leaf explant of *Gmelina arborea* in medium supplemented with IAA. Whereas Patel and Patel (2013) have reported for leaf explant of *T. undulata* that low concentration of IAA (1 and 1.5 mg/l) failed to differentiate callus and less proliferation was observed at increased IAA concentrations (2-3 mg/l).

Addition of NAA in the medium also formed callus in 100% cultures at all the concentrations. An off-white friable callus was observed in all concentrations but the callus

growth was less in lower concentrations of NAA (0.1 and 0.5 µM) as compared to higher concentrations (1 and 2 µM). Whereas earlier study on *H. indicus* reported dark green compact callus in medium containing NAA (Shanmugapriya and Shivakumar, 2011). Whereas in accordance with the present result, Patel and Patel (2013) also observed formation of friable callus from leaf explant of *T. undulata* in NAA fortified medium and its proliferation increased as the concentration increased. Lubaina and Murugan (2012) observed that leaves of *Plumbago zeylanica* formed only callus but it failed to differentiate shoots in NAA fortified medium. Similarly leaves of *Justicia gendarussa* and *Ophiorrhiza japonica* also formed only callus in medium fortified with NAA (Agastian et al., 2006; Kai et al., 2008). Both IAA and NAA failed to induce shoots, which is also observed in leaf explant of *P. rosea* and *P. zeylanica* (Das and Rout, 2002).

Fortifying MS medium with 2,4-D induced profuse off-white friable callus in all the concentrations. Earlier report on the same plant by Purohit et al. (2015) documented compact callus in medium having 2,4-D. Whereas Mishra (2015) reported whitish light greeni compact callus in *Ocimum sanctum* with 100% response and the result is comparable to the present study. Similarly leaf explant of *Tylophora indica* (Faisal and Anis, 2003), *Spilanthes acmella* (Singh and Chaturvedi, 2012), *Tinospora cordifolia* (Bhalerao et al., 2013) and *Achyranthes aspera* (Sen et al., 2014) also formed only callus when inoculated on 2,4-D supplemented medium.

In present study when the effect of three different auxins on leaf explant was evaluated, 2,4-D fortified medium was better in terms of callus proliferation as compared to IAA and NAA. Similarly in leaf explant of *Rhodiola sachalinensis* 2,4-D was more efficient for callus induction than other PGRs (Wu et al., 2003). The present findings are similar to earlier report by Shanmugapriya and Shivakumar (2011) for *H. indicus* which suggested that 2,4-D is better for callus induction as compared to IAA and NAA.

BA and Kn when used individually or in combination evoked a similar response and only callus was formed. Similarly leaf explants of *Enicostemma hyssopifolium*, *Embelia ribes* and *Stevia rebaudiana* failed to induce organogenesis from leaf explant in medium having BA and Kn (Seetharam et al., 2002; Raghu et al., 2006; Anbazhagan et al., 2010). This may be due to the antagonistic effect of cytokinins which inhibit the synthesis of IAA oxidase isoenzyme and in turn stops the production of endogenous IAA (Lee, 1971).

In *H. indicus* the leaf explants were unable to differentiate shoots in presence of individual cytokinins or auxins in the medium. Thus a combination of cytokinins with auxins was tried as this ratio is reported to influences the type of organ differentiation as well as the regenerative mode of development (Skoog and Miller, 1957; Bhojwani and Razdan, 1996). Earlier studies also suggest that the main function of cytokinin in *in vitro* organogenesis is to control the cell cycle and 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., 1996, 2003). Pierik (1997) states that cytokinins are known to induce growth and development but if added with auxins it facilitates the cell division.

Indirect organogenesis was observed when combinations of BA at 20 and 25 µM with IAA (0.1-2 µM) was used and rest of the combinations induced only callus. Similarly Wadl et al. (2011) also reported that only BA at 2.2 µM and 8.8 µM with 11.4 µM IAA induced regeneration from leaves of *Pityopsis ruthii*. The correct ratio of cytokinin to auxin is a prerequisite as their interaction is known induce shoot development under *in vitro* conditions (Kamat and Rao, 1978; Thorpe et al., 1980). In the present study optimum shoots regenerated from medium supplemented with BA (20 µM) and IAA (1 µM) i.e. high cytokinin to auxin ratio which is generally reported beneficial for differentiation of shoots (Krikorian, 1995). Low auxin level is beneficial for shoot regeneration is due to high endogenous levels of auxins in tissue which inhibit the shoot formation (Christison and Warnick, 1988). This is also reported for medicinal plants like *Cichorium intybus* and *Streptocarpus rexii* (Velayutham et al., 2006; North and Ndakidemi, 2012). Similarly combination of BA and IAA has been reported optimum for shoot regeneration in many other medicinal plants like *Brunfelsia calycina* (Liberman et al., 2010), *Arnica montana* (Petrova et al., 2011), *Spilanthes acmella* (Singh and Chaturvedi, 2012), *Ajuga bracteosa* (Kaul et al., 2013), *Tylophora indica* (Haque and Ghosh, 2013) and *Bacopa monnieri* (Kumari et al., 2015).

Replacing IAA with NAA in combination with BA formed only green compact callus in all the combinations within eight weeks. Observations revealed that proliferation of callus was less in lower concentrations of NAA (0.1-0.5 µM), similarly Kala et al. (2014) reported green compact callus in leaves of *Biophytum sensitivum.* Whereas earlier study on *H. indicus* by Sreekumar et al. (2000) is contrary to our findings in which the combination of BA (2.22 µM) and NAA (1.07 µM) induœd 2.75 shoots. Mandal and

Laxminarayana (2014) also reported only callus formation in *Adhatoda vasica* when placed on medium fortified with combinations of BA and NAA. Kabir et al. (2008) observed callus of different morphology i.e. compact and friable, white and green callus from leaf explant of *Abelmoschus esculentus*. Many studies on plants like *Artemisia vulgaris*, *Cichorium intybus*, *Decalepis hamiltoni* and *Piper longum* reported only callus formation in medium having BA and NAA (Soniya and Das, 2002; Giridhar et al., 2004; Velayutham et al., 2006; Borzabad et al., 2010).

Combinations of Kn with IAA when tried, induced only callus with varying morphology i.e. greenish white compact and brown friable-nodular callus. The variation in callus type and colour was also reported in *Cannabis sativa* (Slusarkiewicz-Jarzina et al., 2005). This variation in callus morphology may be due to the varying endogenous auxin concentrations which determine callus induction ability (Lane, 1978). In line with the present study, green compact callus was reported by Preethi et al. (2011) for *Stevia rebaudiana*. Similarly leaf explant of *S. rebaudiana* and *Withania coagulans* also induced only callus which failed to differentiate shoots (Sivaram and Mukundan, 2003; Jain et al., 2011).

Similarly combinations of Kn and NAA also failed to induce shoots and till eight weeks only callus with varying proliferation was observed in which lower concentrations of Kn (5-15 µM) are beneficial. The change in callus formation is may be due the concentration of the PGR which affect negatively to the explant (Mineo, 1990). Similarly *Tecomella undulate* leaves also evoked moderate growth of callus in medium supplemented with low concentrations of Kn (Patel and Patel, 2013). In plants like *Stevia rebaudiana* (Sivaram and Mukundan, 2003), *Euphorbia nivulia* (Sunandakumari et al., 2005) and *Populus ciliate* (Thakur and Srivastava, 2006) also leaves formed only callus when placed on medium fortified with Kn and NAA.

Amongst different PGR combinations tried, regeneration was observed only in medium fortified with BA and IAA. This may due to the fact that the tissues undergo dedifferentiation and redifferentiation only when both exogenous as well as endogenous PGRs in the medium interact and differentiate shoots (Schwarz and Beaty, 1996; Huang et al., 2012; Lee and Huang, 2013). Earlier report by Norstrog (1970) has suggested that endogenous levels of the hormones are also responsible for the regeneration. Cytokinins, derivatives of adenine, are the class of PGR which regulates the synthesis of proteins involved in formation and functioning of mitotic spindles, hence are required for

stimulation of cell division and shoot formation (Chawla, 2002; George et al., 2008). On the other hand auxins make more methylation to DNA, which helps in reprogramming of cells and initiate the division of differentiated cells (George et al., 2008). Therefore synergism of both cytokinins and auxins are known to help in progression through the cell cycle, which is prerequisite for cell proliferation followed by growth and development of plants (Stals and Inzé, 2001). In the present study, shoot regeneration was observed only in medium fortified with BA, which may be due to the reason that the cultures may easily metabolize BA rather than Kn and also the former is reported to control and/or synthesize other cytokinins within the tissues (Mercier et al., 2003; Rai et al., 2009). Another reason is difference in their uptake, translocation rates and variation in metabolic processes (Blakesey, 1991; Kaminek, 1992). Mercier et al. (2003) stated that cytokinins are necessary for cell division and auxins are known to affect division, expansion and differentiation of cells.

After optimization of protocol for shoot regeneration from leaf explant, the next objective was to optimize the medium for shoot regeneration from nodal explant using different combinations of cytokinins and auxins.

4.1.2 Shoot Regeneration from Nodal Explant

Nodes of *H. indicus* were inoculated in basal MS medium as well as in medium fortified with different concentrations of cytokinins (BA, Kn) and auxins (IAA, NAA) individually and in combinations, When nodes were inoculated in basal medium 0.58±0.14 shoot was observed with 41.67% cultures at the end of eight weeks (Table 7). To achieve multiple shoot formation, basal MS medium was fortified with different concentration and combinations of cytokinins (BA and Kn) and auxins (IAA and NAA), and the results are summarised below.

4.1.2.1 Individual cytokinins (BA and Kn)

In the first experiment, effect of two commonly used cytokinins i.e. BA and Kn was evaluated when incorporated individually at different concentrations (5-20 µM). When BA was added to the medium, bud break was observed from one side of the node within a week with simultaneous bud break from opposite node during second week. These buds elongated into shoots in subsequent weeks and the explant was subcultured in fresh

medium at the end of four weeks which facilitated shoot growth as well as branching of shoots in fifth week. Formation of multiple shoots started in sixth week and further elongation, multiplication and branching of shoots was observed till eight weeks. However the explants differentiated green compact callus at the base during fifth week in presence of higher concentrations of BA (15-20 µM) which proliferated till eight weeks. At all the concentrations of BA, 100% response was observed but it varied in shoot number. Lower concentration of BA (5 µM) induced 1.58±0.14 shoots, which increased to 2.00±0.20 shoots at 10 µM (Fig. 18a). Further increase in BA levels to 15 and 20 µM failed to enhance the response, and the shoot number decreased to 1.67±0.14 and 1.42±0.14 respectively (Table 7).

Replacing BA with Kn in the medium evoked almost a similar response and 100% cultures formed shoots in all the concentrations but the numbers were less. The observations also revealed that the shoots which regenerated in media fortified with Kn had longer internodes as compared to shoots which were formed in media supplemented with BA. At lower level of Kn 1.25±0.13 shoots were observed, which slightly increased to 1.67±0.14 at 10 µM concentration (Fig. 18b). Further increase in Kn concentration to 15 µM failed to increase the shoot number as only 1.42±0.14 shoots were formed and the number further decreased to 1.17±0.11 at 20 µM (Table 7). Higher concentrations (15-20 µM) induced callus at the base of explant after subculture.

Both the cytokinins induced axillary bud to proliferate and develop into shoots but BA was better as compared to Kn in terms of shoot regeneration when used individually. Although 100% response was observed in both the cytokinins, none of them induced multiple shoots.

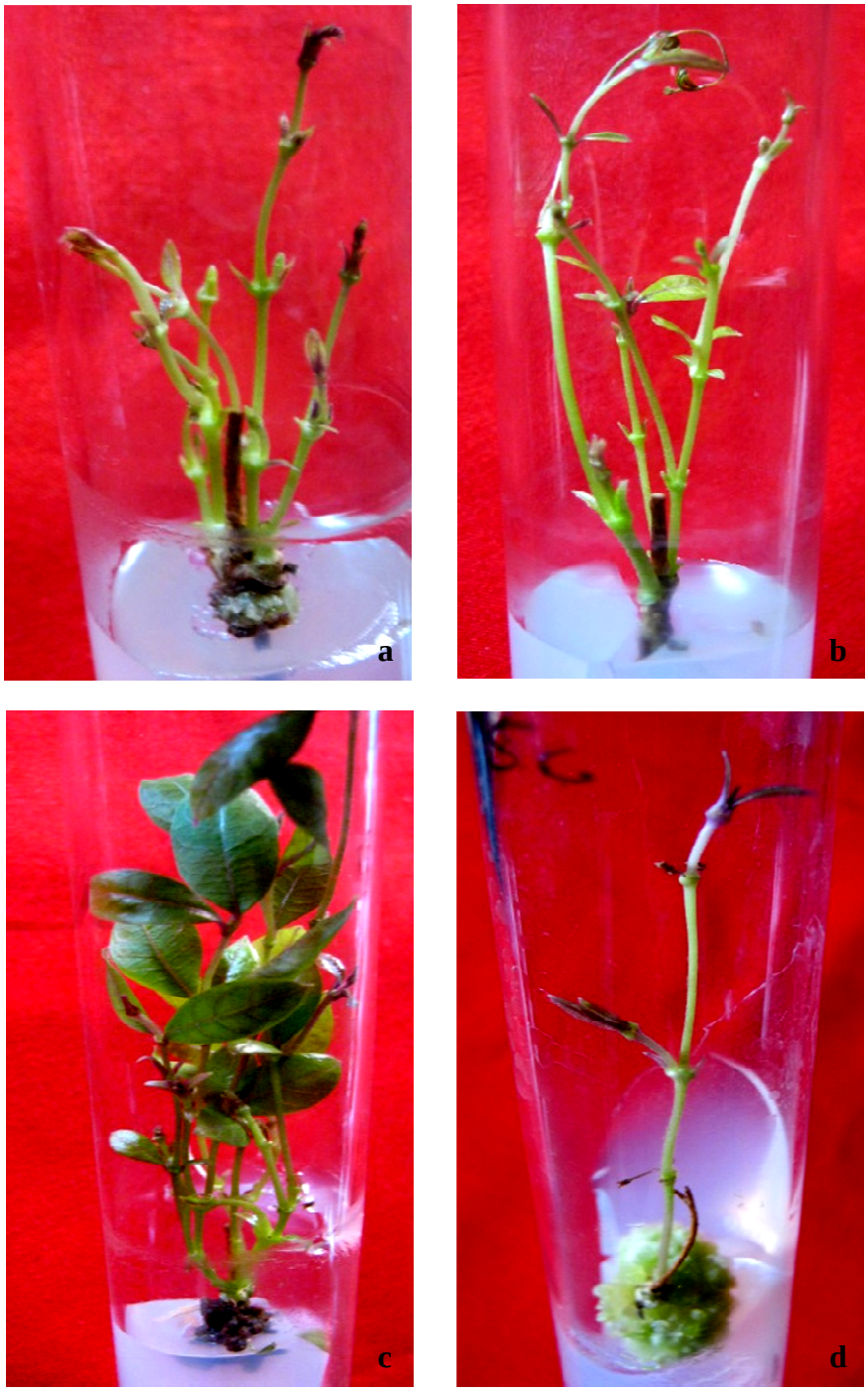

Figure 18: Effect of individual cytokinin or auxin on shoot regeneration from nodal explant after eight weeks- (a) shoot formation from both the axillary buds in presence of BA (10 µM), (b) elongated shoots in Kn (10 µM) fortified medium, (c) shoots with large leaf in presence of IAA (2 µM) and (d) formation of callus at the base offshoot in NAA (0.5 µM).

4.1.2.2 Individual auxins (IAA and NAA)

The MS medium was also fortified with individual auxins and the effect on nodal explant was evaluated. Fortifying the medium with IAA initially induced only one bud to proliferate at the end of one week whereas the other bud proliferated in second week. They developed into shoots by fourth week and upon subculture to a fresh medium further growth was achieved along with multiple shoot formation were observed in fifth week. Further elongation and branching of shoots were observed in sixth week and this continued in seventh and eighth week. In all the concentrations of IAA, compact callus was formed in fourth week at the base of node which simultaneously proliferated till eight weeks.

Presence of IAA in the medium induced axillary bud to develop into shoots and at lower concentrations (0.5 and 1 µM) 1.25±0.27 (66.67%) and 1.42±0.25 (75%) shoots formed respectively. This number increased to 2.17±0.31 at 2 µM and the response was enhanced to 83.33% (Fig. 18c, Table 7). At all the concentrations of IAA, expansion of leaf lamina was observed which resulted in formation of large leaves with simultaneous increase in the length of shoots. Eight weeks observations revealed that the nodal explants responded in a similar manner when medium was fortified with BA.

Replacing IAA with NAA in the medium evoked similar response but the number of shoots were less in all the concentrations. This may be due to formation of friable callus during fourth week at base of the explant. This callus grew simultaneously with shoots and proliferated till eight weeks. As a result shoots failed to elongate and remained stunted without forming multiples. NAA at 0.5 µM concentration, formed only 1.00±0.31 shoots (Fig. 18d) in 50% cultures and at 1 and 2 µM the response failed to enhance as there was decrease in shoot regeneration (Table 7). IAA proved to be better in terms of shoot formation as compared to NAA but none of the auxin induced multiple shoots even after eight weeks. Hence in the further studies the synergistic effects of a combination of cytokinins and cytokinins with auxins on nodal explants was evaluated.

4.1.2.3 Synergism of BA and Kn

BA and Kn individually induced 100% response in terms of shoot regeneration in MS medium, and hence both were added together at same concentration and their synergistic effect on shoot regeneration was observed. BA and Kn at 5 µM concentrations induced 5.75±0.13 shoots and also the length was less as compared to shoots from medium having BA (10 µM) and Kn (5 µM) (Fig. 20a). Keeping BA constant and increasing Kn

concentration to 10 µM slightly enhanced the number of shoots to 5.92±0.34. Whereas Kn at 15 and 20 µM the shoots decreased to 5.58±0.36 and 5.08±0.28, respectively (Table 7).

Table 7: Effect of cytokinins (BA/Kn), auxins (IAA/NAA) and combinations of cytokinins (BA and Kn) on shoot regeneration from nodal explant of *H. indicus* (8 weeks)

BA (µM)	Kn (µM)	IAA (µM)	NAA (µM)	No. of shoots/explant (Mean ± SE)*	% Response
0	0	0	0	0.58±0.14 lm	41.67
5	0	0	0	1.58 ±0.14 ijk	100
10	0	0	0	2.00±0.20 ij	100
15	0	0	0	1.67±0.14 ijk	100
20	0	0	0	1.42 ±0.14 ijkl	100
0	5	0	0	1.25±0.13 ijklm	100
0	10	0	0	1.67±0.14 ijk	100
0	15	0	0	1.42±0.14 ijkl	100
0	20	0	0	1.17±0.11 jklm	100
0	0	0.5	0	1.25±0.27 ijklm	66.67
0	0	1	0	1.42±0.25 ijkl	75
0	0	2	0	2.17±0.31 i	83.33
0	0	0	0.5	1.00±0.31 klm	50
0	0	0	1	0.83±0.28 klm	41.67
0	0	0	2	0.42±0.22 m	25
5	5	0	0	5.75±0.13 fg	100
5	10	0	0	5.92±0.34 efg	100
5	15	0	0	5.58±0.36 fg	100
5	20	0	0	5.08±0.28 gh	100
10	5	0	0	11.00±0.24 a	100
10	10	0	0	9.17±0.50 b	100
10	15	0	0	8.83±0.33 bc	100
10	20	0	0	8.17±0.31 c	100
15	5	0	0	6.42±0.38 def	100
15	10	0	0	6.75±0.46 de	100
15	15	0	0	7.08±0.46 d	100
15	20	0	0	5.92±0.22 efg	100
20	5	0	0	5.00±0.29 gh	100
20	10	0	0	5.67±0.34 fg	100
20	15	0	0	5.50±0.28 fgh	100
20	20	0	0	4.67±0.27 h	100

*Means (n=12) followed by same letter are not significantly different ($p \leq 0.05$) using Duncan's multiple range test.

Increasing the BA concentration to 10 µM evoked a better response with all the Kn concentrations. An early response was observed early in all the combinations as bud break occurred within a week from one node in all the cultures and callus was induced from the base of explant which hindered further growth of shoots (Fig. 19a). The other bud break from the opposite node was by the end of second week and shoot formation was observed from both nodes (Fig. 19b). During third week these shoots elongated and branching was also observed which continued in fourth week along with multiple shoot formation (Fig. 19c). Subculturing of this clusters helped in elongation and proliferation of shoots during fifth week. In sixth and seventh week, further elongation, multiplication and branching of shoots was observed which continued till eight weeks and healthy shoots were observed at the end of eight weeks (Fig. 19d). BA (10 µM) with Kn (5 µM) proved to be the optimum concentration for nodal cultures as 11.00±0.24 shoots were formed per explant. The shoot number slightly decreased to 9.17±0.50 as Kn level reached to 10 µM (Fig. 20b). The number further decreased to 8.83±0.33 and 8.17±0.31 at Kn 15 µM and 20 µM, respectively (Table 7).

Increasing the BA concentration to 15 µM failed to enhance the number of multiples and total of 6.42±0.38 shoots were formed in medium supplemented with BA (15 µM) and Kn (5 µM). This number increased to 6.75±0.46 when 10 µM Kn was used, which again increased at Kn 15 µM concentration and formed 7.08±0.46 shoots. However these shoots were stunted and also brown friable callus was observed at the base of the explant (Fig. 20c). In 20 µM Kn concentration 5.92±0.22 shoots were observed within eight weeks. Further increase in BA level to 20 µM evoked least response and maximum 5.67±0.34 shoots were observed with Kn (10 µM) (Table 7). At BA 20 µM with Kn (10 µM) concentration also friable callus was observed at the base of the node and only few elongated shoots were formed (Fig. 20d).

BA at 10 µM and Kn at 5 µM (MS-1 medium) were synergistic as maximum number of shoots were formed in this concentrations. However the shoots regenerated in a medium with two cytokinins developed smaller leaves and hence were transferred to medium containing IAA (2 µM) for further broadening of leaves (MS-2 medium).

Figure 19: Shoot regeneration from nodal explants in optimized MS medium [BA (10 µM) and Kn (5 µM)]- (a) bud break from one node after one week, (b) elongated axillary shoots from both the nodes after two weeks, (c) multiple shoots at the end of four weeks and (d) healthy elongated shoots with branching at the end of eight weeks.

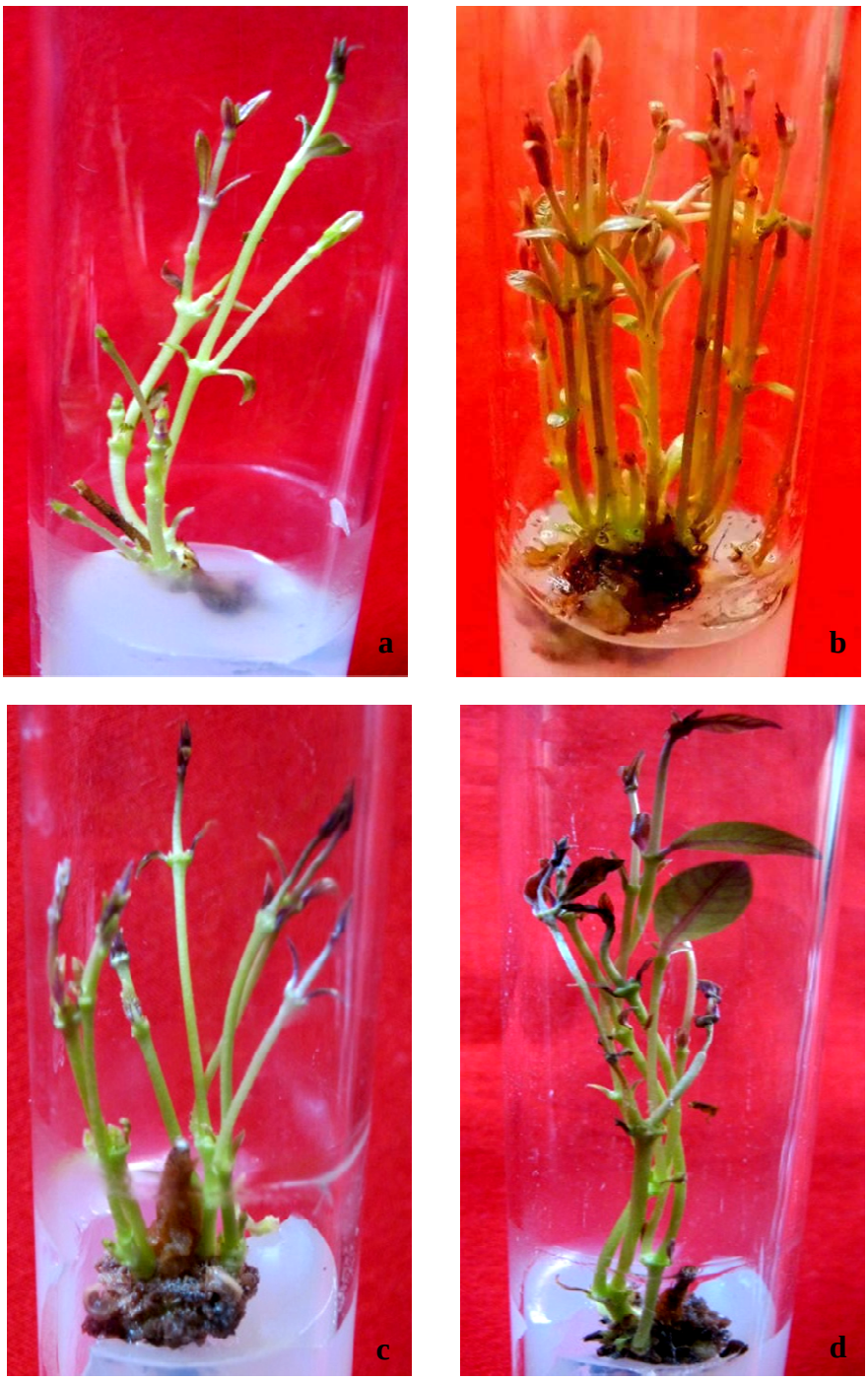

Figure 20: Effect of synergism of cytokinins on shoot regeneration from nodal explant after eight weeks- (a) formation of few shoots in presence of BA (5 µM) and Kn (5 µM), (b) formation of callus at the base of multiple shoots in BA (10 µM) and Kn (10 µM), (c) stunted shoots in presence of BA (15 µM) and Kn (15 µM) and (d) few elongated shoots with friable callus formation at base in medium supplemented with BA (20 µM) and Kn (20 µM).

4.1.2.4 Synergism of BA with IAA/NAA

As a combination of two cytokinins improved the number of shoots, the effect of cytokinins and auxins together was also studied. In all the combinations of BA with IAA/NAA, bud break from one node was observed within a week. Shoot regeneration pattern was similar to the earlier combinations tried but with the growth of shoots, callus differentiated at the base of the node after four weeks and it continued to grow till eight weeks.

In the combinations of BA with IAA, when BA (5 µM) was supplemented with IAA (0.5 µM) it regenerated 4.08±0.56 shoots in 83.33% cultures. Increasing the IAA concentration to 1 µM, this shoot number slightly increased to 4.42±0.79 but the response decreased to 75%. IAA concentration further reached to 2 µM, only 3.58±0.90 multiples developed with 58.33% response which may be due to profuse callusing at base. This shoot number increased (5.17±0.88) when BA was at 10 µM and IAA at 0.5 µM with 75% response (Fig. 21a). Further increase in IAA level to 1 and 2 µM adversely affected the shoot formation and the response further decreased when BA (15 and 20 µM) was combined with different concentrations of IAA (Table 8).

Combinations of BA with NAA failed to enhance the shoot number as profuse callusing at base of the node was observed during fourth week. BA at 5 µM with NAA at 0.5 µM formed only 3.00±0.75 shoots (58.88% response), which decreased to 2.67±0.93 (41.67% response) and 2.00±0.82 (33.33% response) when NAA level increased to 1 and 2 µM respectively. Increasing BA concentration to 10 µM increased the response and 3.67±0.77 shoots (66.67%) developed in combination with 0.5 µM NAA. This was accompanied by greenish callus at the base of explant and due to this shoots faild to elongate (Fig. 21b). But this number decreased at higher NAA concentrations. Fortifying medium with higher BA levels (15 and 20 µM) decreased the response with all NAA concentrations (Table 8).

Thus it was concluded that BA with IAA or NAA induced multiple shoots from nodes in many combinations, however the number was less as compared to shoots which regenerated in medium fortified with BA (10 µM) and Kn (5 µM).

4.1.2.5 Synergism of Kn with IAA/NAA

Individually when Kn was used it formed shoots with 100% response at all concentrations and hence it was combined with IAA/NAA to observe whether it has

synergistic effect on shoot formation from nodal explant. Combination of Kn (5 µM) with IAA (0.5 µM) evoked poor response and it regenerated only 2.58±0.81 shoots from 50% cultures. Increasing the concentration of IAA increased the response, and at 1 µM concentration it formed 3.42±0.79 shoots (66.67%) but this number again decreased to 3.25±0.85 (58.33%) when it reached to 2 µM concentration. The shoot number increased when Kn 10 µM was used and with IAA (0.5 µM) as it regenerated 4.08±0.70 shoots in 75% cultures. The number of shoots slightly increased to 4.25±0.65 in 83.33% cultures when combination of Kn (10 µM) with IAA (1 µM). However multiple shoots were induced but their growth was slow and compact callus was observed at the base of explant which resulted in stunted shoots (Fig. 21c). Further increasing the IAA concentration to 2 µM decreased the shoot number to 3.58±0.82 (66.67% response). Increasing Kn level to 15 and 20 µM adversely affected the shoot regeneration potency of nodes and less shoots were formed in all combinations (Table 8).

When NAA was used in combination with Kn, the least morphogenic response was observed and it failed to form multiple shoots in many of the combinations. A combination of Kn (5 µM) and NAA (0.5 µM) induced meagre response and only 1.50±0.52 shoots were observed within eight weeks from 41.67% cultures. This number again decreased to 1.17±0.48 (33.33%) and 1.00±0.50 (25%) as the NAA concentration was increased to 1 and 2 µM respectively. Increasing the Kn concentration to 10 µM also induced very less response and total 1.67±0.45 (58.33%) shoots were observed with NAA (0.5 µM). Increasing Kn level to 15 µM increased the shoot number to 2.33±0.48 in 75% cultures, but it again decreased when NAA concentration was raised to 1 and 2 µM. Kn at 20 µM induced maximum shoots i.e. 3.25±0.45 (83.33%) with NAA at 0.5 µM (Fig. 21d). However further increase in NAA concentration failed to increase the shoot number (Table 8).

BA or Kn when coupled with IAA and NAA induced multiple shoots but the number was less as compared to shoots developed in medium fortified with a combination of cytokinins. Thus cytokinins i.e. BA (10 µM) and Kn (5 µM) proved to be optimum for shoot regeneration from nodal explant and this combination was further utilized for secondary metabolite studies (MS-1).

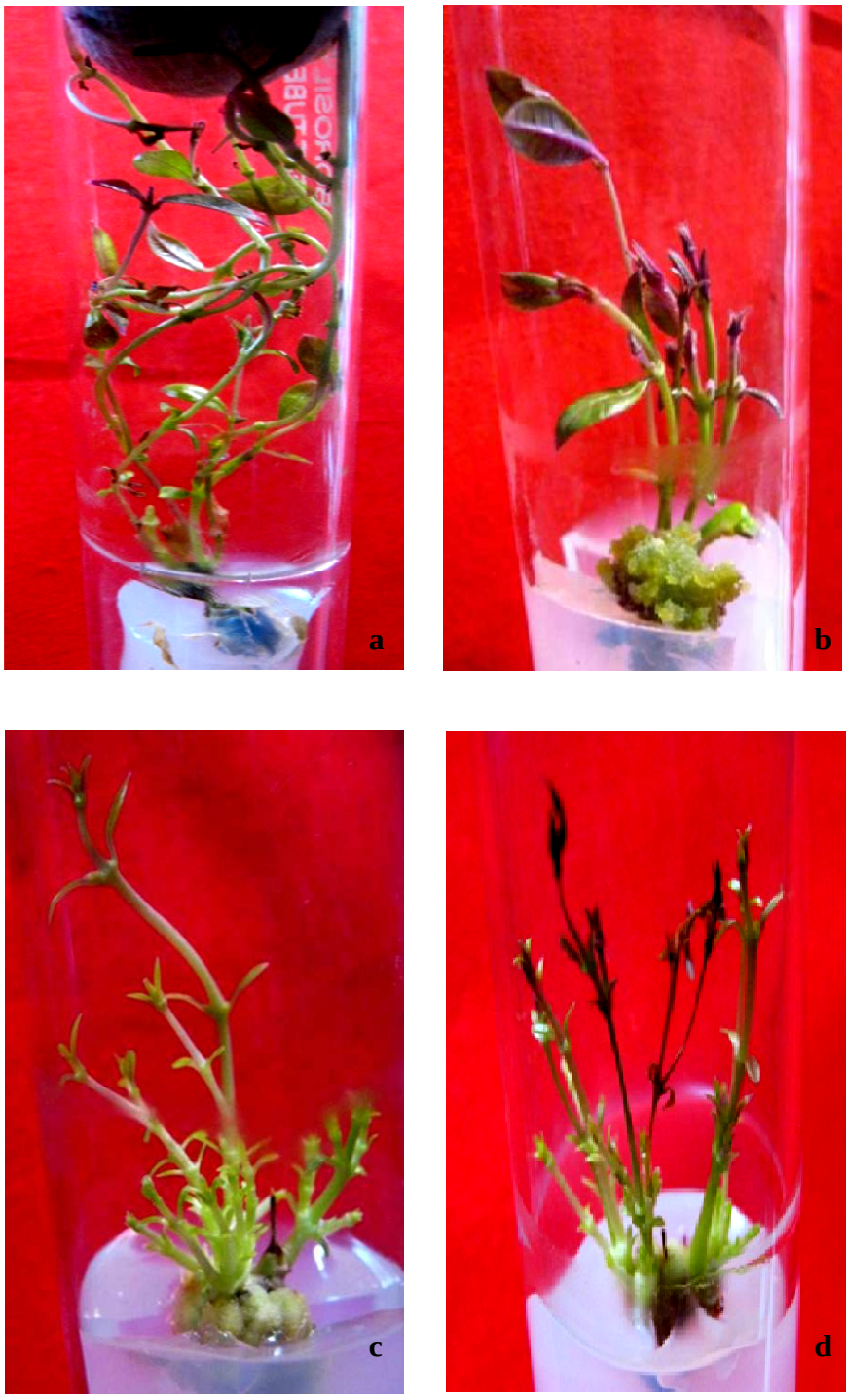

Figure 21: Effect of combination of cytokinin and auxin on shoot formation from nodal explant after eight weeks- (a) few elongated shoots in presence of BA (10 µM) and IAA (0.5 µM), (b) green friable callusing at the base of stunted shoots in medium with BA (10 µM) and NAA (0.5 µM), (c) green compact callus at the base of stunted shoots in Kn (10 µM) and IAA (1 µM) containing medium and (d) formation of weak shoots in medium fortified with Kn (20 µM) and NAA (0.5 µM).

Table 8: Effect of different combinations of cytokinins with auxins on shoot regeneration from nodal explant of *H. indicus* (8 weeks)

BA (µM)	Kn (µM)	IAA (µM)	NAA (µM)	No. of shoots/explant (Mean ± SE)*	% Response
0	0	0	0	0.58±0.14 g	41.67
5	0	0.5	0	4.08±0.56 abcd	83.33
5	0	1	0	4.42±0.79 abc	75
5	0	2	0	3.58±0.90 abcdef	58.33
10	0	0.5	0	5.17±0.88 a	75
10	0	1	0	4.67±0.99 ab	66.67
10	0	2	0	4.33±0.89 abcd	66.67
15	0	0.5	0	4.17±1.05 abcd	58.33
15	0	1	0	4.00±1.05 abcde	58.33
15	0	2	0	3.83±0.98 abcdef	58.33
20	0	0.5	0	4.08±1.06 abcd	58.33
20	0	1	0	3.42±0.72 abcdefg	66.67
20	0	2	0	3.00±0.91 abcdefg	50
5	0	0	0.5	3.00±0.75 abcdefg	58.33
5	0	0	1	2.67±0.93 abcdefg	41.67
5	0	0	2	2.00±0.82 bcdefg	33.33
10	0	0	0.5	3.67±0.77 abcdef	66.67
10	0	0	1	3.17±0.58 abcdefg	75
10	0	0	2	3.08±0.67 abcdefg	66.67
15	0	0	0.5	3.42±1.01 abcdefg	50
15	0	0	1	2.75±0.95 abcdefg	41.67
15	0	0	2	2.50±1.02 abcdefg	33.33
20	0	0	0.5	2.33±0.84 abcdefg	41.67
20	0	0	1	2.25±0.92 bcdefg	33.33
20	0	0	2	1.67±0.84 cdefg	25

Contd…

BA (µM)	Kn (µM)	IAA (µM)	NAA (µM)	No. of shoots/explant (Mean ± SE)*	% Response
0	5	0.5	0	2.58±0.81 abcdefg	50
0	5	1	0	3.42±0.79 abcdefg	66.67
0	5	2	0	3.25±0.85 abcdefg	58.33
0	10	0.5	0	4.08±0.70 abcd	75
0	10	1	0	4.25±0.65 abcd	83.33
0	10	2	0	3.58±0.82 abcdef	66.67
0	15	0.5	0	3.25±0.59 abcdefg	75
0	15	1	0	3.08±0.85 abcdefg	75
0	15	2	0	2.42±0.56 abcdefg	66.67
0	20	0.5	0	3.50±1.02 abcdef	50
0	20	1	0	3.17±1.09 abcdefg	41.67
0	20	2	0	2.08±0.86 bcdefg	33.33
0	5	0	0.5	1.50±0.52 cdefg	41.67
0	5	0	1	1.17±0.48 efg	33.33
0	5	0	2	1.00±0.50 fg	25
0	10	0	0.5	1.67±0.45 cdefg	58.33
0	10	0	1	1.42±0.49 defg	41.67
0	10	0	2	1.08±0.38 fg	41.67
0	15	0	0.5	2.33±0.48 abcdefg	75
0	15	0	1	2.08±0.45 bcdefg	66.67
0	15	0	2	1.83±0.41 cdefg	66.67
0	20	0	0.5	3.25±0.45 abcdefg	83.33
0	20	0	1	3.08±0.57 abcdefg	75
0	20	0	2	1.92±0.57 bcdefg	50

*Means (n=12) followed by same letter are not significantly different ($p \leq 0.05$) using Duncan's multiple range test.

When nodes of *H. indicus* were inoculated in basal MS medium (control), a meagre response was observed and only 0.58±0.14 shoots were formed in 41.67% cultures. Similar reports are documented for nodal cultures of *Ceropegia candelabrum* (Beena et al., 2003), *Tylophora indica* (Faisal et al., 2007) and *C. hirsute* (Nikam et al., 2008) where basal MS medium without PGRs induced less response.

Sachs and Thimann (1964) reported that cytokinin regulates apical dominance and later on Wang and Charle (1991) suggested that BAP acts as an apical dominance inhibitor. The beneficial effect of cytokinin is also due to promotion of woody tissue formation near vascular tissues of bud which facilitated easy translocation of water and nutrients (Mohammed and Al-Younis, 1991). Supplementing basal medium with BA

increased the response to 100% in all the concentrations, but maximum of only 2.00±0.20 shoots were observed at 10 µM concentration. Similarly nodal explants of *Vitex negundo* (Chandramu et al., 2003) and *Tinospora cordifolia* (Gururaj et al., 2007) are less responsive in BA fortified medium. Earlier reports on same plant by Siddique and Bari (2006) and Singh et al. (2015) have also reported less shoot formation in BAP fortified medium. Whereas other reports on *H. indicus* reported maximum shoot regeneration in BAP fortified medium (Sreekumar et al., 2000; Shanmugapriya and Sivakumar, 2011; Shekhawat and Manokari, 2016).

Supplementing medium with Kn also regenerated less shoots (1.67±0.14 in 100% cultures) in medium supplemented with 10 µM level and similarly earlier reports on same plant also stated less shoots formation in Kn supplemented media (Siddique and Bari, 2006; Shekhawat and Manokari, 2016). Similarly in other plants like *Vitex trifolia* (Hiregoudar et al., 2006), *Ceropegia pusilla* (Kondamudi et al., 2010) and *T. cordifolia* (Sivakumar et al., 2014) also Kn has been reported to be less responsive. It was also observed medium fortified with Kn induced less number of shoots as compared to BA which in line with earlier report on *H. indicus* (Sreekumar et al., 2000; Singh et al., 2015; Shekhawat and Manokari, 2016), *Cunila galioides* (Fracaro and Echeverrigaray, 2001) and *Aristolochia indica* (Pattar and Jayaraj, 2012). However shoots regenerated in Kn supplemented medium had longer internodes and similar observation has been recorded in other Asclepiadaceae plants like *Gymnema sylvestre* (Komalavalli and Rao, 2000), *C. candelabrum* (Beena et al., 2003) and *Tylophora indica* nodes (Faisal et al., 2007).

The higher concentrations (15 and 20 µM) of both the cytokinins formed basal callus which is also reported by Sreekumar et al. (2000) for the same plant as well as in *Ceropegia juncea* (Nikam and Savant, 2009) and *Pogostemon cablin* (Swamy et al., 2010). Both the cytokinins induced shoots but failed to induce optimum regeneration and is also reported in earlier reports of *H. indicus* (Siddique et al., 2006; Sundarmani and Hasina, 2015), *Commiphora wightii* (Tejovathi et al., 2011) and *Sophora tonkinensis* (Jana et al., 2013).

After cytokinins, the effects of individual auxins were assessed for formation of multiple shoots. IAA at all the concentrations induced shoots from nodes but a maximum of 2.17±0.31 shoots (83.33% response) were formed at 2 µM concentration. Unlike cytokinins, this concentration of IAA failed to induce 100% response which is in line with earlier report on same plant by Shanmugapriya and Sivakumar (2011) in which IAA (1

mg/l) induced maximum 70% response. Similarly Sain and Sharma (2013) also reported maximum 2.42±0.39 shoots from IAA fortified medium in *Catharanthus roseus*. The nodes placed in medium containing IAA formed green compact callus at the base which is also reported by Siddique and Bari (2006) in *H. indicus*.

When NAA was used individually in the medium, only 1.00±0.31 shoots with 50% response was observed at 0.5 µM. Siddique and Bari (2006) observed that NAA (0.5-2 mg/l) induced only callus from nodes of the same plant till four weeks. Similarly Nagahatenna and Peiris (2007) have reported that individually when NAA (0.1 mg/l) was used, the axillary bud of *H. indicus* failed to proliferate into shoot. This result is also in line with earlier report of Shanmugapriya and Sivakumar (2011) for the same plant and they have reported only 45% culture response in medium fortified with 3 mg/l NAA. Similarly Seetharam et al. (2007) also reported less response in NAA fortified medium for *Vernonia cinerea* nodal explant. Mei-Yin and Sani (2018) have reported that when NAA was used individually in the medium, it formed very less shoots from nodes of *Vernonia amygdalina* and also callusing at the base of the node was observed.

IAA and NAA evoked shoot formation but it was accompanied with callus formation at base of nodes, which is also reported in *Prosopis cineraria* (Kumar and Singh, 2010). Presence of auxins in medium failed to induce multiple shoots and similar observations are reported by Kumari and Singh (2012) for *Salvadora persica*. Whereas callus induced on nodes of *Aristolochia indica* and *H. indicus* failed to regenerate shoots when transferred to medium containing IAA and NAA alone (Siddique et al., 2006). The observations thus revealed that the shoots regenerated from auxin fortified medium have less branching of shoots as compared to cytokinin based medium. This may be due to inhibition of bud break by auxins of the medium (Thimann and Skoog, 1933). The overall response of cytokinins is better as compared to auxin in terms of shoot formation and is comparable to earlier report for *Catharanthus roseus* (Sain and Sharma, 2013).

Both the cytokinins failed to induce multiples when used individually but when combined together they induced an optimum response in presence of BA (10 µM) and Kn (5 µM) with 11.00±0.24 shoots from 100% cultures. Similar observations have been reported in nodal explant of *Enicostemma hyssopifolium* (Seetharam et al., 2002), *Leptadenia reticulata* (Sudipta et al., 2011) and *Chlorophytum borivilianum* (Ashraf et al., 2014). Earlier study on the same plant (Patnaik and Debata, 1996) reported combination of BA and Kn to be optimum for multiples but shoots developed small leaves and similar

findings are reported in the present study. Similarly Malathy and Pai (1998) also reported maximum shoot proliferation from nodal explants of same plant in combination of BA (13.32 µM) and Kn (2.32 µM). Whereas combination of BAP (1.0 mg/l) with Kn (2.0 mg/l) induced optimum 8-10 shoots with 80% response in *H. indicus* (Rama Devi et al., 2014). The interaction of BA and Kn has been reported to be optimum for shoot regeneration in nodal explant of *Piper longum* (Soniya and Das, 2002), *Eclipta alba* (Baskaran and Jayabalan, 2005), *Crataeva nurvala* (Walia et al., 2007), *Swertia chirata* (Balaraju et al., 2009), *Andrographis paniculata* (Dandin and Murthy, 2012) and *Catharanthus roseus* (Sain and Sharma, 2013).

After combination of cytokinins, combinations of cytokinins and auxins were tried as they are known to facilitate shoot regeneration as well as to maintain the meristem activity (George, 1993; Dello et al., 2007). BA coupled with IAA induced only 5.17±0.88 shoots when the former was at 10 µM and the later at 0.5 µM. Earlier report by Siddique and Bari (2006) documents that only 2.18±0.52 shoots (15% response) regenerated in presence of BA (2.5 mg/l) with IAA (0.5 mg/l) for the same plant. Although multiple shoots were observed in these combinations, the number of shoots was less as compared to medium supplemented with BA and Kn and is also reported by Rama Devi et al. (2014) for *H. indicus*. Recently Najar et al. (2018) also reported that combinations of BA with IAA failed to enhance number of shoots in nodal explant of *Tylophora indica*. The decrease in shoot number may be due to the formation of callus, which is also reported for same plant by Siddique et al. (2006). Similarly Abhyankar and Reddy (2007) observed that when nodes of *Adhatoda vasica* were cultured in BA-IAA combinations, shoots failed to grow due to callus formation. In *Acmella calva* (Amudha and Shanthi, 2011) and *Salvadora persica* (Kumari and Singh, 2012) also, callus was observed in combinations of BA and IAA. The % response for shoot regeneration varied as the concentrations changes and similar observations were also reported by Srilatha et al. (2014) for *Vigna mungo* nodal explants. The response of combination of BA with IAA is better as compared to medium containing BA only which is in line with earlier reports on *Cassia angustifolia* (Siddique and Anis, 2007), *Celastrus paniculatus* (Phulwaria et al., 2013a) and *Toddalia asiatica* (Anand et al., 2015).

Replacing IAA with NAA and combined with BA also evoked multiple shoot formation but the shoot number was decreased to 3.67±0.77 (66.67% response) in medium fortified with BA (10 µM) and NAA (0.5 µM). Earlier report on *H. indicus* by Misra et al.

(2003) documented 4.82 shoots when medium was fortified with BAP (1.0 mg/l) and NAA (0.5 mg/l). Other studies on *H. indicus* (Siddique and Bari, 2006; Nagahatenna and Peiris, 2007) reported that combination of BAP and NAA induced multiple shoots but it failed to enhance the shoot number as compared to optimum combination for regeneration. Similar reports have been documented for nodal explant of *Cineraria maritime* and *Catharanthus roseus* (Banerjee et al., 2004; Sain and Sharma, 2013). Whereas Saha et al. (2003) reported optimum number of shoots (7.4±0.22) in nodes of same plant when placed in BA (2.0 mg/1) and NAA (0.1 mg/l). This combinations formed profuse callus at the base which is also reported in earlier report on same plant (Sreekumar et al., 2000; Misra et al., 2003; Nagahatenna and Peiris, 2007) and in *Tylophora indica* (Faisal et al., 2007). Siddique et al. (2006) and Amudha and Shanthi (2011) documented callus formed in nodes of *H. indicus* and *Acmella calva* respectively which differentiated shoots upon subculture, whereas in the present study the callus proliferated without any shoot bud formation. BA and NAA induced better regeneration as compared to medium supplemented with individual BA and is reported in *Holarrhena antidysenterica* (Ahmed et al., 2001) and *Vernonia cinerea* (Seetharam et al., 2007).

When Kn was added at 10 µM with 1 µM IAA induced 4.25±0.65 shoots which is less as compared to earlier combinations. Similarly earlier studies on the same plant also stated less shoots in medium fortified with Kn and IAA (Siddique and Bari, 2006; Siddique et al., 2006). These combinations induced multiple shoots but they failed to increase the shoot number as compared to optimum combination, and is recently reported for *Tylophora indica* nodal explant (Najar et al., 2018). However the response of cultures varied as the concentrations of Kn and IAA varied and is also reported for *Vigna mungo* (Srilatha et al., 2014). Combinations of Kn with IAA evoked less shoot proliferation as compared to BA-IAA combinations, which is in accordance with earlier reports on *Vitex negundo* (Chandramu et al., 2003) and *Salvadora persica* (Kumari and Singh, 2012).

Combinations of Kn and NAA induced multiples and maximum 3.25±0.45 shoots were formed in Kn (20 µM) and NAA (0.5 µM). Misra et al. (2003) also stated that combination of Kn with NAA is not favourable for shoot regeneration and reported only 3.83 shoots in *H. indicus*. Another studies by Siddique et al. (2006), Siddique and Bari (2006) and Sundarmani and Hasina (2015) also reported 3-4 shoots from the same plant in Kn and NAA fortified medium. Whereas Saha et al. (2003) reported only 1.6±0.22 shoots for the same plant, which may be due to profuse callusing in medium containing Kn and

NAA was observed which is also observed in the present study as well as in earlier studies for the same plant (Patnaik and Debata, 1996; Misra et al., 2003; Siddique and Bari, 2006). Studies on *Ceropegia juncea* (Nikam and Savant, 2009) and *Prosopis cineraria* (Kumar and Singh, 2010) nodal explant also observed hindrance of shoot proliferation due to callusing. Whereas contrary result has been documented by Patnaik and Debata (1996) in which the combination of Kn (1.15 µM) and NAA (0.054 µM) formed optimum response with 8.2±0.4 shoots in *H. indicus*. Medium fortified with Kn and NAA induced less number of multiples as compared to combination of Kn and IAA as is also reported for *Ceropegia hirsute* (Nikam et al., 2008), *C. juncea* (Nikam and Savant, 2009) and *Aegle armelos* (Yadav and Singh, 2011).

Combinations of cytokinin with auxin revealed that the overall response of BA with auxins is better as compared to Kn, which is also reported for *H. indicus* (Misra et al., 2003), *Momordica cymbalaria* (Nikam et al., 2009) and *Salvadora persica* (Kumari and Singh, 2012). In the present study, addition of auxins with cytokinin failed to improve the number of shoots which is also reported for *Spilanthes acmella* (Haw and Keng, 2003) and *Veronica anagallis-aquatica* (Shahzad et al., 2011). This may be due to endogenous level of auxins which alters the response by decreasing cytokinin levels as it promotes metabolic inactivation of BA by N-glucosylation or regulates cytokinin oxidase genes which encode proteins capable of degrading the cytokinin (Kaminek et al., 1997; Kieber and Schaller, 2014). Reduction in shoot number is also be due to callusing at base which is due to the accumulation of auxin in the tissue (Marks and Simpson, 1994; Pierik, 1997).

This regeneration study suggested that nodal cultures responded well when placed in medium fortified with combination of cytokinins and branching of shoots was also observed. In the further work the potency of *in vitro* nodes to form shoots was evaluated in cytokinin supplemented medium. This study was carried out to compare the regenerative potency of both the explants for shoot formation.

4.1.2.6 Shoot culture establishment using in vitro nodes

Nodes of *H. indicus* were excised from eight weeks old healthy *in vitro* shoots grown in optimized medium (MS-1). These nodes were placed in basal medium and media fortified with individual cytokinin and a combination of cytokinins which were tried earlier for *in vivo* nodal explants. When *in vitro* nodes were placed in basal MS medium which

induced only 0.58±0.22 shoots with 41.67% response within eight weeks (Table 9). The medium was hence supplemented with individual cytokinins and its effect was observed.

- **Individual cytokinins**

When BA was incorporated into the medium, bud break was observed from one side of node at the end of a week which elongated into shoot with simultaneous bud break from opposite node during second week. The response was exactly similar to *in vivo* buds, and these shoots further elongated till fourth week and upon subculture, multiple shoot formation started during fifth week. Branching of the shoots was observed during sixth week which elongated till eight weeks. The shoot number varied at different concentrations of BA (5-20 µM) and at 5 µM formed a total 1.17±0.16 shoots (91.67% response) within eight weeks. Further increase in concentrations to 10 µM helped axillary buds to proliferate and formed maximum 1.83±0.16 shoots with 100% response. BA at 15 and 20 µM failed to enhance the shoot number (Table 9).

Replacing BA with Kn in the medium evoked a similar response but the number of shoots was less as compared to BA concentrations. Out of the different concentrations (5-20 µM) of Kn tried, only 10 µM was able to form 1.58±0.28 shoots in 83.33% cultures (Table 9).

Individually both the cytokinins induced shoot regeneration but the effectiveness of BA was better as compared to Kn. Thus in further study the effect of both the cytokinins together was evaluated.

- **Synergism of cytokinins**

MS medium when fortified with different combinations of BA (5 µM) and Kn (5 µM) induced 2.00±0.51 shoots in 58.33% cultures. This number was slightly increased to 2.17±0.47 with increased response to 66.67% when 10 µM concentration of Kn was used. However increasing the Kn level to 15 and 20 µM decreased the shoot number to 1.75±0.47 (66.67% response) and 1.08±0.32 (50% response), respectively.

When BA concentration was increased to 10 µM, multiple shoot formation was observed with all concentration of Kn. BA (10 µM) with Kn (5 µM) proved to be optimum as axillary bud break from one side was observed within a week of inoculation (Fig. 22a). This bud elongated and formed shoot during second week along with simultaneous bud break from opposite node (Fig. 22b). Both the shoots elongated during third week and

branching of axillary shoots was observed during fourth week (Fig. 22c). The shoots branched as well as formed multiples in sixth week and this continued till eight weeks and optimum 5.42±0.36 shoots with 100% response was observed. However the shoots failed to elongate and callusing was observed at the base (Fig. 22d). Increasing the Kn concentration to 10 µM failed to increase the shoot number and at the end of total eight weeks a total of 4.67±0.54 shoots (100% response) were observed. The shoot number decreased to 4.33±0.48 (91.67%) and 3.83±0.79 (83.33%) when BA was 10 µM with Kn at 15 and 20 µM respectively. The combinations of BA (15 and 20 µM) with Kn (5-20 µM) also regenerated multiple shoots, however the number was less as compared to earlier combinations (Table 9).

Hence the observations revealed that the *in vitro* node has the potency to regenerate shoots in cytokinin based medium. BA and Kn when added together in medium facilitated multiple shoot formation and combination of BA (10 µM) with Kn (5 µM) induced optimum response within eight weeks. But these shoots were less in number as well as in growth as compared to shoots regenerated from *in vivo* nodes.

In vitro nodes have been used as an explant for multiple shoot formation in a number of species and when these nodes were placed in basal medium it induced 0.58±0.22 shoots (41.67% culture response) and similarly *in vitro* buds of *Crataeva nurvala* induced 41.67% culture response in basal medium (Walia et al., 2007). Earlier report by Sreekumar et al. (2000) observed no shoot formation in basal medium for the same plant. Placing nodes in medium containing BA induced maximum 1.83±0.16 shoots with 100% response in 10 µM concentration and further increasing or decreasing the concentration failed to increase the shoot number. Similarly Mahadev et al. (2014) also reported that *Solanum viarum* nodes formed varied number of shoots according to the concentration of BA, and this cytokinin was unable to induce optimum shoots which is comparable with the present study. Whereas Nagahatenna and Peiris (2007) reported formation of 4.47±1.64 shoot from *in vitro* nodes of *H. indicus* in medium fortified with BAP (5 mg/l).

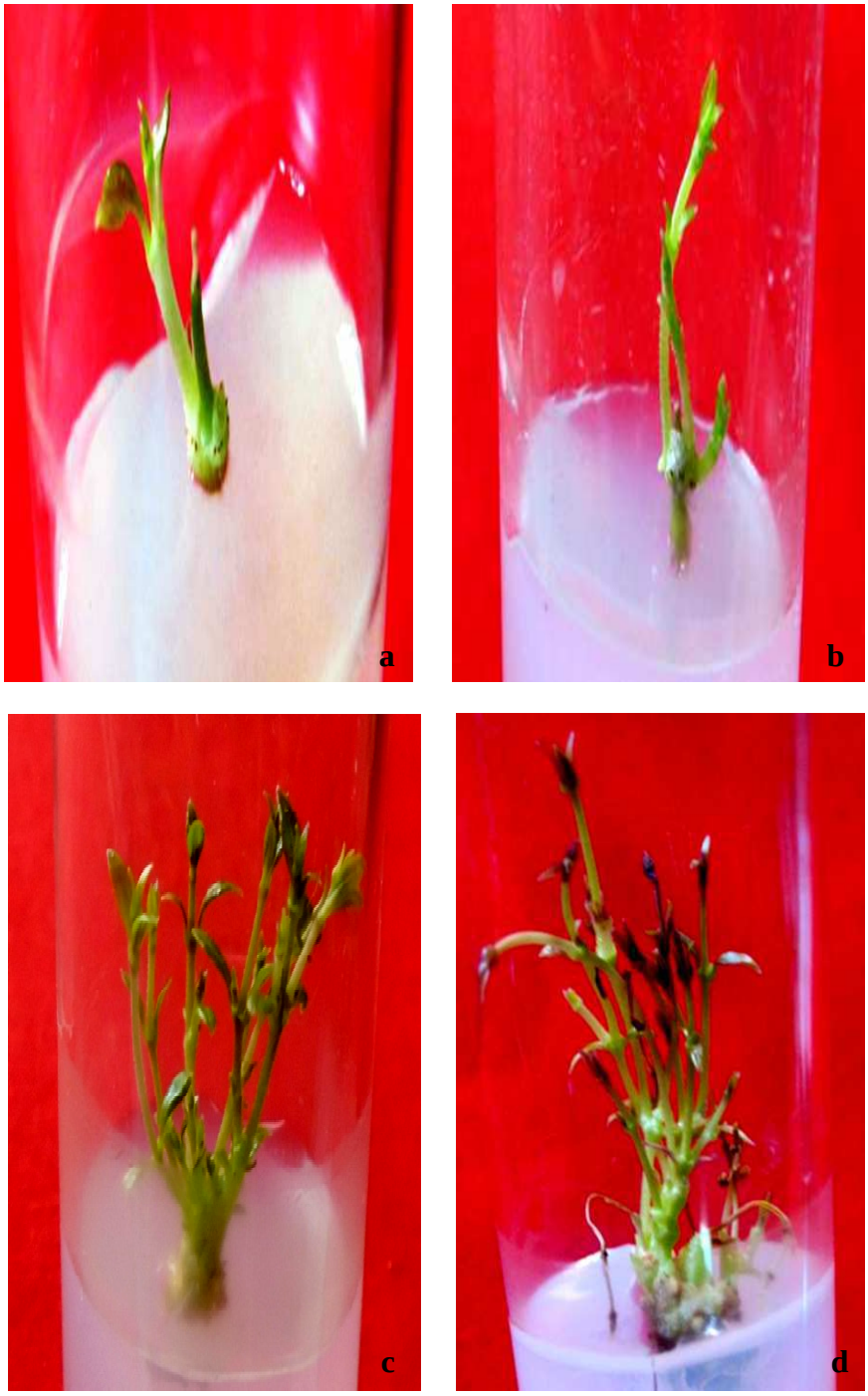

Figure 22: Shoot regeneration from *in vitro* nodes in medium MS + sucrose (3%) + BA (10 µM) + Kn (5 µM)- (a) bud break from at the end of one week, (b) elongated shoots from both nodes at the end of two weeks, (c) multiple shoots with branching after four weeks and (d) healthy elongated shoots at the end of eight weeks.

Table 9: Effect of cytokinins on shoot regeneration from *in vitro* nodes of *H. indicus* (8 weeks)

BA (µM)	Kn (µM)	No. of shoots/explant (Mean ± SE)*	% Response
0	0	0.58±0.22 f	41.67
5	0	1.17±0.16 ef	91.67
10	0	1.83±0.16 cdef	100
15	0	1.42±0.18 def	91.67
20	0	1.08±0.18 f	83.33
0	5	0.83±0.16 f	75
0	10	1.58±0.28 def	83.33
0	15	1.33±0.30 def	83.33
0	20	0.83±0.26 f	58.33
5	5	2.00±0.51 bcdef	58.33
5	10	2.17±0.47 bcdef	66.67
5	15	1.75±0.47 cdef	66.67
5	20	1.08±0.32 f	50
10	5	5.42±0.36 a	100
10	10	4.67±0.54 ab	100
10	15	4.33±0.48 abc	91.67
10	20	3.83±0.79 abcde	83.33
15	5	3.92±0.61 abcd	83.33
15	10	3.25±0.72 abcdef	75
15	15	3.17±0.79 abcdef	75
15	20	2.58±0.59 bcdef	75
20	5	3.00±0.61 abcdef	83.33
20	10	3.25±0.59 abcdef	75
20	15	2.83±0.73 abcdef	66.67
20	20	2.25±0.68 bcdef	50

*Means (n=12) were subjected to ANOVA and means followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test.

Replacing the cytokinin in the medium decreased the shoot number to 1.58±0.28 in 83.33% cultures at 10 µM Kn, which is less in comparison to BA fortified medium. Similar result has been reported for *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012), *Pogostemon cablin* (Jin et al., 2014) and *Paederia foetida* (Behera et al., 2018).

Combining BA and Kn induced better response in comparison and multiple shoots were formed in which combination of BA (10 µM) with Kn (5 µM) formed optimum 5.42±0.36 shoots in 100% cultures. Similarly earlier study on *in vitro* nodes of *Vanilla planifolia* has documented that BA and Kn individually induced shoots but maximum

response was observed when medium was fortified with combination of BA and Kn (Abebe et al., 2009). In *Pogostemon cablin*, Swamy et al. (2010) have also reported that combining BA and Kn induced optimum shoots. The observations of the present study also revealed that the regeneration potency of *in vitro* nodes is less as compared to *in vivo* nodes which is contrary to the earlier results of Sreekumar et al. (2000) and Nagahatenna and Peiris (2007) for the same plant. *In vitro* nodes formed less callus at base as compared to *in vivo* node, which is opposite to earlier report on *Lavandula pedunculata* (Zuzarte et al., 2010).

In vivo nodal explants regenerated optimum shoots in medium supplemented with BA (10 µM) and Kn (5 µM). Similarly when *in vitro* nodes were placed in cytokinin fortified medium, optimum shoot regeneration was observed in medium having same PGR concentration. This also suggested that *in vitro* nodes have potency to form shoots, however the potency is less in comparison to *in vivo* nodal explant.

After shoot regeneration the shoots regenerated from leaf and nodal explants were utilized for rooting studies.

4.1.3 *In Vitro* Rooting

The elongated microshoots were placed in full, half and quarter strengths of liquid MS medium were fortified with sucrose (1%) for rooting. They failed to survive after second week in different strength basal media, thus it was fortified with different concentrations (1-25 µM) of auxins like IBA and NAA, and their effect on root induction was observed till four weeks.

4.1.3.1 IBA

When different concentrations of IBA (1-25 µM) were tried in full strength medium lower concentrations of IBA (1-6 µM) failed to induce any roots and shoots failed to survive after two weeks. Increasing the IBA concentration to 8 µM increased the shoot survival rate to 25% but they failed to induce roots. IBA at 10 µM was able to induce only few roots (1.42±0.45) with 50% response, but only 33.33% shoots survived. The root number increased when IBA was at 15 µM as 1.67±0.50 roots (50% response) were formed with 41.67% shoots survival rate. Further increase in IBA concentration to 20 and 25 µM adversely affected the rooting and at 20 µM only 0.92±0.38 roots (33.33% response) with 33.33% shoot survival was observed. Whereas the shoots turned brown

after two weeks without any root formation in medium fortified with 25 µM of IBA (Table 10).

Table 10: Effect of different strengths of MS medium fortified with IBA on root induction from *H. indicus* shoots (4 weeks)

IBA (µM)	No. of roots/shoot (Mean ± SE)*	% Rooting	% Shoot survival
MS			
0	0 d	0	0
1-6	0 d	0	0
8	0 d	0	25
10	1.42±0.45 a	50	33.33
15	1.67±0.50 a	50	41.67
20	0.92±0.38 ab	33.33	33.33
25	0 d	0	0
½MS			
0	0 d	0	0
1-6	0 d	0	0
8	2.00±0.87 ab	33.33	50
10	3.33±0.89 abc	58.33	58.33
15	3.75±0.96 abc	58.33	75
20	4.17±0.90 abc	66.67	83.33
25	2.17±0.75 abcd	41.67	66.67
¼MS			
0	0 d	0	0
1-6	0 d	0	0
8	3.58±0.93 abcd	58.33	66.67
10	4.67±1.05 acd	66.67	91.67
15	6.00±0.89 cef	83.33	100
20	8.83±0.28 e	100	100
25	4.33±0.83 abcdf	75	83.33

*Means (n= 12) followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD test.

When the strength was reduced to half, it evoked a slight better response but the lower concentrations of IBA (1-6 µM) failed to show rhizogenesis in this strength, whereas but 8 to 20 µM induced roots in shoots. Further increase in IBA concentration to 25 µM proved to adversely affect the rooting of shoots. In ½MS medium fortified 8 µM IBA, 50% of shoots survived till four weeks out of which 33.33% shoots formed a total of 2.00±0.87 roots. This root number increased to 3.33±0.89 at 10 µM with 58.33% shoot survival and

rooting response. Further increase in IBA level to 15 and 20 µM evoked a better response and at 15 µM as 3.75±0.96 roots were observed with 75% shoot survival and 58.33% rooting response. IBA at 20 µM induced better rooting with 4.17±0.90 (66.67% response) roots and 83.33% shoot survival. Increasing the IBA concentration to 25 µM declined the root number to 2.17±0.75 as well as % of shoot survival (66.67%) and rooting response (41.67%) (Table 10).

Further reduction in strength of medium to quarter evoked early and better rooting as compared to earlier strengths. In this, swelling of nodes along with increase in shoot length was observed in first week. Root induction started in second week along with further increase in shoot length. In the next week, growth of root and shoot was observed which continued till fourth week. Similar to above two strengths, ¼MS medium fortified with lower IBA concentrations (1-6 µM) failed to induce roots and turned brown. A 8 µM IBA induced 3.58±0.93 roots with 58.33% rooting response and 66.67% shoots survival. Number of roots was increased to 4.67±1.05 (66.67%) with 91.67% shoots survival in the medium fortified with IBA (10 µM). Increasing IBA level to 15 µM proved to be beneficial for shoot survival as 100% shoots survived and induced 6.00±0.89 roots in 83.33% cultures. Optimum rooting was observed at 20 µM IBA which formed 8.83±0.28 roots with 100% rooting and shoot survival rate (Fig. 23a). Further increase in IBA concentration to 25 µM failed to increase the rooting of shoots (Table 10).

4.1.3.2 NAA

NAA is another auxin which is known to induce roots in microshoots and hence different strengths of MS medium fortified with 1-25 µM concentrations of NAA were tried. This auxin evoked a less and delayed rooting as compared to medium containing IBA and at lower concentrations i.e. 1 and 2 µM only 0.33±0.14 and 0.83±0.26 roots were formed respectively with less % response (33.33 and 50%). In both the combinations, the shoot survival rate is also less which was only 25% and 33.33% for 1 and 2 µM respectively. Increasing the NAA to 4 µM slightly increased the root formation and 1.17±0.28 roots with 66.67% shoot survival and rooting response was observed. The root number was increased to 2.08±0.30 along with 83.33% shoot survival and rooting response at NAA 6 µM. Optimum number of roots i.e. 3.42±0.55 in 91.67% cultures with 100% shoot survival was observed when NAA was increased to 8 µM (Fig. 23b). Further increase in NAA concentration from 10-20 µM decreased the rooting in shoots and NAA at

25 µM adversely affected the shoots as they turned brown after second week without root induction (Table 11).

Reducing the strength to ½MS failed to increase the rooting in shoots and only 0.92±0.36 roots were observed with 50% shoot survival and 41.67% rooting response at 10 µM NAA. In this strength also shoots turned brown after two weeks and no root formation was observed at 25 µM concentration (Table 11). Further reduction in MS strength to quarter also induced meagre response and only 0.50±0.25 roots with 33.33% rooting and shoot survival response was observed in medium fortified with 6 µM NAA. In this strength, 15-25 µM NAA concentrations adversely affected the shoots and they failed to survive within two weeks in this strength (Table 11).

Basal MS medium of different strengths (full, half and quarter) failed to induce rooting and is also reported for *Ajuga bracteosa* (Kaul et al., 2013) and *Rubia cordifolia* (Khadke et al., 2013) microshoots. *In vitro* rooting can be achieved in medium fortified with auxins like IBA and NAA (George and Sherrington, 1984) and it is documented for plants like *Astragalus cariensis* (Erisen et al., 2010), *Achyranthes aspera* (Sen et al., 2014) and *Allamanda cathartica* (Khanam and Anis, 2018).

Fortifying the media with different IBA concentrations induced rhizogenesis and optimum rooting with 8.83±0.28 roots (100% response) was observed in ¼MS medium fortified with 20 µM of IBA. Earlier studies on the same plant by Sreekumar et al. (2002) also reported ¼MS medium with IBA at 9.80 µM to be optimum for rooting (12 roots with 98% frequency). Similarly Shekhawat and Manokari (2016) also reported this strength supplemented with IBA (3 mg/l) to be optimum for rooting in *H. indicus*. Promotory effect of ¼MS media has been well reported in other medicinal plants like *Wrightia tinctoria* (Purohit and Kukda, 2004), *Bacopa monnieri* (Mehta et al., 2012b), *Terminalia bellerica* (Mehta et al., 2012c), *Catharanthus roseus* (Sain and Sharma, 2013) and *Leptadenia reticulata* (Rathore et al., 2013; Patel et al., 2014).

Earlier studies also documented IBA for rooting in *H. indicus* (Saha et al., 2003; Rama Devi et al., 2014; Sundarmani and Hasina, 2015), *Tylophora indica* (Faisal and Anis, 2003), *Stevia rebaudiana* (Patel and Shah, 2009) and *Camellia sinensis* (Bidarigh and Azarpuor, 2013) shoots. Whereas report Misra et al. (2003) stated that IBA is less responsive and optimum rooting (5.20±0.84 roots, 98% response) was reported when ½MS medium was fortified with 0.75% sucrose, IBA (2.0 mg/l), NAA (1.0 mg/l) and activated charcoal (100 mg/l) in *H. indicus*. Whereas combination of Kn with IBA was reported in

other studies on same plant (Patnaik and Debata, 1996; Siddique et al., 2006; Siddique and Bari, 2006).

Table 11: Effect of different strengths of MS medium fortified with NAA on root induction from *H. indicus* shoots (4 weeks)

NAA (µM)	No. of roots/shoot (Mean ± SE)*	% Response	% Shoot survival
MS			
0	0 d	0	0
1	0.33±0.14 cd	33.33	25
2	0.83±0.26 bcd	50	33.33
4	1.17±0.28 bcd	66.67	66.67
6	2.08±0.30 b	83.33	83.33
8	3.42±0.55 a	91.67	100
10	1.33±0.40 bc	50	83.33
15	1.08±0.38 bcd	41.67	66.67
20	0.83±0.39 bcd	33.33	50
25	0 d	0	25
½MS			
0	0 d	0	0
1	0.33±0.18 cd	25	25
2	0.42±0.22 cd	25	25
4	0.50±0.22 cd	33.33	33.33
6	0.67±0.30 cd	33.33	41.67
8	0.75±0.17 cd	66.67	41.67
10	0.92±0.36 bcd	41.67	50
15	0.58±0.25 cd	33.33	33.33
20	0.17±0.11 cd	16.67	16.67
25	0 d	0	0
¼MS			
0	0 d	0	0
1	0.17±0.16 cd	8.33	16.67
2	0.25±0.17 cd	16.67	25
4	0.33±0.18 cd	25	25
6	0.50±0.25 cd	33.33	33.33
8	0.42±0.18 cd	33.33	41.67
10	0.17±0.11 cd	16.67	33.33
15-25	0 d	0	0

*Means (n=12) were subjected to ANOVA and means followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 23: Rooting of shoots after four weeks- (a) formation of multiple roots in ¼MS medium fortified with sucrose (1%) and IBA (20 µM) and (b) formation of few roots in MS medium fortified with sucrose (1%) and NAA (8 µM).

Amongst different concentrations of NAA, maximum of 3.42±0.55 roots were formed in medium containing 10 µM NAA and further increase in NAA level adversely affected the root formation as it may increase ethylene biosynthesis which affects root formation and/or elongation (Riov and Yang, 1989; Kollmeier et al., 2000; Taiz and Zeiger, 2003). However the number of roots is less in NAA fortified medium as compared to IBA fortified medium which is comparable with earlier report on same plant (Sreekumar et al., 2002; Nagahatenna and Peiris, 2007; Sundarmani and Hasina, 2015). Superiority of IBA over NAA has been reported in *T. indica* (Thomas and Philip, 2005), *Munronia pinnata* (Gunathilake et al., 2008) and *Rubia cordifolia* (Khadke et al., 2013). The reason is due to the slow movement and degradation of IBA which facilitates its localization near the site of application and in turn increases the rooting (Martin, 2002). Whereas in plants like *Decalepis hamiltonii* (Anitha and Pullaiah, 2002), *Vitex trifolia* (Hiregoudar et al., 2006) and *S. rebaudiana* (Thiyagarajan and Venkatachalam, 2012) optimum rooting was reported in NAA supplemented medium. Whereas Sahai et al. (2010) reported same

number of root formation in *T. indica* when IBA (0.5 µM) and NAA (5 µM) was used as a rooting hormone.

IBA and NAA induced rooting in *H. indicus* shoots, however IBA was proved to be better as compared to NAA as it induced more roots with better response for shoot survival.

In the regeneration studies on *H. indicus* optimization of protocol for shoot formation from both leaf and nodal explant regenerated shoots, but the mode or regeneration is indirect for leaf explant, which can generate of somaclonal variants. Whereas nodal explants regenerate direct shoot regeneration from axillary buds without callus intervention and hence further secondary metabolite studies were carried out using shoot cultures developed from nodal explants.

4.2 SECONDARY METABOLITE STUDIES

In this study, *in vitro* shoots regenerated from nodal explants were first qualitatively analyzed for their potential to synthesize metabolites using HPTLC fingerprinting. After that, quantification of lupeol and rutin in shoots was done followed by elicitation of same using yeast extract, salicylic acid and methyl jasmonate.

MS medium fortified with sucrose (3%), BA (10 µM) and Kn (5 µM) regenerated optimum shoots from nodal explants (MS-1), but maximum shoot length and leaf broadening in shoots was observed in medium fortified with IAA (2 µM). Thus the shoots from optimized medium were transferred to medium containing IAA (2 µM) and allowed to grow for another eight weeks (MS-2). *In vitro* shoots from both these media were utilized for HPTLC fingerprinting to observe their effect on synthesis of metabolites.

4.2.1 HPTLC Fingerprint of Shoots

The chemical profiling of three samples i.e. *in vivo* and *in vitro* shoots from MS-1 and MS-2 media were extracted 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 respective mobile phases followed by derivatization using anisaldehyde-sulphuric acid reagent. Then the plates were scanned at 525 nm for densitometry analysis and the results are summarized below.

Hexane extracts of all samples were spotted on TLC plates and developed in mobile phase toluene:methanol (9:1 v/v). The plates were derivatized to obtain a fingerprint as shown in Fig. 24a, b which revealed almost similar profiling in both the samples in comparison to extracts of *in vivo* shoots. However scanning of the plates was done to obtain the chromatograms of all the samples (Fig. 24c, d, e), which revealed variation in peak numbers, their heights and areas. Extract of *in vivo* shoots revealed total 13 peaks of which peaks at Rf 0.14, 0.24, 0.29, 0.33, 0.37, 0.47, 0.53, 0.58, 0.82 and 0.90 were unique and absent in extracts of shoots from MS-1 and MS-2. Peak at Rf 0.58 (11^{th} peak) had maximum area of 20049.4 which was 24.17% of total area of the chromatogram in extract of *in vivo* shoots, however this peak was absent in extracts of both MS-1 and 2. Whereas area of peak at Rf 0.02 decreased in extract of MS-1 and was absent in extract of MS-2 (Table 12). When extract of *in vitro* shoots from MS-1 was separated, total 19 bands were observed, of which bands at Rf 0.04, 0.11, 0.13, 0.21, 0.25, 0.34, 0.38, 0.48, 0.54, 0.60, 0.77, 0.86 and 0.93 were unique in this extract. In this extract, peak at 0.60 Rf (15^{th} peak) covered the maximum area i.e. 12926.2 (17.67%) which was absent in other two extracts. Separating extract of shoots from MS-2 revealed total 16 peaks. Out of 16 peaks, peaks at Rf 0.08, 0.15, 0.18, 0.30 and 0.74 is common between MS-1 and 2, whereas peaks at 0.05, 0.10, 0.22, 0.42, 0.52, 0.66, 0.75, 0.85 and 0.92 were unique in this extract. Maximum 19371.2 area (21.44%) was covered by peak at Rf 0.42, which was not present in *in vivo* and *in vitro* shoot from MS-1 (Table 12). In hexane extract, none of the peaks were common for all three samples, which might be due to effect of PGRs of the medium which not only affects the peak areas but also affects the type of secondary metabolites.

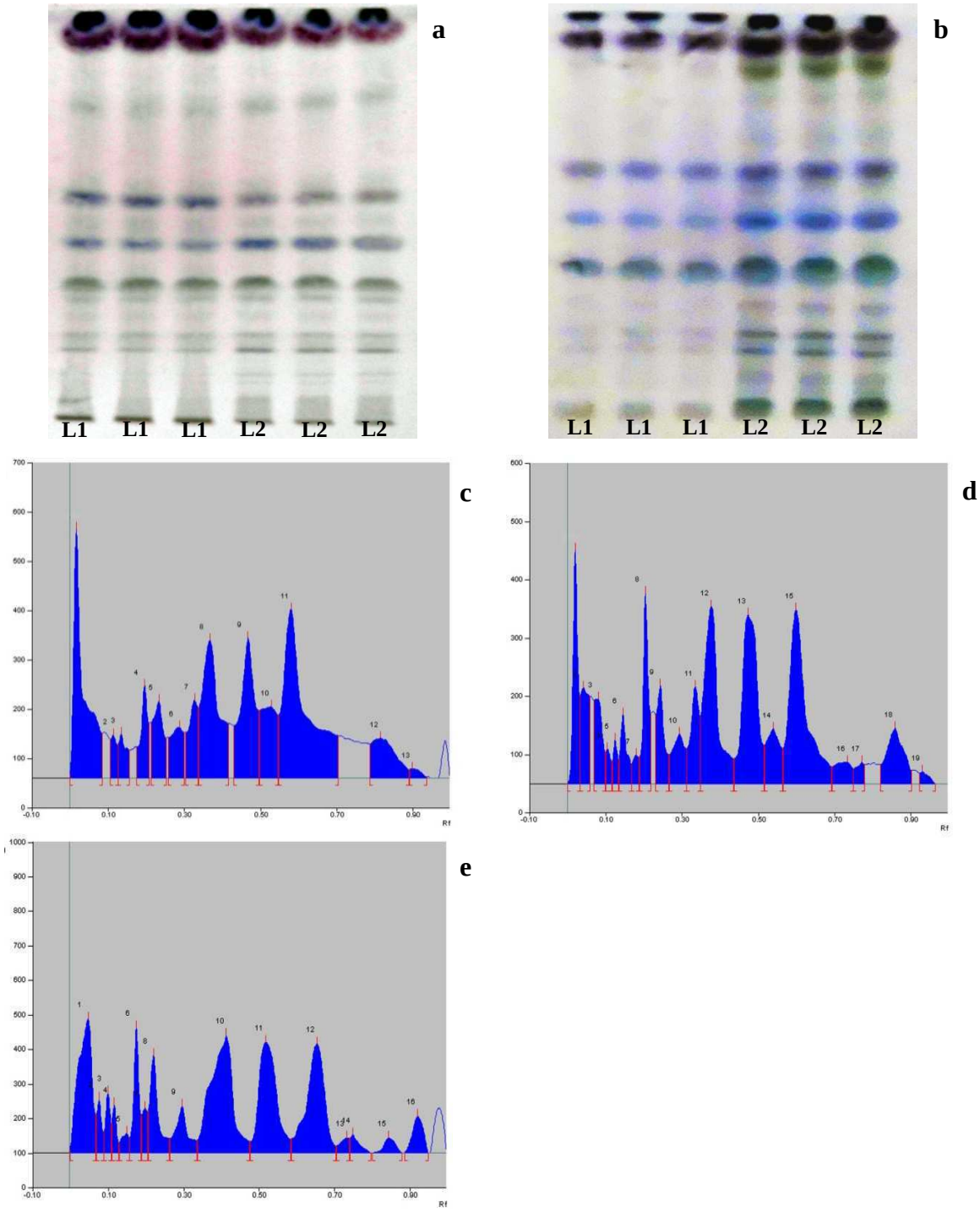

Figure 24: HPTLC fingerprint of hexane extracts- (a) *in vivo* shoots (L1) and *in vitro* shoots from MS-1 (L2), (b) *in vivo* shoots (L1) and *in vitro* shoots from MS-2 (L2). Densitometry scanning of plates at 525 nm and respective peaks of (c) *in vivo* shoots, (d) *in vitro* shoots from MS-1 and (e) *in vitro* shoots from MS-2.

Table 12: Fingerprint of different samples for hexane extracts

Rf value	*In vivo*		*In vitro* (MS-1)		*In vitro* (MS-2)	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	12946.7	15.61	4774.0	6.53	-	-
0.04	-	-	3455.1	4.72	-	-
0.05	-	-	-	-	13331.2	14.76
0.08	-	-	3027.1	4.14	1887.0	2.09
0.10	-	-	-	-	2061.2	2.28
0.11	-	-	713.9	0.98	-	-
0.12	1401.0	1.69	-	-	1422.2	1.57
0.13	-	-	799.6	1.09	-	-
0.14	1726.5	2.08	-	-	-	-
0.15	-	-	1883.3	2.57	1063.9	1.18
0.18	-	-	715.8	0.98	4782.3	5.29
0.20	3236.0	3.90	-	-	1695.8	1.88
0.21	-	-	4292.2	5.87	-	-
0.22	-	-	-	-	5757.2	6.37
0.24	4087.7	4.93	-	-	-	-
0.25	-	-	3124.6	4.27	-	-
0.29	3295.5	3.97	-	-	-	-
0.30	-	-	2589.0	3.54	4045.6	4.48
0.33	3599.9	4.34	-	-	-	-
0.34	-	-	3507.1	4.80	-	-
0.37	11627.3	14.02	-	-	-	-
0.38	-	-	10376.7	14.19	-	-
0.42	-	-	-	-	19371.2	21.44
0.47	9783.0	11.79	-	-	-	-
0.48	-	-	10936.0	14.95	-	-
0.52	-	-	-	-	14498.5	16.05
0.53	5719.3	6.90	-	-	-	-
0.54	-	-	3137.8	4.29	-	-
0.58	20049.4	24.17	-	-	-	-
0.60	-	-	12926.2	17.67	-	-
0.66	-	-	-	-	14171.2	15.69
0.74	-	-	1562.1	2.14	1030.2	1.14
0.75	-	-	-	-	1127.0	1.25
0.77	-	-	758.6	1.04	-	-
0.82	4955.6	5.97	-	-	-	-
0.85	-	-	-	-	1222.5	1.35
0.86	-	-	4103.6	5.61	-	-
0.90	514.5	0.62	-	-	-	-
0.92	-	-	-	-	2879.3	3.19
0.93	-	-	457.7	0.63	-	-

Another extract was prepared using ethyl acetate and were separated in mobile phase toluene:ethyl acetate:formic acid (6.5:2.5:1 v/v). The plates were derivatized to obtain bands (Fig. 25a, b) followed by scanning at 525 nm for development of chromatograms (Fig. 25c, d, e). It was observed that total 16 peaks were present in extract of *in vivo* shoots out of which peak at Rf 0.62 have maximum i.e. 34096.8 area which covered 28.58% of total area which was absent in other two extracts. Amongst all, the peaks appearing at Rf 0.32, 0.36 and 0.62 were only present in extract of *in vivo* shoots, whereas peak at Rf 0.53 is present in all samples (Table 13).

When extract of shoots from MS-1 were separated total 16 bands were developed in plate as well as respective peaks in chromatogram. Out of 16 peaks, total 5 peaks at Rf 0.03, 0.06, 0.35, 0.75 and 0.84 were observed only in this extract. Maximum peak area was observed for 14^{th} peak (Rf = 0.75) having 15495.9 area (13.03%) in the chromatogram, but was absent in extracts of *in vivo* and *in vitro* shoots from MS-1 (Table 13). Whereas extract of *in vitro* shoots from MS-2 had total 15 peaks out of which peak no. 13^{th} (Rf 0.72) had maximum peak area (18962.6, 19.65%) which was reduced in extract of *in vivo* shoots i.e. 8363.8 (7.01%), and was absent in extract of MS-1 (Table 13). Similarly peaks at Rf 0.02, 0.19, 0.25, 0.27, 0.33, 0.38, 0.47 and 0.99 were observed only in this extract and hence unique for it. Peaks at 0.31 and 0.63 were common between both the samples of *in vitro* shoots, whereas peak at 0.53 was common in all three samples (Table 13).

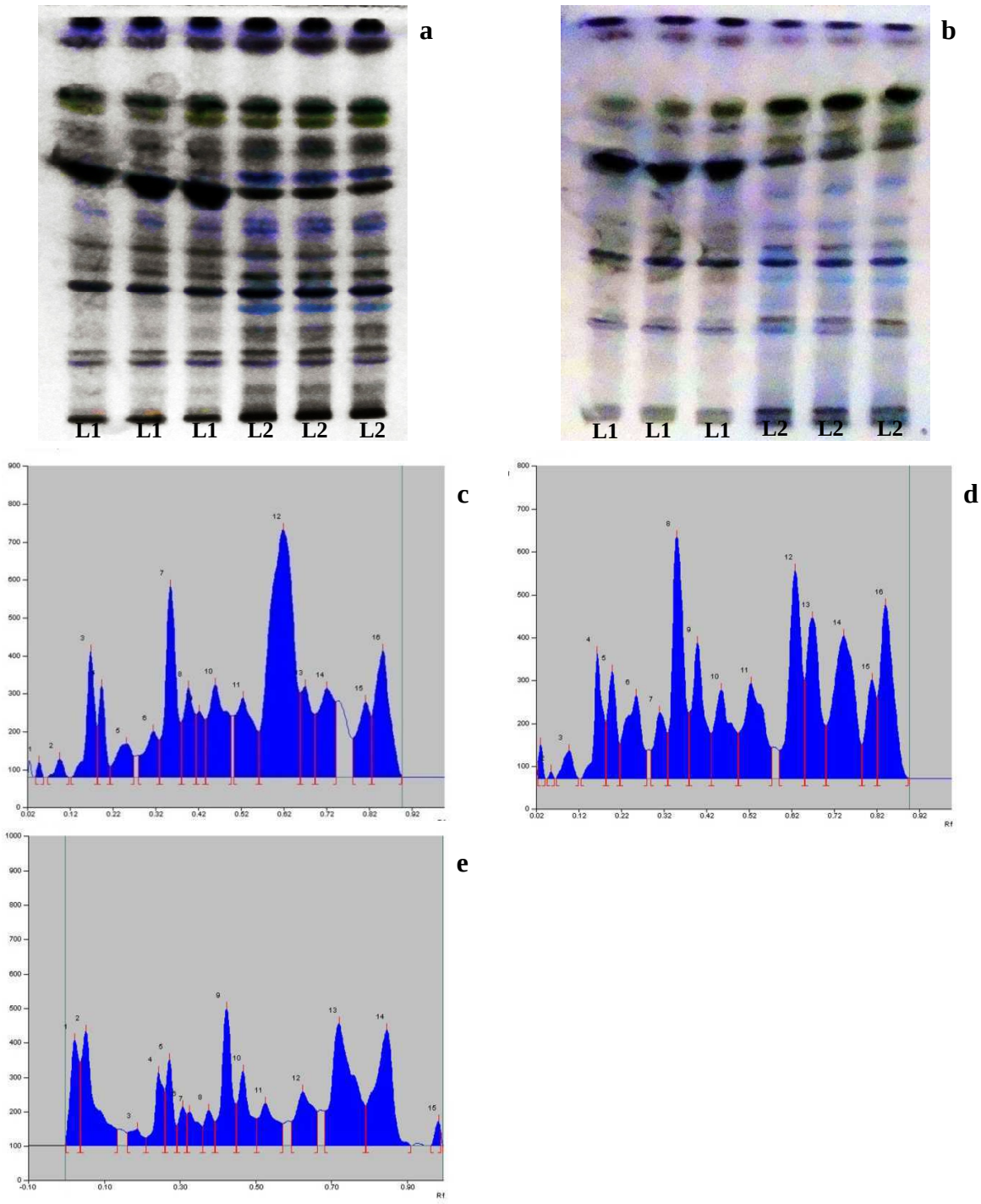

Figure 25: HPTLC fingerprint of ethyl acetate extracts- (a) *in vivo* shoots (L1) and *in vitro* shoots from MS-1 (L2), (b) *in vivo* shoots (L1) and *in vitro* shoots from MS-2 (L2). Densitometry scanning of plates at 525 nm and respective peaks of (c) *in vivo* shoots, (d) *in vitro* shoots from MS-1 and (e) *in vitro* shoots from MS-2.

Table 13: Fingerprint of different samples for ethyl acetate extracts

Rf value	*In vivo*		*In vitro* (MS-1)		*In vitro* (MS-2)	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.02	-	-	-	-	6488.0	6.72
0.03	-	-	616.6	0.56	-	-
0.05	346.1	0.29	-	-	11279.4	11.69
0.06	-	-	127.5	0.11	-	-
0.10	826.1	0.69	1424.9	1.20	-	-
0.17	5950.3	4.99	5259.7	4.42	-	-
0.19	-	-	-	-	1535.0	1.59
0.20	3480.2	2.92	4733.9	3.98	-	-
0.25	-	-	-	-	4468.5	4.63
0.26	3083.4	2.58	6832.1	5.75	-	-
0.27	-	-	-	-	4244.4	4.40
0.31	-	-	3868.6	3.25	2036.2	2.11
0.32	3609.2	3.02	-	-	-	-
0.33	-	-	-	-	2581.6	2.67
0.35	-	-	13710.4	11.53	-	-
0.36	11639.3	9.76	-	-	-	-
0.38	-	-	-	-	2244.4	2.33
0.40	5480.9	4.59	8322.0	7.00	-	-
0.43	2954.3	2.48	-	-	9694.9	10.04
0.46	9333.3	7.82	7557.9	6.36	-	-
0.47	-	-	-	-	5821.9	6.03
0.53	8136.5	6.82	9739.3	8.19	5024.3	5.21
0.62	34096.8	28.58	-	-	-	-
0.63	-	-	13381.1	11.25	6761.5	7.01
0.67	5791.4	4.85	10876.0	9.15	-	-
0.72	8363.8	7.01	-	-	18962.6	19.65
0.75	-	-	15495.9	13.03	-	-
0.81	5706.1	4.78	5043.3	4.24	-	-
0.84	-	-	11865.4	9.98	-	-
0.85	10513.3	8.81	-	-	14475.6	15.00
0.99	-	-	-	-	900.6	0.93

Separating methanolic extracts of all shoots in toluene:ethyl acetate:formic acid (6.8:2.5:0.7 v/v) revealed bands having almost similar profile in all samples (Fig. 26a, b). However scanning of the plates at 525 nm developed chromatograms which depicted change in peak number and areas (Fig. 26c, d, e). When extract of *in vivo* shoots was separated on TLC, total 14 bands were observed in which 12^{th} peak at Rf 0.74 has maximum peak area of 31886.0 which covered 27.37% area of the chromatogram. In this extracts total 7 unique peaks were recorded which were at Rf 0.02, 0.31, 0.37, 0.48, 0.57, 0.61 and 0.88 (Table 14).

Scanning the extract of MS-1 shoots confirmed total 16 bands of which peaks appeared at Rf 0.04, 0.08, 0.14, 0.20, 0.34, 0.50, 0.59, 0.65, 0.78 and 0.95 were only observed in this extract. In this extract also, peak at 0.74 Rf had maximum area i.e. 13517.6 (19.23%) which was less as compared to area of the same peak in extract of *in vivo* shoots (Table 14). Lastly extract of *in vivo* shoots from MS-2 was scanned and it was observed that it contained total 18 peaks. Out of these, peaks at Rf 0.45 and 0.74 were present in all three samples whereas peak at Rf 0.39 was present in both the *in vitro* cultures (Table 14). In this extract also peak at 0.74 Rf had maximum area but this area (13517.6, 19.23%) was decreased in comparison to extract of *in vivo* shoots but increased when compared to *in vitro* shoots from MS-1. However peaks at Rf 0.01, 0.03, 0.13, 0.25, 0.30, 0.35, 0.49, 0.54, 0.60, 0.62, 0.64, 0.85 and 0.89 were only present in this extract (Table 14).

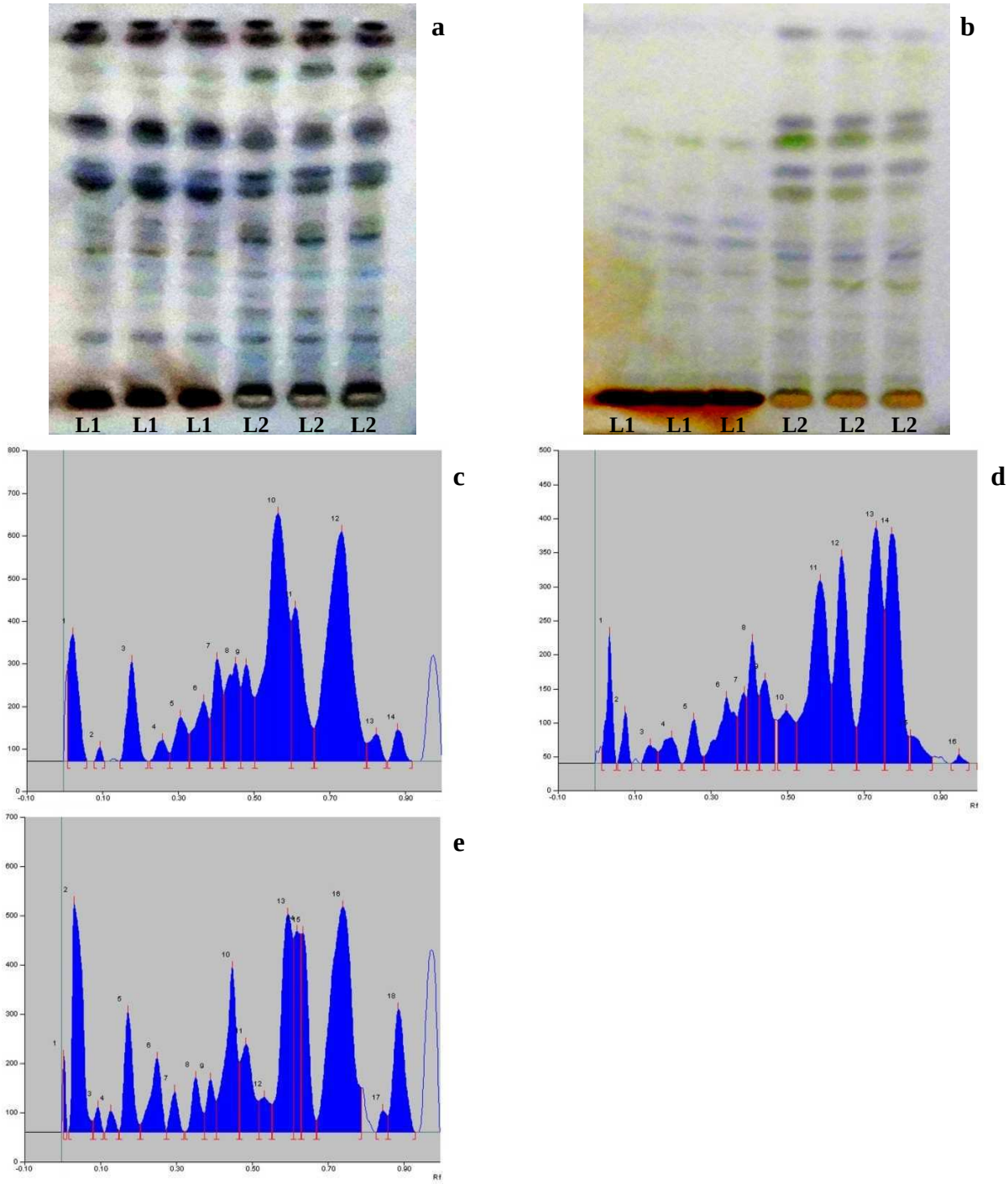

Figure 26: HPTLC fingerprint of methanol extracts- (a) *in vivo* shoots (L1) and *in vitro* shoots from MS-1 (L2), (b) *in vivo* shoots (L1) and *in vitro* shoots from MS-2 (L2). Densitometry scanning of bands at 525 nm and respective peaks of (c) *in vivo* shoots, (d) *in vitro* shoots from MS-1 and (e) *in vitro* shoots from MS-2.

Table 14: Fingerprint of different samples for methanol extracts

Rf value	*In vivo*		*In vitro* (MS-1)		*In vitro* (MS-2)	
	Peak area	Peak area (%)	Peak area	Peak area (%)	Peak area	Peak area (%)
0.01	-	-	-	-	686.7	0.67
0.02	7483.0	6.42	-	-	-	-
0.03	-	-	-	-	11287.3	10.97
0.04	-	-	2515.4	3.58	-	-
0.08	-	-	1034.4	1.47	-	-
0.10	380.1	0.33	-	-	689.0	0.67
0.13	-	-	-	-	715.0	0.70
0.14	-	-	671.3	0.95	-	-
0.18	5379.4	4.62	-	-	4850.8	4.72
0.20	-	-	1287.9	1.83	-	-
0.25	-	-	-	-	4012.0	3.90
0.26	1253.9	1.08	1353.5	1.93	-	-
0.30	-	-	-	-	1509.3	1.47
0.31	2972.7	2.55	-	-	-	-
0.34	-	-	3855.4	5.48	-	-
0.35	-	-	-	-	2304.0	2.24
0.37	4628.2	3.97	-	-	-	-
0.39	-	-	1841.5	2.62	2032.7	1.98
0.41	5562.6	4.77	3635.0	5.17	-	-
0.45	7293.6	6.26	3365.5	4.79	9043.3	8.79
0.48	5697.0	4.89	-	-	-	-
0.49	-	-	-	-	5334.0	5.19
0.50	-	-	2811.1	4.00	-	-
0.54	-	-	-	-	1808.9	1.76
0.57	29374.4	25.21	-	-	-	-
0.59	-	-	12000.4	17.07	-	-
0.60	-	-	-	-	12683.4	12.33
0.61	11040.3	9.48	-	-	-	-
0.62	-	-	-	-	6444.4	6.27
0.64	-	-	-	-	7239.2	7.04
0.65	-	-	9617.4	13.68	-	-
0.74	31886.0	27.37	13517.6	19.23	24496.5	23.82
0.78	-	-	11367.5	16.17	-	-
0.83	1773.4	1.52	1226.6	1.74	-	-
0.85	-	-	-	-	762.2	0.74
0.88	1774.7	1.52	-	-	-	-
0.89	-	-	-	-	6953.1	6.76
0.95	-	-	202.6	0.29	-	-

Thus chemical profiling of shoots revealed that the shoots were able to synthesize metabolites, however change in band/peak number and area depicted the variation amongst the samples. Reports suggested that HPTLC fingerprinting of the plants can be compared on the basis of total number of separated bands, its position, sequence and colour

(Mammen et al., 2011; Kamboj and Saluja, 2013). This fingerprinting technique has been approved by American and European pharmacopoeias as well as by WHO for qualitative and quantitative evaluation of herbal drugs (Waksmundzka-Hajnos et al., 2008; Kulkarni et al., 2014; Elela et al., 2015). In the present study three solvents with increasing polarities have been used as this is done when the compound of interest is unknown (Harborne, 1984; Williamson et al., 1996). It was revealed that chemical profiling of shoots from cytokinin based medium is nearly similar to *in vivo* samples as compared to shoots from auxin fortified medium. This may be due to influential effect of cytokinins on metabolites (Sangwan et al., 2001; Arikat et al., 2004). Report of Palenius and Alejo (2005) have suggested that the synthesis of a metabolite under *in vitro* conditions depends on many factors but the composition of the medium especially PGRs is one of the critical factor. Srivastava and Shrivastava (2008) have reported a similar variation in HPTLC profiling between wild and *in vitro* shoots of *Bacopa monnieri*.

Alagar et al. (2014) reported that *Curculigo orchioides* regenerated via different pathways showed slight variation in fingerprint. This is also reported for *in vitro* regenerated plants of *Curcuma longa* (Kharade et al., 2014), *Arnicae folium* and *A. caulis* (Stefanache et al., 2014) and *Celastrus paniculatus* (Anusha et al., 2016). *In vitro* callus, shoots and roots of *Oroxylum indicum* synthesized metabolites like wild plant parts but slight variation was observed in HPTLC profiling between them (Rojsanga et al., 2017). Similarly storage temperatures of synthetic seeds affected the secondary metabolite synthesis in *in vitro* plants of *Rauvolfia serpentina* (Gantait and Kundu, 2017). Whereas *in vitro* derived shoots and plants of *Passiflora caerulea* (Busilacchi et al., 2008), *Munronia pinnata* (Gunathilake et al., 2008) and *Sarcandra glabra* (Zhu et al., 2011) had similar HPTLC profiling as wild plants. This method has also been used for comparison between wild and *in vitro* cultures of other important medicinal plants like *Asparagus adscendens* (Mehta and Subramanian, 2005), *Bacopa monnieri* (Patni et al., 2010), *Chlorophytum borivilianum* (Basu and Jha, 2013) and *Curcuma angustifolia* (Jena et al., 2018).

Thus HPTLC fingerprinting revealed that *in vitro* cultures were able to synthesize metabolites like *in vivo* plants, however variation in peak areas depicted variation in synthesis of metabolites. This study also revealed that traditionally roots of *H. indicus* is being used and gained importance however the shoots also synthesizes large number of metabolites which needs to be further investigated. In the next experiments two important

metabolites reported for the plants viz. lupeol and rutin were quantified in *in vivo* as well as *in vitro* shoots (MS-1 and MS-2).

4.2.2 HPTLC Analysis for Lupeol in Shoot Cultures

Firstly the standard curve of lupeol was prepared to obtain the straight line equation followed by quantification of the same using HPTLC.

4.2.2.1 Standard curve of lupeol

The plate was loaded with 100-600 ng concentration of standard lupeol followed by development in toluene:methanol (9:1 v/v). They were derivatized using anisaldehyde-sulphuric acid reagent and scanned at 525 nm for standard curve preparation. The correlation coefficient (R^2) of the line was 0.995 and the equation of straight line was y = 11.18x+579.1 (Fig. 27).

4.2.2.2 Quantification of lupeol

Hexane extracts of *in vivo* as well as *in vitro* shoots from MS-1 and MS-2 were separated on TLC plats. When extract of *in vitro* shoots harvested from MS-1 media i.e. BA (10 µM) and Kn (5 µM), presence of lupeol was observed after derivatization in extracts of both *in vivo* as well as *in vitro* shoots (Fig. 28a). When the plates were analyzed at 525 nm for chromatogram development, peak of lupeol was observed (Fig. 28b). The AUC depicted the quantity of lupeol as 185±0.00 µg/gm in *in vivo* shoots, whereas in shoots from MS-1 (cytokinin fortified medium) it slightly increased to 187±0.01 µg/gm (Table 15). *In vitro* shoots were transferred from MS-1 to MS-2 (medium fortified with 2 µM IAA), and the TLC plates showed presence of lupeol in comparison to standard and *in vivo* shoots (Fig. 28c). Similarly densitometric scanning of plate revealed peaks of the same confirming the lupeol presence in cultures. However calculating the lupeol content revealed that contains extracts of *in vitro* shoots from MS-2 synthesized less amount of lupeol i.e. 53±0.00 µg/gm, in comparison to *in vivo* and *in vitro* shoots from MS-1 (Table 15).

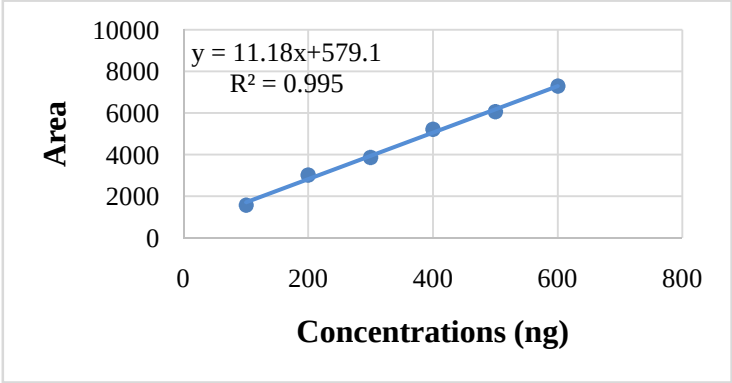

Figure 27: Standard curve of lupeol

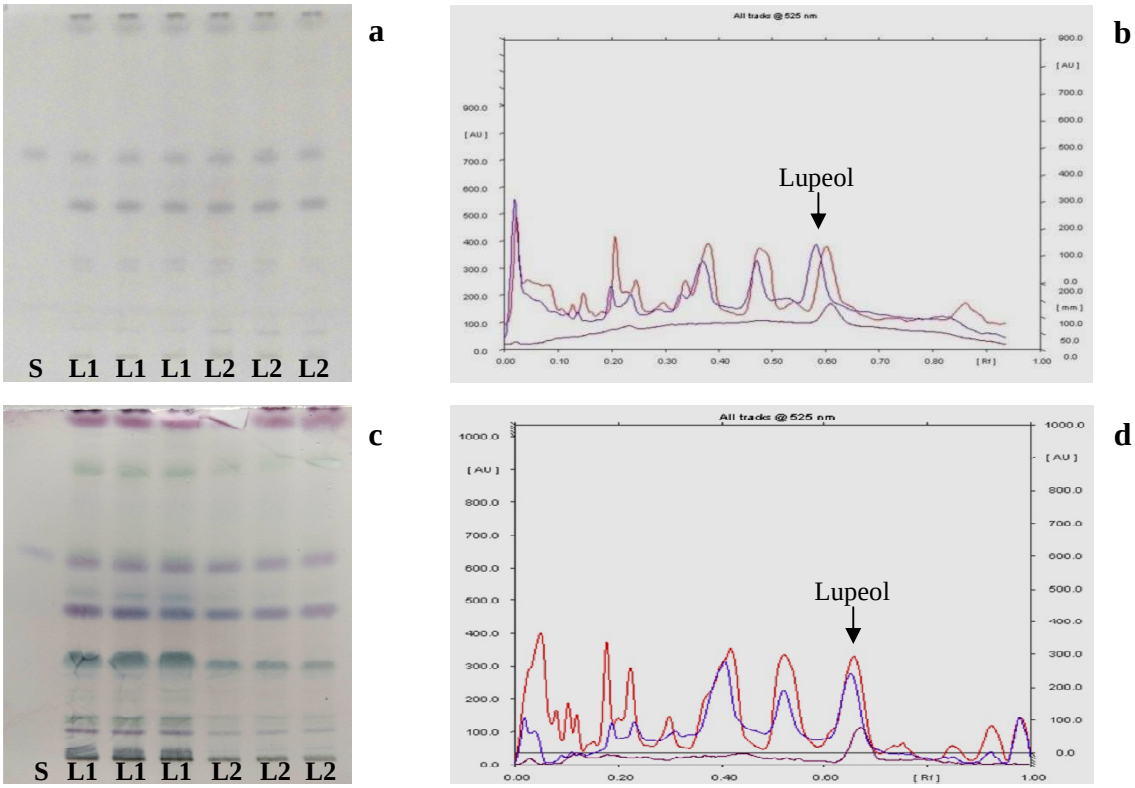

Figure 28: Quantification of lupeol in shoot cultures- (a) TLC plates containing lupeol standard (S), *in vivo* shoots (L1) and *in vitro* shoots from MS-1 (L2) in triplicate, (b) chromatogram of *in vivo* and *in vitro* shoots (MS-1) after scanning at 525 nm, (c) TLC plates containing lupeol standard (S), *in vivo* shoots (L1), *in vitro* shoots from MS-2 (L2) in triplicate and (d) chromatogram of *in vivo* and *in vitro* shoots (MS-2) after scanning at 525 nm. Red line- *in vivo* sample, blue line- *in vitro* samples.

Table 15: Lupeol content in different samples of *H. indicus*

Sample	Lupeol (µg/gm)* (Mean ± SE)
In vivo shoots	185±0.00 a
In vitro shoots (From MS-1)	187±0.01 a
In vitro shoots (From MS-2)	53±0.00 b

*Means (n=3) followed by same letter are not significantly different ($p \leq 0.05$) using Duncan's multiple range test.

The differentiated cells generally synthesize high amount of metabolites as compared to dedifferentiated cultures like callus (Lorence and Nessler, 2004; Liu et al., 2006; Pasqua et al., 2006). Palazon et al. (2006) reported that organogenesis is essential for steroidal saponin production in *Ruscus aculeatus* cultures. Similarly picroside was absent in dedifferentiated callus cultures and was only synthesized in redifferentiated cultures of *Picrorhiza kurroa* (Sood and Chauhan, 2009). Observations of the present study revealed that lupeol quantity varied in MS-1 and MS-2, and the reason may be the presence of PGRs in the medium which are known to affect the secondary metabolite synthesis under *in vitro* conditions (Dornenburg and Knorr, 1995; Lee et al., 2011). This result is in accordance with earlier report for the callus cultures of the same plant in which lupeol amount changes according to combinations and concentrations of cytokinins and auxins (Misra et al., 2005). Effect of PGRs on synthesis of bacoside-A has also been reported in shoot cultures of *Bacopa monnieri* (Parale and Nikam, 2009).

Amongst the two media tested, cytokinin based medium (eight weeks old shoots) had high content of lupeol as compared to *in vivo* shoots, which is comparable with earlier report for the same plant (Misra et al., 2003; Purohit et al., 2015). Medium fortified with BA and Kn had beneficial effect on synthesis of lupeol, similarly in shoots of *Hypericum hirsutum* and *H. maculatum* hypericin and pseudohypericin content increased in presence of these cytokinins (Coste et al., 2011). Dandin and Murthy (2012) also reported that synthesis of andrographolide was more in shoot cultures of *Andrographis paniculata* in same PGR combination. Presence of cytokinins in the medium helped to enhance secondary metabolite synthesis in shoot cultures of *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., 2017). Recently Cioć et al. (2018) has reported that BA significantly increased the content of different phenolic acids in shoot cultures of *Myrtus communis*.

When the shoots were transferred from cytokinin (MS-1) to auxin (MS-2) based medium, it adversely affected the lupeol synthesis. Similarly Grover et al. (2012) have reported that synthesis of terpenoids varies according to PGRs in suspension cultures of *Camellia sinensis*. Report of Gadzovska et al. (2005) states that hypericin and pseudohypericin content increased in shoots of *Hypericum perforatum* in BA fortified medium, which decreased when shoots were transferred to IAA or IBA fortified medium. Whereas Singh and Chaturvedi (2013) reported that presence of auxin along with cytokinin adversely affected the synthesis of azadirachtin in callus culture of *Azadirachta indica*.

Another reason which may decrease the content of lupeol is age of the culture, as the synthesis of many metabolites is influenced by number of subcultures (Bourgaud et al., 2001). Analysis of 16 weeks old shoots (MS-2 media) revealed that the lupeol content decreased and similar variation in lupeol content for eight weeks callus cultures of *Cryptostegia grandiflora* as compared to six weeks is reported by Singh et al. (2011a). The age of culture also affected the synthesis of lupeol in *Euphorbia tirucalli* (Uchida et al., 2010) as well as other metabolites like vincristine in *Catharanthus roseus* (Aslam et al., 2009), cerpegin in *Ceropegia juncea* (Nikam and Savant, 2009) and galanthamine in *Leucojum aestivum* (Ivanov et al., 2013).

It was concluded that *in vitro* shoots from both MS-1 and MS-2 were able to synthesize lupeol, and the content in shoots grown on cytokinin based medium (MS-1) in comparison to shoots from auxin fortified medium (MS-2) was high. Thus it was confirmed that the auxin based medium facilitated the growth of shoots but it adversely affected lupeol synthesis. Further elicitation experiments were carried out on eight weeks old shoots grown in medium fortified with BA (10 µM) and Kn (5 µM).

4.2.2.3 Elicitation studies for lupeol

In vitro shoots developed in MS-1 medium were fed with elicitors as they are known to enhance the secondary metabolites. The cultures were kept on shaker at 100 rpm and harvested after first, second and third week. Fresh and dry weights (FW/DW) were recorded and dried shoots were powdered and extracted for analysis of lupeol and rutin content.

MS liquid medium fortified with BA (10 µM) and Kn (5 µM) was used as a control and shoot cultures were grown in this medium for a period of 3 weeks. The observation revealed that the shoots started to grow during second week and elongated by the end of

three weeks (Fig. 29a, b, c). FW was 3.54±0.42 gm at the end of three weeks and DW reached to 0.468±0.05 gm (Fig. 31). The extracts of different weeks were prepared and separated on TLC plate which confirmed the presence of lupeol in all the samples after derivatization (Fig. 30a). Densitometric scanning of the plate was done at 525 nm which generated chromatogram of the same (Fig. 30b). Lupeol quantity was calculated using respective peak area for each sample and it was 99.37±0.00 µg/gm in shoots harvested after first week, which increased to 260.58±0.00 µg/gm at the end of second week. However the lupeol content decreased in shoot cultures to 241.42±0.01 µg/gm in the third week (Table 16).

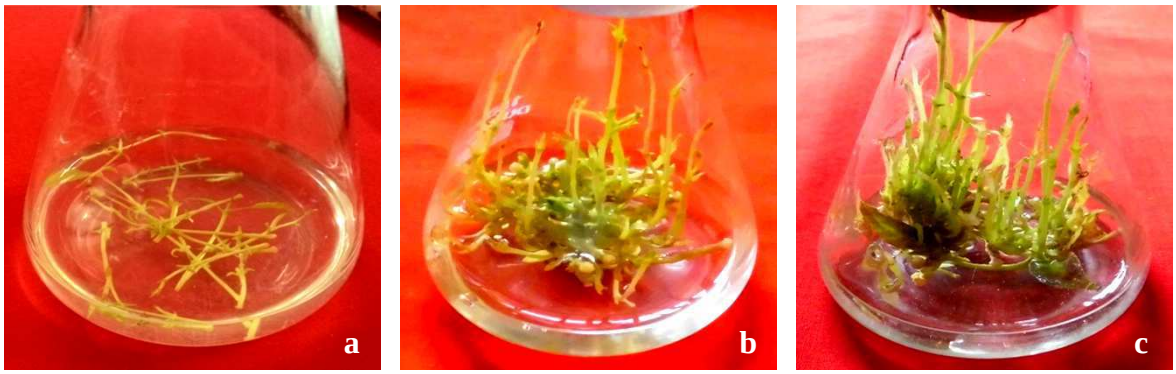

Figure 29: Shoot cultures in liquid medium fortified with BA (10 µM) and Kn (5 µM)- (a-c) after 1 week, (b) after 2 weeks and (c) after 3 weeks.

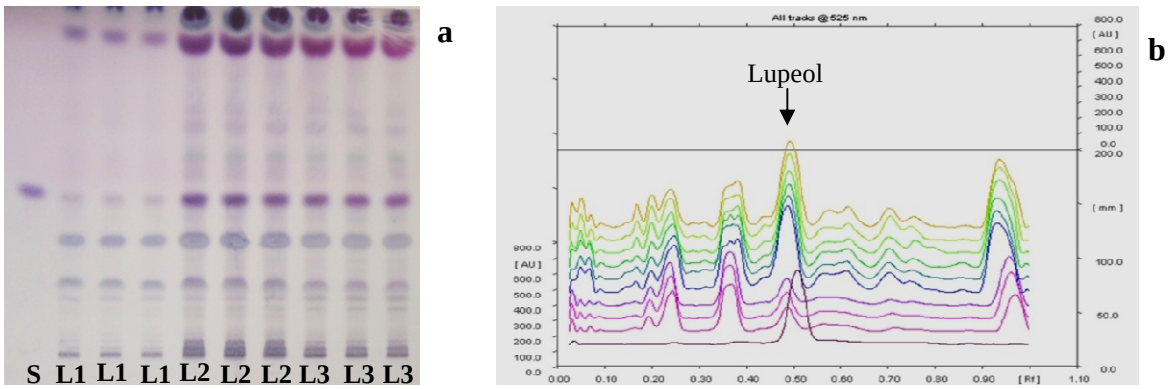

Figure 30: Lupeol quantification in liquid shoot cultures- (a) TLC plate of control samples. S- lupeol standard, L1- 1 week, L2- 2 weeks and L3- 3 weeks samples and (b) chromatogram of the control samples after scanning at 525 nm.

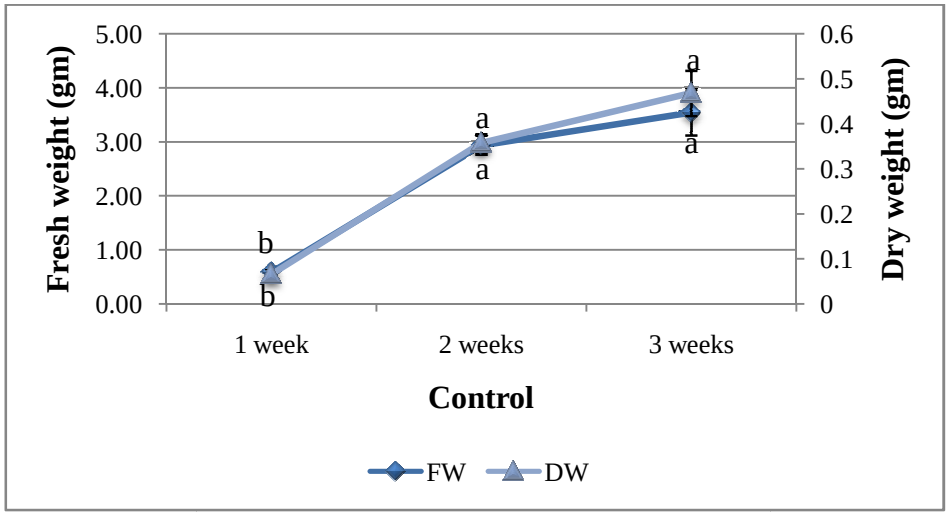

Figure 31: FW-DW of shoots from control medium [BA (10 µM) + Kn (5 µM)]. Each line shows the mean values (n= 5) and error bar as standard error. Means followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

- **Yeast extract**

Commonly used elicitor YE was added at different concentrations (25-200 mg/l) and in all the concentrations normal shoot growth was observed up to three weeks (Fig. 32a-l). There was simultaneous increase in FW and DW respectively, however variation in lupeol content was observed. At 25 mg/l YE, FW of the shoots increased to 3.70±0.06 gm in third week which is higher in comparison to control, however DW was less (0.372±0.01 gm) as compared to control (Fig. 33). Lupeol appeared after derivatization in all the samples (Fig. 34a) followed by densitometric analysis (Fig. 34b). Lupeol quantity was 102.43±0.00 µg/gm in shoots treated for one week with 25 mg/l which was more as compared to control. However the quantity of lupeol decreased in the second (235.32±0.00 µg/gm) and three weeks (109.38±0.00 µg/gm) (Table 16).

Increasing YE concentration to 50 mg/l evoked better response for shoot growth as well as lupeol synthesis as compared to control and 25 mg/l concentration. The FW-DW of the shoots were 3.85±0.10 and 0.479±0.00 gm respectively after three weeks treatment (Fig. 33). All the extracts when separated on plates showed lupeol band (Fig. 34c) and the chromatograms of the same showed peak of lupeol in all the samples (Fig. 34d). The lupeol content was 216.80±0.00 µg/gm after one week treatment, which increased to 221.64±0.01 µg/gm after two weeks and finally reached to 275.56±0.00 µg/gm after three weeks (Table 16) and it was highest as compared to all the other concentrations of YE.

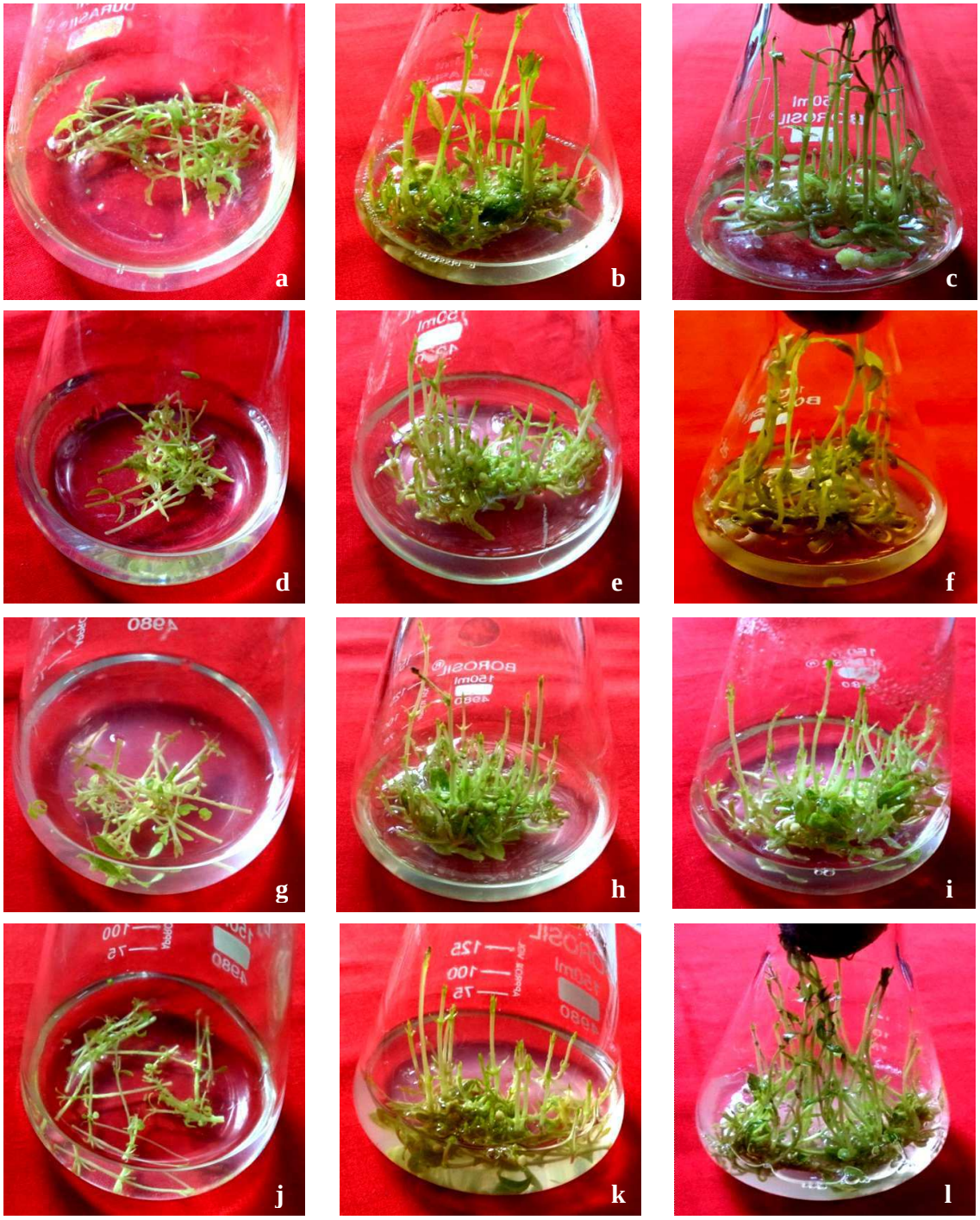

Figure 32: Elicitation of shoots in medium fortified with different concentrations of YE: (a-c) 25 mg/l, (d-f) 50 mg/l, (g-i) 100 mg/l and (j-l) 200 mg/l. (a, d, g, and j) after 1 week, (b, e, h and k) after 2 weeks and (c, f, i and l) after 3 weeks treatment.

Increasing YE level to 100 mg/l failed to increase the biomass and lupeol quantity in shoot cultures. The FW at the end of three weeks was 2.93±0.27 gm and DW was 0.337±0.03 gm (Fig. 33). The presence of lupeol was observed as a band in samples for all weeks (Fig. 34e) and chromatogram depicted the respective peaks (Fig. 34f). Peak areas revealed that the quantity of lupeol was less as compared to earlier concentrations, and maximum lupeol recorded was 154.32±0.01 µg/gm in samples treated for two weeks which was least in comparison to other samples (Table 16).

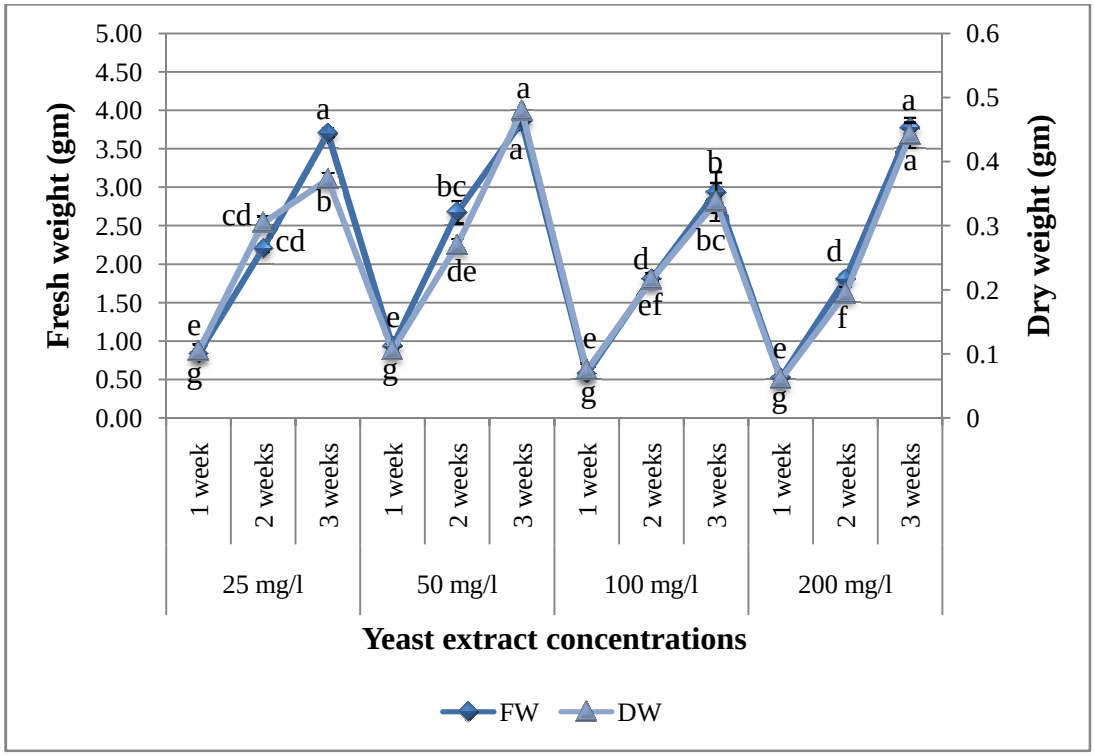

Figure 33: FW-DW of shoots from YE treated samples. Each line shows the mean values (n= 5) and error bar as standard error. Means followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Further enhancing YE concentration to 200 mg/l increased the biomass and lupeol content in comparison to 100 mg/l concentration but was less as compared to 50 mg/l. FW and DW of treated cultures was 3.77±0.13 and 0.441±0.02 gm respectively after three weeks (Fig. 33). TLC plates (Fig. 34g) and the chromatograms (Fig. 34h) of every week's sample revealed lupeol's presence and it was less as compared to YE 50 mg/l (Table 16).

The above findings suggest that *H. indicus* shoots were able to synthesize lupeol in liquid medium (control) as well as in elicitated cultures. However variation was observed and YE at 50 mg/l induced highest biomass as well as lupeol content as compared to

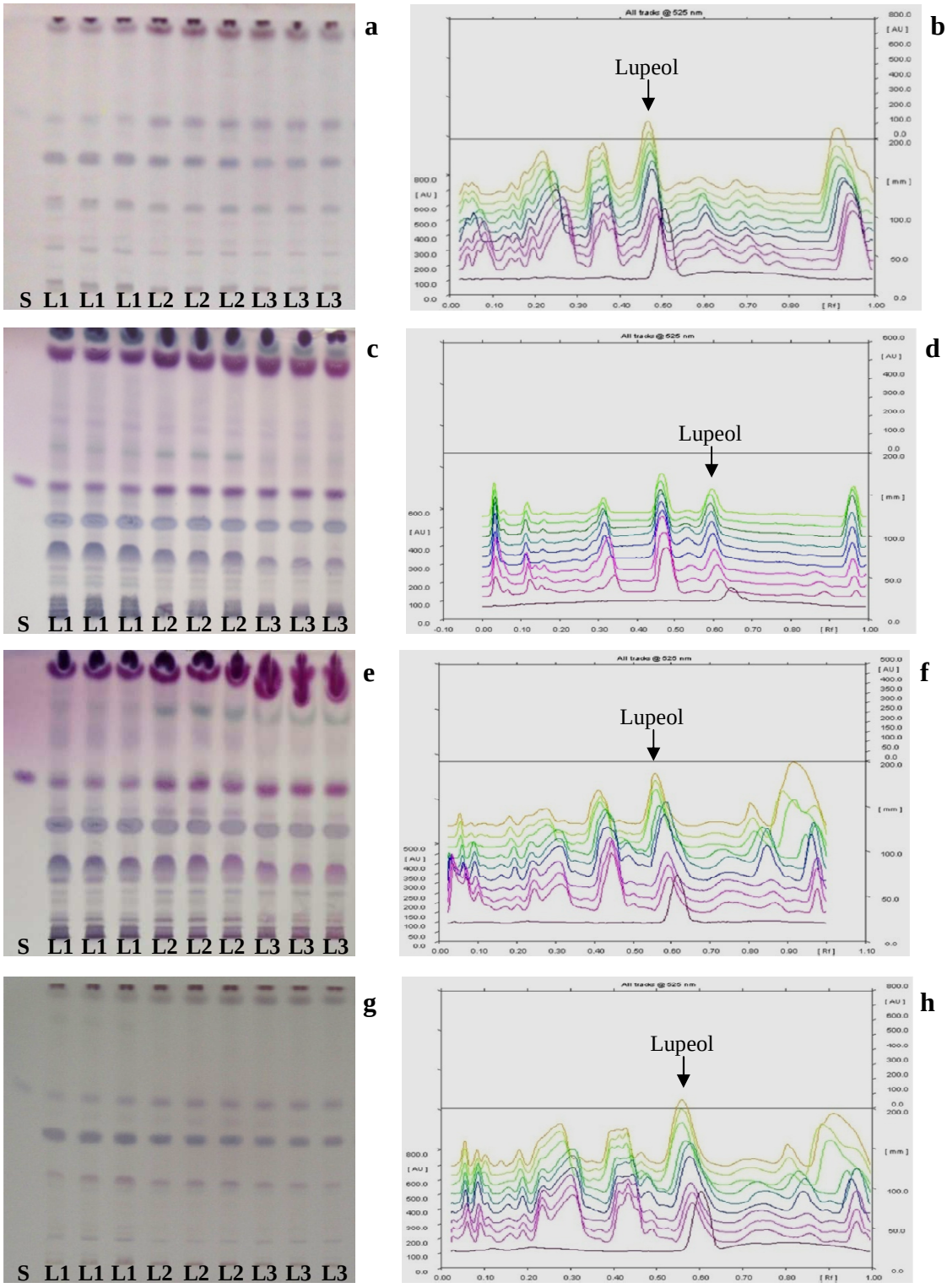

Figure 34: TLC plates and respective chromatograms of hexane extracts of shoots treated with different concentrations of YE- (a, b) 25 mg/l, (c, d) 50 mg/l, (e, f) 100 mg/l and (g, h) 200 mg/l. S- lupeol standard, L1- 1 week sample, L2- 2 weeks sample and L3- 3 weeks sample. Scanning of plates was done at 525 nm.

control and other concentrations. Higher concentrations of YE (100 and 200 mg/l) adversely affected the shoot biomass as well as lupeol synthesis.

Table 16: Lupeol content in shoot cultures of *H. indicus* after elicitation using yeast extract

YE (mg/l)	Lupeol (µg/gm) (Mean ± SE)*		
	1 week	2 weeks	3 weeks
Control	99.37±0.00 fg	260.58±0.00 ab	241.42±0.01 bc
25	102.43±0.00 fg	235.32±0.00 cd	109.38±0.00 f
50	216.80±0.00 d	221.64±0.01 cd	275.56±0.00 a
100	83.40±0.00 gh	154.32±0.01 e	113.70±0.00 f
200	67.56±0.00 h	167.63±0.00 e	227.60±0.00 cd

*Means (n=3) followed by same letter are not significantly different ($p \leq 0.05$) using Tukey's test.

- **Salicylic acid**

After biotic elicitor, commonly used abiotic elicitor SA was fed to shoot cultures at different concentrations (25-200 µM). Presence of SA in the medium improved shoots growth during second week which was similar to control and YE fortified media. Observations revealed that the shoot growth was normal and healthy shoots were observed in all concentrations (Fig. 35a-l). Feeding the cultures with 25 µM SA increased the FW (2.99±0.12 gm) as well as DW (0.506±0.02 gm) at the end of three weeks as compared to control (Fig. 36). The extracts of different weeks were separated and plates were derivatized to confirm lupeol (Fig. 37a) and the chromatograms identified the same peaks (Fig. 37b). Calculating the lupeol content in shoots for all the samples revealed that it was less during first and second week and it increased to 197.28±0.00 µg/gm in shoots treated for three weeks (Table 17).

The biomass of shoots increased (FW/DW: 3.17±0.09/0.589±0.02 gm) when the shoot cultures were treated with 50 µM SA for three weeks (Fig. 36). The lupeol presence was depicted as a band on TLC plate (Fig. 37c) and peaks (Fig. 37d). Lupeol was 138.51±0.00 µg/gm after one week, which again increased to 221.69±0.00 µg/gm after two weeks treatment. Maximum lupeol content amongst all the SA concentrations was observed after three weeks treatment which was 335.40±0.04 µg/gm and is optimum in comparison to control and YE fortified media (Table 17).

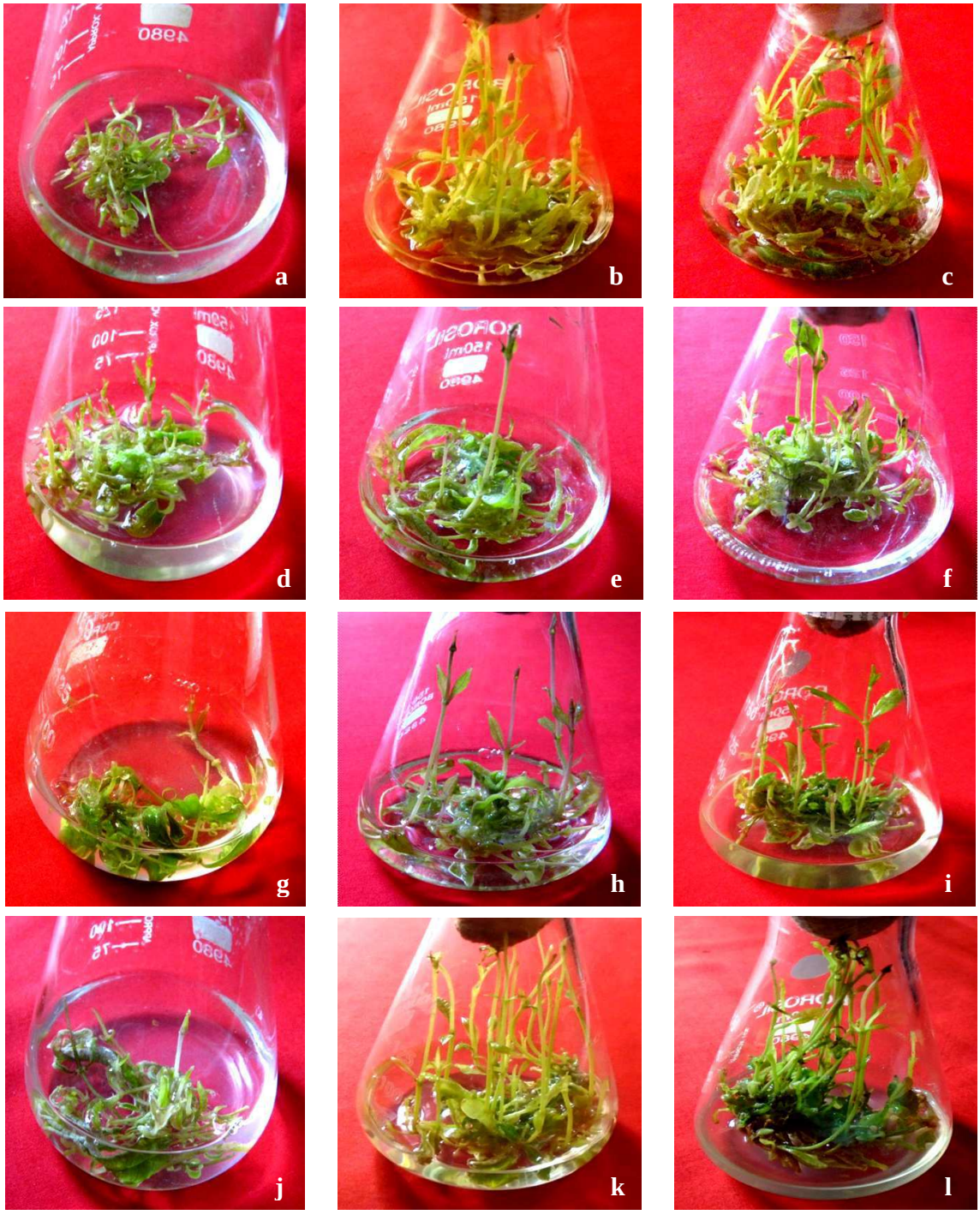

Figure 35: Elicitation of shoots in medium fortified with different concentrations of SA- (a-c) 25 µM, (d-f) 50 µM, (g-i) µM and (j-l) 200 µM. (a, d, g, and j) after 1 week, (b, e, h and k) after 2 weeks, (c, f, i and l) after 3 weeks.

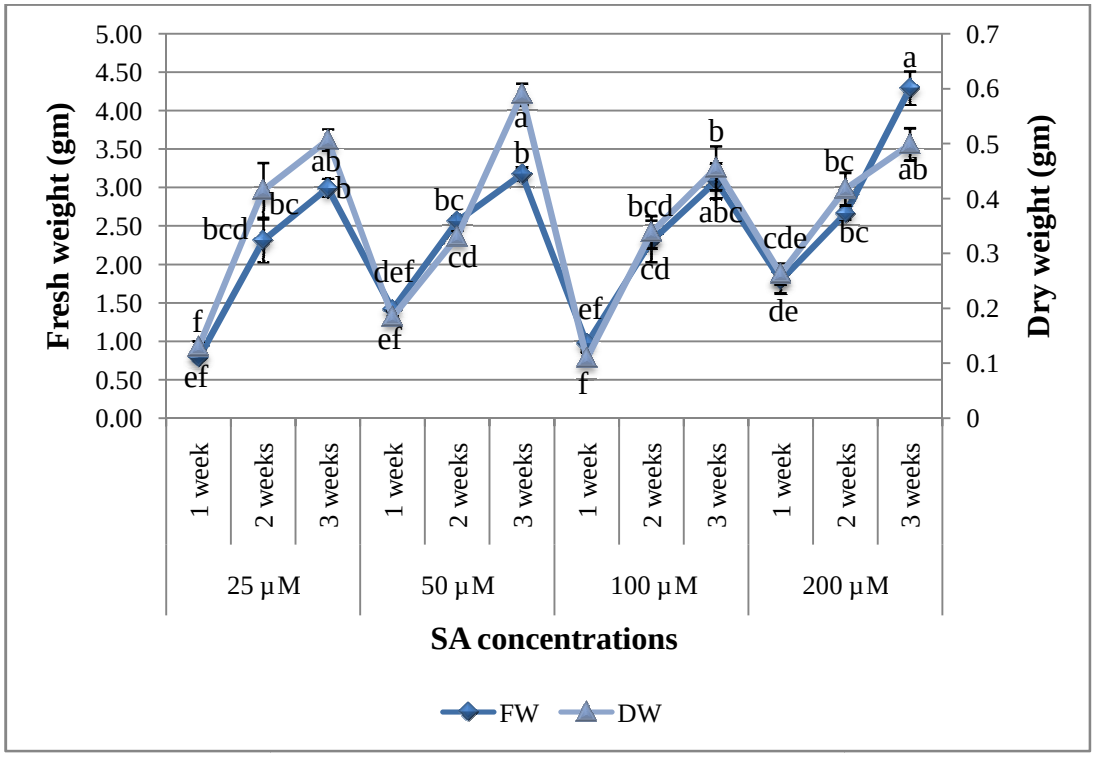

Figure 36: FW-DW of shoots from SA treated samples. Each line shows the mean values (n= 5) and error bar as standard error. Means followed by same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Further increase in SA level to 100 µM adversely affected the shoot biomass in all the weeks as compared to earlier concentrations (Fig. 36). Presence of lupeol was observed as a bands and peaks on TLC plate (Fig. 37e) and in chromatogram (Fig. 37f). However the lupeol content decreased in comparison to 50 µM concentration. At SA 200 µM the FW/DW was 4.29±0.22/0.498±0.03 gm in third week, which is maximum amongst all other concentrations of SA, YE and control (Fig. 36). TLC plate (Fig. 37g) as well chromatogram (Fig. 37h) confirmed the presence of lupeol but only 130.75±0.00 µg/gm was recorded, which is least in comparison to other SA concentrations after three weeks treatment (Table 17).

Elicitation of shoot cultures with SA revealed that it facilitated shoot growth along with lupeol biosynthesis in shoots of *H. indicus*. 50 µM of SA was the optimum concentration for lupeol synthesis in the present study.

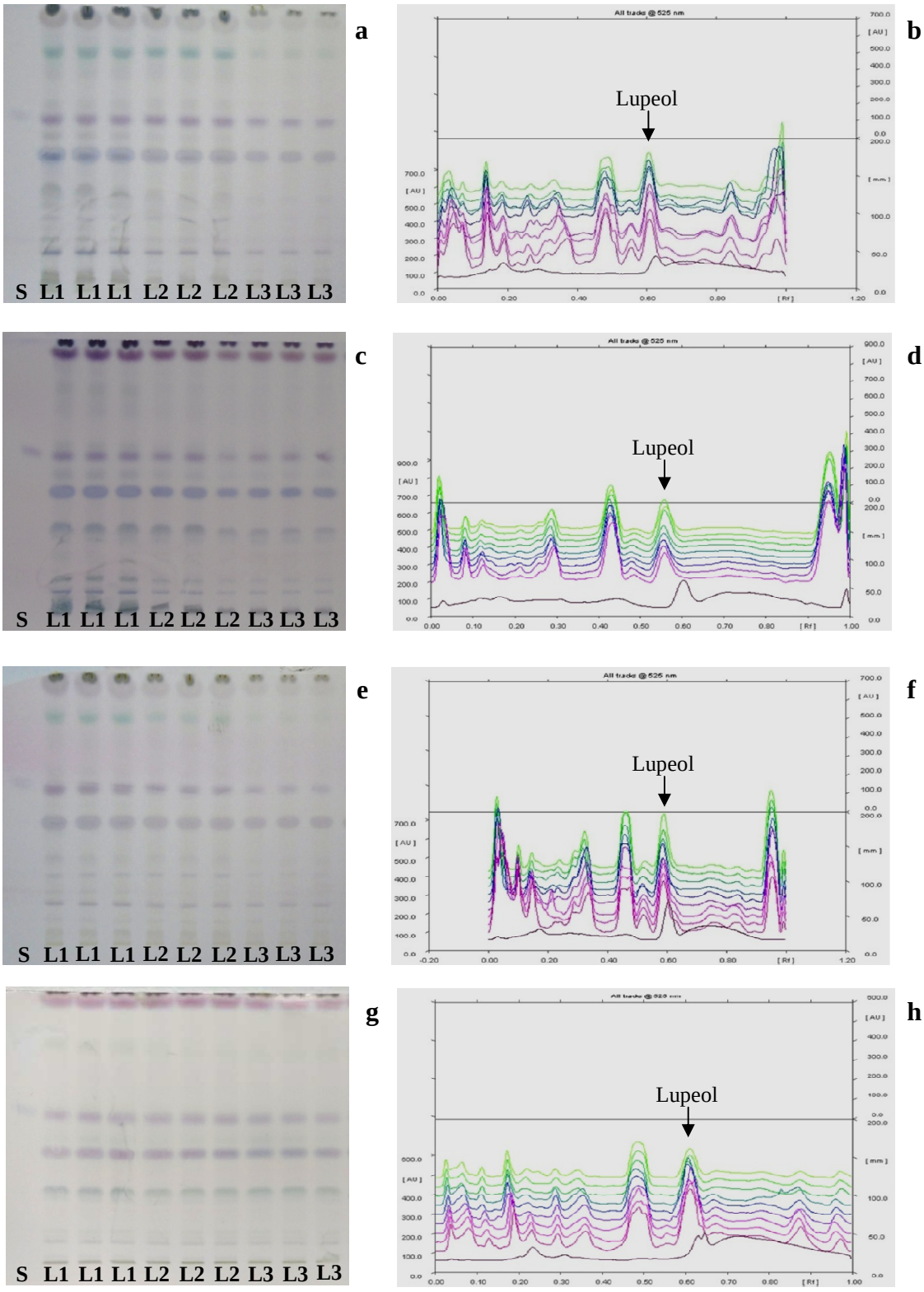

Figure 37: TLC plates and respective chromatograms of hexane extracts of shoots treated with different concentrations of SA- (a, b) 25 µM, (c, d) 50 µM, (e, f) 100 µM and (g, h) 200 µM. S- lupeol standard, L1- 1 week sample, L2- 2 weeks sample and L3- 3 weeks sample (in triplicate). Scanning of plates was done at 525 nm.

Table 17: Lupeol content in shoot cultures of *H. indicus* after elicitation using salicylic acid

SA (µM)	Lupeol (µg/gm) (Mean ± SE)*		
	1 week	2 weeks	3 weeks
Control	99.37±0.00 fgh	260.58±0.00 ab	241.42±0.01 b
25	54.39±0.00 gh	44.40±0.00 h	197.28±0.00 bcde
50	138.51±0.00 cdefg	221.69±0.00 bc	335.40±0.04 a
100	212.40±0.00 bcd	176.51±0.02 bcdef	191.21±0.02 bcde
200	114.44±0.00 efgh	106.84±0.00 fgh	130.75±0.00 defg

*Means (n=3) followed by same letter are not significantly different ($p \leq 0.05$) using Tukey's test.

- **Methyl jasmonate**

Another elicitor MJ was also commonly used abiotic type of elicitor which known to enhance metabolites and it was added to the media to evaluate its effect on lupeol. However in the presence of MJ, shoots failed to survive within a week and turned brown, hence lupeol content was not estimated.

Thus from elicitation studies it was concluded that lupeol synthesis can be enhanced by treating the cultures with YE and SA but the shoots failed to survive in MJ fortified medium. Among different concentrations of YE and SA, 50 µM concentration of SA was effective in enhancing lupeol content in shoot cultures.

Secondary metabolites are produced by plants mainly as a defence mechanism towards pathogenic attacks hence their production can be increased by feeding them with compounds called as elicitors and this process is known as elicitation (Brooks and Watson, 1986; He, 1996). In the present studies growth of shoots was observed in control as well as media fortified with YE and SA, and this is due to nutrient uptake from liquid medium as there is a close contact of tissue with medium as well as continuous shaking minimizes apical dominance (Largia et al., 2015). Earlier report on lupeol enhancement in callus culture of *H. indicus* has been reported after treatment with γ-rays (Misra and Mehrotra, 2006). Whereas lupeol quantity was increased using ergosterol in *Cryptostegia grandiflora* (Singh et al., 2011a), UV-B exposure in *Vitis vinifera* (Gil et al., 2012) and jasmonic acid in *Jatropha curcas* (Zaragoza-Martinez et al., 2016).

Addition of YE in the medium increased lupeol quantity in the present study and similarly this elicitor has been reported to increase expression of genes related to triterpenoid biosynthesis in *Medicago truncatula* (Suzuki et al., 2002). However quantity of lupeol is dependent on concentrations of YE which is reported for vinblastine and vincristine synthesis in *Catharanthus roseus* (Maqsood and Abdul, 2017). In the present study optimum lupeol was observed in shoots treated with 50 mg/l and increased concentrations failed to enhance the growth as well as lupeol synthesis in shoots. This is in agreement with fact that lower concentrations of YE is beneficial for shoot cultures (Vasil and Hilderbrandt, 1966). Low concentration of YE was beneficial for growth and phenolic content whereas higher concentration inhibited both in *Curcuma mangga* plantlets (Abraham et al., 2011). Similarly lower levels of YE proved to be beneficial for synthesis of plumbagin in *Drosera burmanii* (Putalun et al., 2010) and rosmarinic acid in *Melissa officinalis* (Nasiri-Bezenjani et al., 2014). YE also increased the production of p-coumaric acid and furanocoumarins in *Glehnia littoralis* (Ishikawa et al., 2007), pseudojujubogenin in *Bacopa monnieri* (Kamonwannasit et al., 2008) and plumbagin in *Drosera indica* (Thaweesak et al., 2011).

Improved shoot growth in presence of YE is because it contains amino acid, chitin, N-acetyl-glucosamine oligomers, β-glucan, glycopeptides and ergosterol and is also a rich source of vitamin B-complex (George et al., 2008). Although the exact mechanism of elicitation is unclear but peptide and polysaccharide moieties of YE are known to stimulate metabolite synthesis (Boller, 1995; Menke et al., 1999; Zhao et al., 2011). 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. Whereas another hypothesis depicted that cations of YE (Ca^{2+}, Co^{2+} and Zn^{2+}) stimulate the secondary metabolites synthesis (Eskandari et al., 2012).

SA which is an another elicitor is also reported to be a main signal molecule involved in the systemic acquired resistance (SAR) of plants to pathogens (Raskin, 1992; Hammerschmidt, 1999) and is also activates different defence responses under stress conditions (Cameron, 2000). In *H. indicus* shoot cultures, addition of SA in the medium

increased the shoot biomass as it is known to facilitate growth in plants (Vicente and Plasencia, 2011). However lower concentrations of SA i.e. 25 and 50 µM facilitated shoot growth whereas higher concentrations (100 and 200 µM) hindered the growth, and similar reports are documented for *Andrographis paniculata* (Zaheer and Giri, 2015). Amongst different SA concentrations, 50 µM was proved to elicit maximum lupeol and further increase in concentrations adversely affected the synthesis, which is in line with report of *Matricaria chamomilla* for phenolic acids synthesis (Kovacik et al., 2009). 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). Alex et al. (2000) observed that the expression two important genes of terpenoid biosynthesis pathway i.e. 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) and HMG CoA reductase (HMGR) increased after SA elicitation in *Brassica juncea*. Similarly SA treatment is known to accumulate the precursors of terpenoids biosynthesis in *Glycyrrhiza glabra* (Hayashi et al., 2004).

50 µM SA has been reported to enhance bacoside A in *Bacopa monnieri* (Largia et al., 2015; Sharma et al., 2015) and rosmarinic acid in *Solenostemon scutellarioides* (Sahu et al., 2013). Whereas digitoxin content was optimum when shoots of *Digitalis purpurea* were treated with 50 µM SA but for digoxin 200 µM SA was beneficial (Patil et al., 2013). Similarly production of hypericin and pseudohypericin enhanced at 50 µM for the same elicitor in *Hypericum hirsutum* shoots, whereas in other species (*H. maculatum*) the content of same metabolites increased at 200 µM (Coste et al., 2011). SA also been reported to enhance caffeic acid and rosmarinic acid in *Thymus membranaceus* (Perez-Tortosa et al., 2012), hypericin and pseudohypericin in *H. 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) and carotenoid in *Ruta angustifolia* (Othman et al., 2015).

The exact mechanism of action is unclear, but it triggers SA gene expression and its synthesis in tissue which favours metabolite synthesis (Szalai et al., 2011). Later on Askari and Ehsanzadeh (2015) reported that SA interferes the activity of antioxidant enzymes and inhibit catalase enzyme, which in turn increase H_2O_2 as well as secondary metabolites production.

In the present observation SA was better in terms of lupeol synthesis as compared to YE, which in line with reports on *Digitalis purpurea* (Patil et al., 2013) and *Swertia chirata* (Kumar et al., 2013). The synthesis of lupeol was maximum in 3 weeks old cultures and in optimum concentrations of both YE (50 mg/l) and SA (50 µM). This may be due to more proliferation of shoots during second week in which the energy was utilized for growth rather than metabolite synthesis (de Figueiro et al., 2010).

When MJ was added to the medium in the present study, it inhibited the shoot growth immediately and they turned brown within a week. Elicitors have to coordinate with stress induced after subculturing but combination of stresses from subculture and elicitor feeding sometimes induces rapid cell death (Ciddi et al., 1995; Moreno et al., 1996). Cosio et al. (1990) reported that MJ has inhibitory effects on growth and different metabolic activities in plants. Similarly growth of *S. chirata* shoot was inhibited after treatment with 50 and 75 µM MJ (Kumar et al., 2013). Whereas Largia et al. (2015) reported that 100 µM concentration of MJ adversely affected and 150 µM completely inhibited the growth of *Bacopa monnieri* shoots. Complete inhibition of the growth was reported in *Withania somnifera* shoots upon treatment with 150-250 µM MJ (Sivanandhan et al., 2013). The inhibitory effect of MJ has been documented for *Centella asiatica* (Cheong and Choi, 2003; Kim et al., 2004), *Eryngium planum* (Kikowska et al., 2012) and *Exacum affine* (Skrzypczak-Pietraszek et al., 2014).

Lu et al. (2001) reported that the sensitivity of each plant is different for elicitor which may be the reason that each plant behaves differently in response to elicitors. The efficiency of elicitors varied depending on genotypes, concentration and exposure time of elicitor, type of culture etc. (Zhao et al., 2010). The site and accumulation of secondary metabolites in plants is also dependent on growth, tissue differentiation and development (Collin, 2001). Thus response of each plant to elicitor varies and therefore it becomes crucial to determine suitable concentrations of elicitors for product optimization (Shinde et al., 2009).

HPTLC analysis of lupeol confirmed that shoot cultures developed from nodal explants synthesized lupeol, however PGR affected its synthesis as variation was observed in shoot cultured derived from medium MS-1 and MS-2. Thus shoots from both these media were also analyzed for rutin synthesis followed by elicitation experiment using same elicitors as used for lupeol.

4.2.3 HPTLC Analysis for Rutin in Shoot Cultures

4.2.3.1 Standard curve of rutin

Rutin standard was loaded in the concentration range from 100-500 ng and then developed in ethyl acetate:butanol:formic acid:water (5:3:1:1 v/v) followed by scanning at 254 nm. Standard curve was prepared whose correlation coefficient (R^2) was 0.978 and the equation of straight line was y = 16.92x+1031 (Fig. 38).

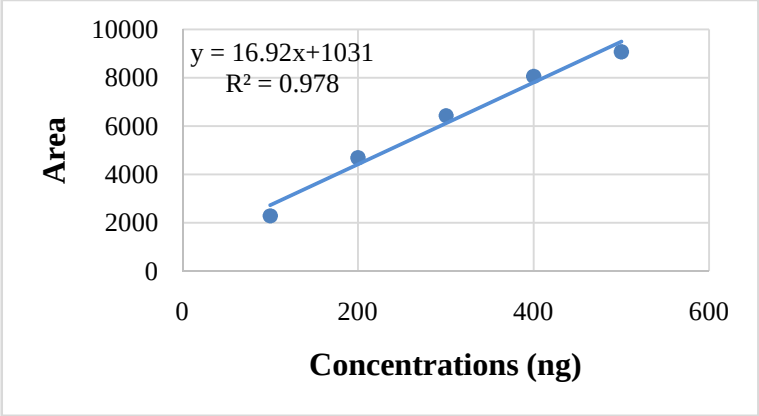

Figure 38: Standard curve of rutin

4.2.3.2 Quantification of rutin

When ethanolic extracts of *in vivo* as well as *in vitro* shoots from MS-1 and MS-2 were analyzed for rutin, band was observed in *in vivo* shoots but was absent in *in vitro* shoots from both MS-1 and MS-2 (Fig. 39a, b). However spectral analysis of *in vivo* sample was carried out to confirm the presence of rutin and it was observed that a spectrum of *in vivo* shoots changed in comparison to standard spectra (Fig. 40a). This may be due to either isomer formation of the rutin or may be an other compound at same Rf. Further analysis of mass spectra also revealed the absence of rutin and even quercetin (from which rutin is synthesized) in *in vivo* as well as *in vitro* samples (Fig. 41a-c).

Similarly rutin was absent in *Hypericum perforatum* and *H. androsaemum in vitro* shoots (Dias et al., 1999). *In vitro* cultures of *Solidago chilensis* (Schmeda-Hirschmann et al., 2005), *Peucedanum japonicum* (Chen et al., 2016) and *Pterospartum tridentatum* (Gonçalves et al., 2018) also failed to synthesize rutin. Whereas shoot and callus of *Fabiana imbricata* synthesized very less amount of rutin as compared to wild plant (Schmeda-Hirschmann et al., 2004). Study on *H. perforatum* cv. Topas revealed that rutin

was absent in callus or cell suspension and synthesized only in plantlets (Pasqua et al., 2003).

PGRs of the medium are known to affect synthesis of metabolites and especially the enzymes of phenylpropanoid pathway (Sangwan et al., 2001). In the present study optimized medium have cytokinins (BA and Kn) which may inhibit rutin synthesis as earlier report by Lee et al. (2011) documented that rutin synthesis is inhibited in callus of *Morus alba* grown in presence of cytokinins. Similarly Karalija et al. (2014) also reported that Kn negatively affected rutin synthesis in shoots of *Knautia sarajevensis*. Inhibition of another flavonoid (luteolin and its 7-O-glucoside) was also reported in shoots of *Scutellaria alpine* when cultured in cytokinin supplemented media (Grzegorczyk-Karolak et al., 2015). This supported the fact that cytokinin significantly suppresses the transport of macronutrients such as nitrate, ammonium, sulfate and phosphate, and nitrate is known to regulate the expression of genes involved in the phenylpropanoid and flavonoid pathways (Sakakibara et al., 2006). However shoots from auxin fortified medium (MS-2) failed to synthesize rutin which may be due to the NH_4^+ to NO_3^- ratio of the medium which affects the secondary metabolite synthesis (Smetanska, 2008) and is also reported to affect rutin synthesis in *M. alba* (Lee et al., 2011). Another reason behind this is the lack of the stress under *in vitro* conditions (Dias et al., 1998) and the function of flavonoids is defence against bacterial and fungal infections, it may not be synthesized in cultures (Dixon and Paiva, 1995).

Although rutin was not detected in *in vitro* shoots from both the media i.e. MS-1 and MS-2, further elicitation experiments were carried out to study whether YE and SA would trigger the synthesis rutin in shoot cultures of *H. indicus*. However for these experiments, shoots developed in MS-1 [BA (10 µM) and Kn (5 µM)] fortified medium were taken up as generally cytokinins are known to facilitate metabolite synthesis.

4.2.3.3 Elicitation of rutin

Both YE and SA were added to MS-1 liquid medium which was control for the study. When ethanol extract of control samples of all three weeks were developed on TLC plate, band corresponding to the band of standard rutin was observed at UV 254 nm (Fig. 39c). However the densitometric scanning of plates revealed very less peak area of the same, thus they were scanned for spectral analysis. It was observed that similarly as *in vivo*

shoots, spectra of all the control samples (1-3 weeks) changed in comparison to standard spectra of rutin (Fig. 40a). Furthermore the shoot cultures were fed with YE (25-200 mg/l), and the extracts of all samples were prepared and separated on TLC plates. Observing the plates at UV 254 nm confirmed the band at similar Rf of rutin standard (Fig. 39d).

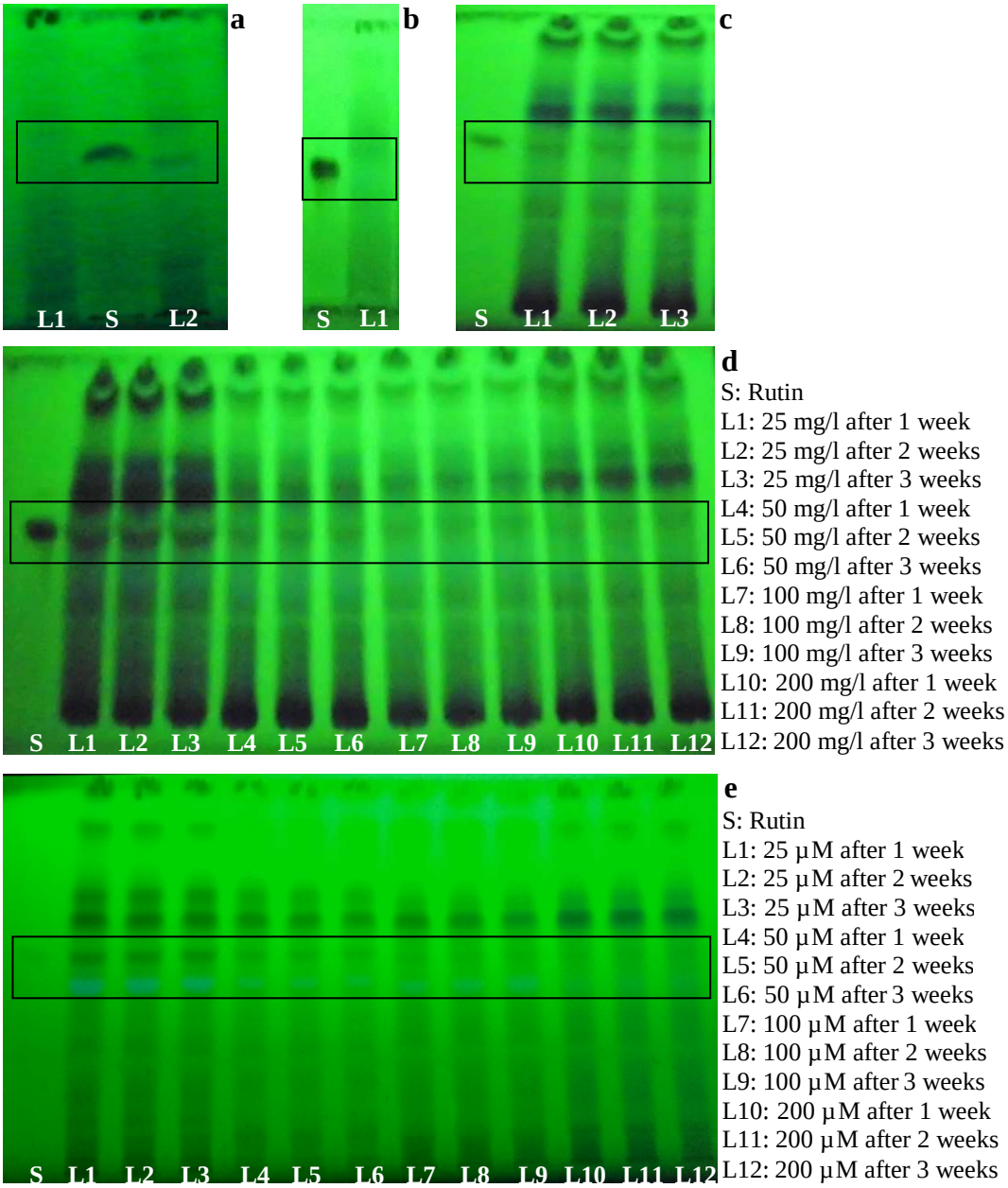

Figure 39: TLC plates of ethanol extract at 254 nm- (a) *in vivo* and *in vitro* shoots (MS-1)- S- rutin standard, L1- *in vitro* shoot (MS-1), L2- *in vivo* shoot, (b) S- rutin standard, L1- *in vitro* shoot (MS-2), (c) *in vitro* shoot (liquid)- L1- 1 week old, L2- 2 weeks old and L3- 3 weeks old *in vitro* shoots, (d) ethanol extract of YE treated shoots and (e) ethanol extract of SA treated shoots.

Densitometic analysis revealed very small peak corresponding to rutin standard. But when spectral analysis of samples was done, it was observed that the spectra changed and representative sample's spectra are given in Fig. 40b.

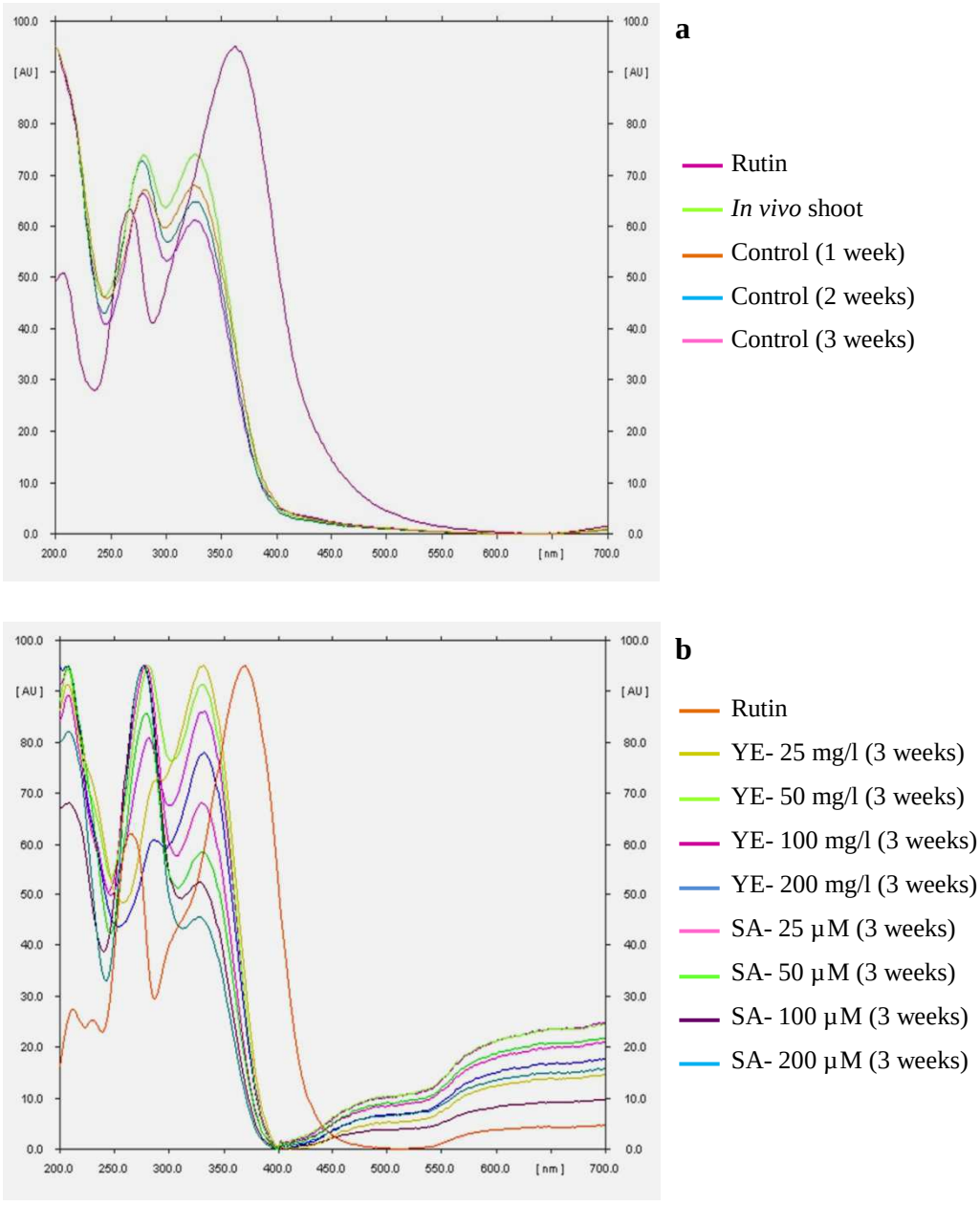

Figure 40: Spectral analysis of all samples for rutin identification at 254 nm- (a) spectra of *in vivo* and *in vitro* control media (1-3 weeks) and (b) spectra of *in vitro* shoots treated with different concentrations of YE and SA.

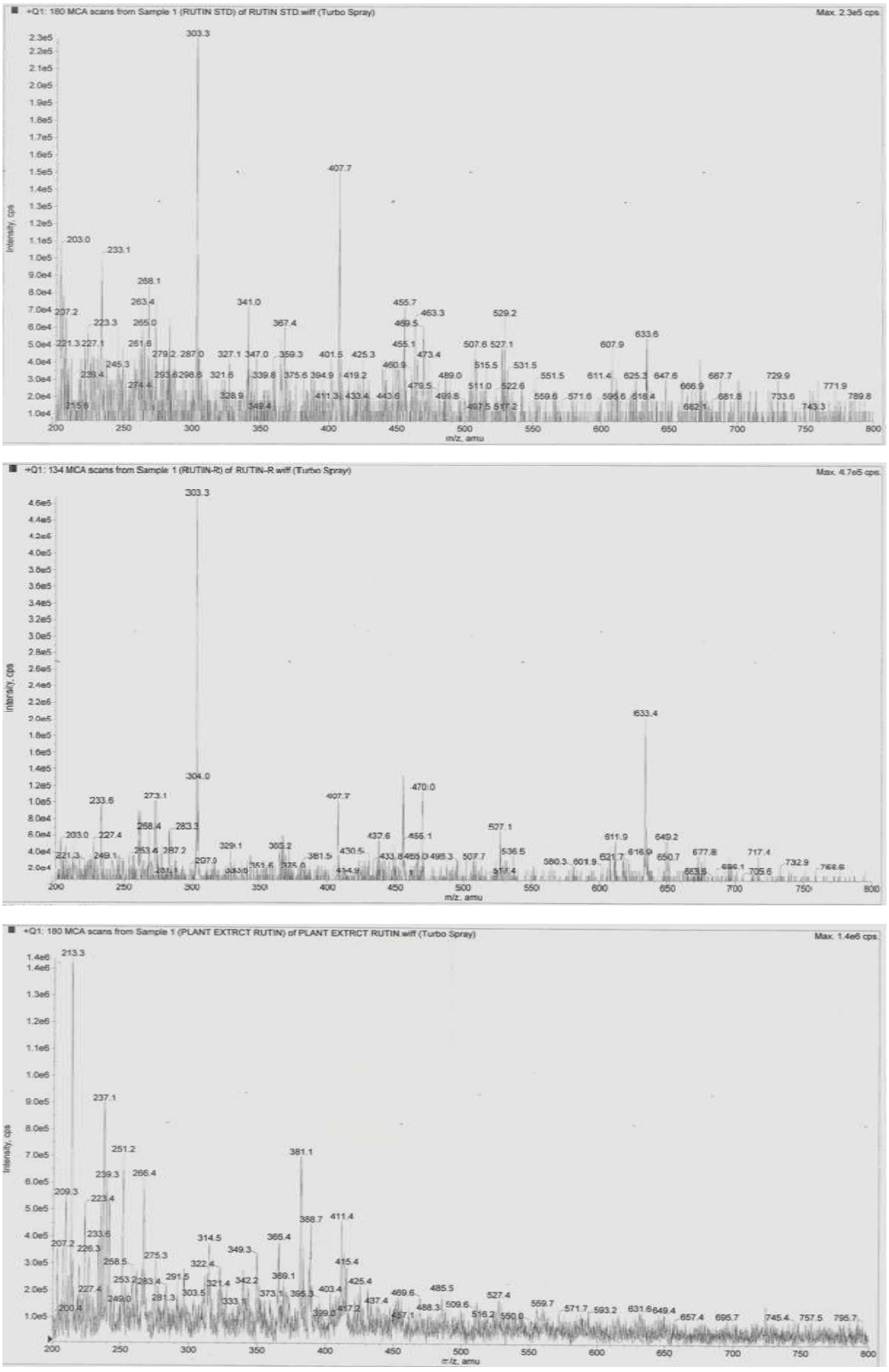

Figure 41: Mass spectra of samples of ethanolic extract- (a) *in vivo* shoots, (b) *in vitro* (MS-1) shoots and (c) *in vitro* (MS-2) shoots

Similarly the shoots were treated with all SA concentrations (25-200 µM) and extracts of the same when separated on TLC plates revealed presence of bands corresponding to rutin standard in all concentrations under UV 254 nm (Fig. 39e). Densitometric analysis of the same at 254 nm revealed that peak appeared in samples in accordance with standard but further spectral analysis depicted change in spectra (Fig. 40b) which confirmed that the compound was different but had the same Rf as rutin.

Thus there was further need to analyze the upstream metabolite of this pathway i.e. kaempferol which is a precursor for quercetin (Fig. 6). Elicitation studies had revealed that maximum biomass of shoots was generated in media fortified with YE (50 mg/l) and SA (50 µM) after 3 weeks. Thus *in vivo* and *in vitro* shoots (MS-1) along with treated shoots with YE (50 mg/l) and SA (50 µM) were selected for qualitative analysis of kaempferol. Separating the extracts on TLC plate in ethyl acetate:butanol:formic acid:water (5:3:1:1 v/v) and observing under UV 366 nm revealed the band of kaempferol in all the samples at corresponding Rf as standard (Fig. 42a). Further confirmation was done by densitometric scanning which revealed very small peak of kaempferol and spectral analysis of the same confirmed that the compound is kaempferol as the spectra of samples matched with standard sample (Fig. 42b).

Earlier study by Dias et al. (1998) reported that flavonoids synthesized under *in vitro* condition have different Rf and UV spectra as compared to standard and *in vivo* plant. Whereas Suri et al. (2002) reported that YE and SA adversely affected rutin synthesis in callus cultures of *Citrus hystrix*. Similarly Gomez-Vasquez et al. (2004) have reported that cell cultures of *Manihot esculenta* failed to enhance rutin content when YE was added to it. Similarly Kuo et al. (2015) reported that SA failed to increase rutin content in comparison to control in callus culture of *Saussurea involucrate*. Other elicitors like abscisic acid, phenylalanine, sodium acetate and jasmonic acid were also unable to increase the content.

The spectra of *in vivo* shoots was different from standard rutin spectra which revealed that *in vivo* shoots are unable to synthesize rutin which may be due to the variation in climatic conditions. da Silva et al. (2006) reported that differences in *Casearia sylvestris* biomes showed major variation in the rutin content and it might be due to different aspects such as difference in genetic makeup, environmental conditions, developmental stage of the plants (vegetative and reproductive), soil characteristics etc. The rutin content is high in places at higher altitude as it known to be an UV protector for

plant (Jogia, 1984; Shirley, 1996; Atroch, 1999). Similarly variation in rutin content was observed according to geographic variation in *Hypericum perforatum* (Hamoudova et al., 2006) and *Capparis spinosa* (Musallam et al., 2012). Another possible reason may be the phenotype plasticity of the species which is the ability of a genotype to produce specific phenotypes depending on the environmental context and thus they are dependent with each other (Scheiner, 1993).

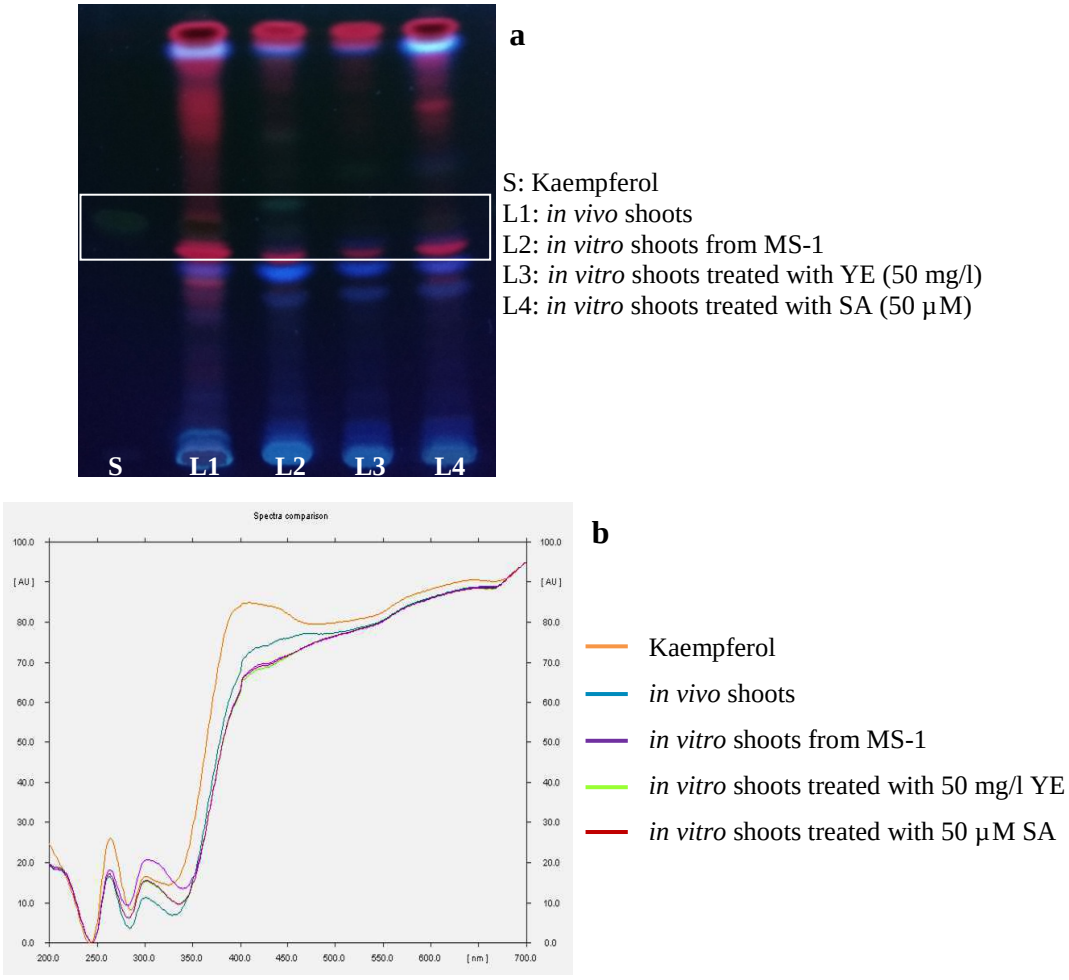

Figure 42: Qualitative analysis for kaempferol in *in vivo* and *in vitro* shoots at 366 nm- (a) TLC plate containing standard and samples and (b) spectral analysis of all the samples.

Thus secondary metabolite studies revealed that content of lupeol increased after elicitation with YE and SA, amongst which later was better as compared to former. Whereas MJ completely inhibited the growth of shoots and hence it was considered unfavourable for elicitation. However when ethanolic extract was analyzed for rutin it was observed that rutin was absent in *in vivo* shoots and *in vitro* cultures, also treating the cultures with elicitors failed to synthesize rutin. Mass spectra of ethanolic extract of *in vivo* and *in vitro* (MS-1) shoots also confirmed that both rutin as well as its precursor quercetin were absent in the extract. In rutin biosynthetic pathway (KEGG) it was observed that the upstream metabolite of quercetin is kaempferol (Fig. 6), and thus it was analyzed qualitatively in selected samples. This analysis revealed presence of kaempferol in shoot cultures which was also confirmed by spectral analysis and thus there was a need that further investigation on gene expression study should be carried out.

4.2.3.4 Gene expression analysis for F3'5'H and F3'H genes

As the results of phytochemical analysis of shoots revealed that kaempferol was synthesized but its downstream metabolites like quercetin and rutin was absent. Thus genes converting kaempferol to quercetin were selected for further analysis. Flavonoid pathway from KEGG depicted that flavonoid 3',5'-hydroxylase (*F3'5'H*) and flavonoid 3'-monooxygenase (*F3'H*) are the two genes which are responsible for conversion of kaempferol to quercetin (Fig. 6). *F3'5'H* and *F3'H* belongs to cytochrome P450 family which catalyses hydroxylation of flavonol B-ring at 3'5' and 3' positions respectively. These genes were analyzed in shoots of *H. indicus* at transcriptional level, and primers for respective genes were synthesized as follows.

(1) Primer synthesis for selected genes

First step in gene expression studies was primer designing using different tools as mentioned in Chapter 2 section 3.2.6.10. Primers were synthesized by selecting other plant species in which the sequences for the selected genes were available. Primers for gene 1 (*F3'5'H*) were synthesized from two different plant species: *Vitis vinifera* (NCBI accession no. vvi_100261319) and *Eustoma grandiflorum* (NCBI accession no. U72654.2). Sequence alignments after CLUSTALW analysis for both the plants is given below along with selected primer sequences.

- **G1P1 primer**


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vvi_100232896 ATGGCCATAGATACAAGCCTCTTGCTTGAGTTTGCTGCAGCAACTCTACTCTTCTTCATC
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spen_107005054 ATGACGTTACGTATTAGTGAGTTATTTGTCGCGGCTATAATATATATCATAGTACATATT
vvi_100261319 ACCCGCTTCTTCATTCGTTCTCTCCTTCTCAAATC-----TTCCCGGAA--GCTCCCTCC
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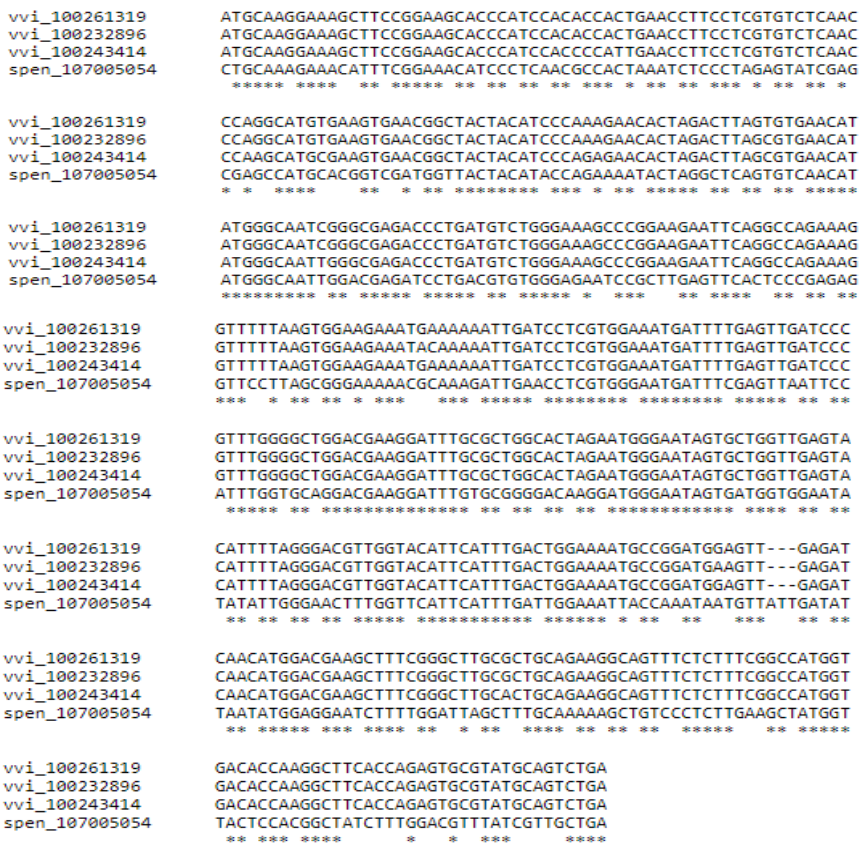
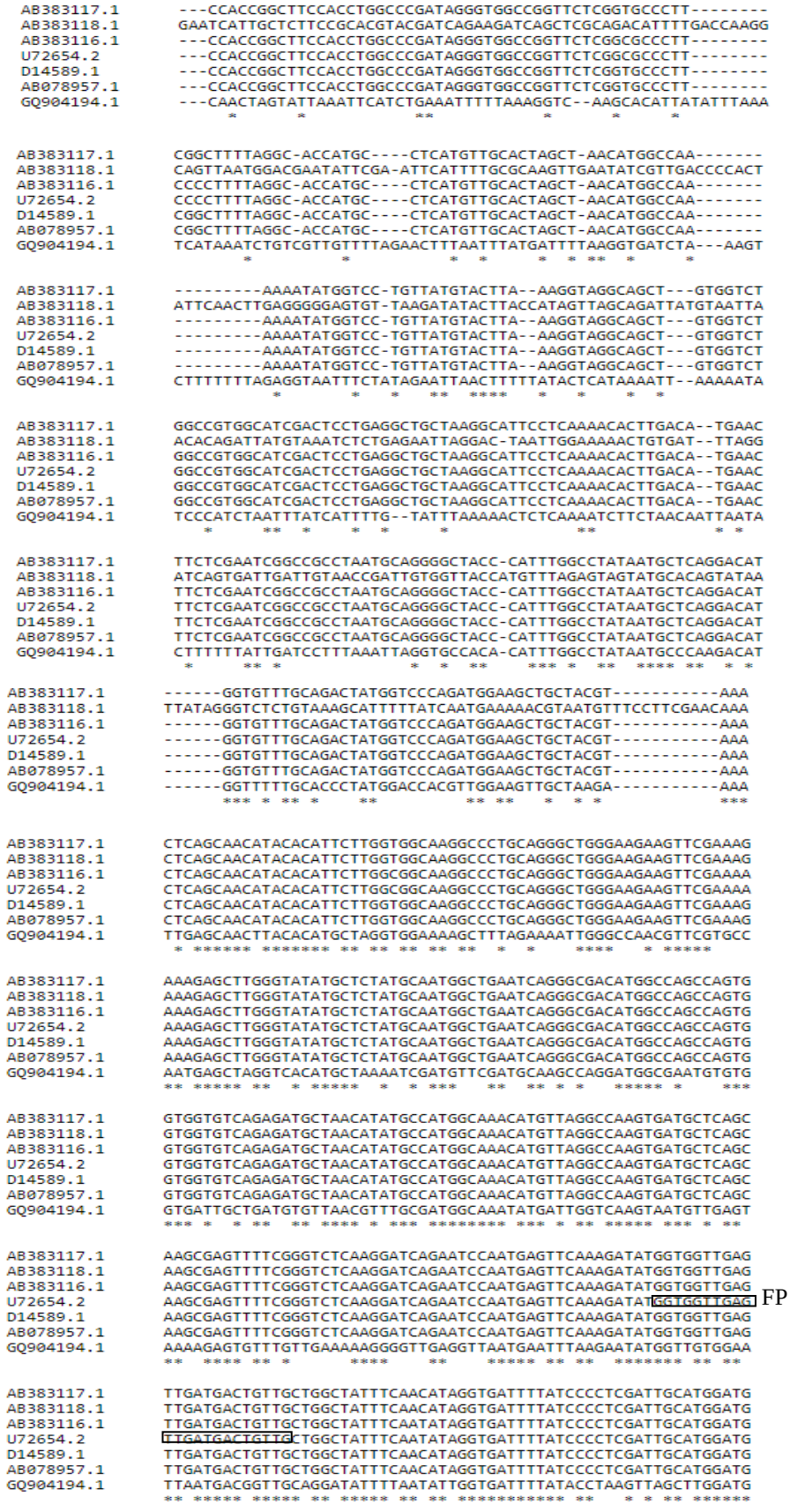


Figure 43: Multiple sequence alignment of *F3'5'H* using CLUSTALW analysis for G1P1 primer. FP- Forward primer, RP- reverse primer and primer sequence is in the box.

- **G1P2 primer**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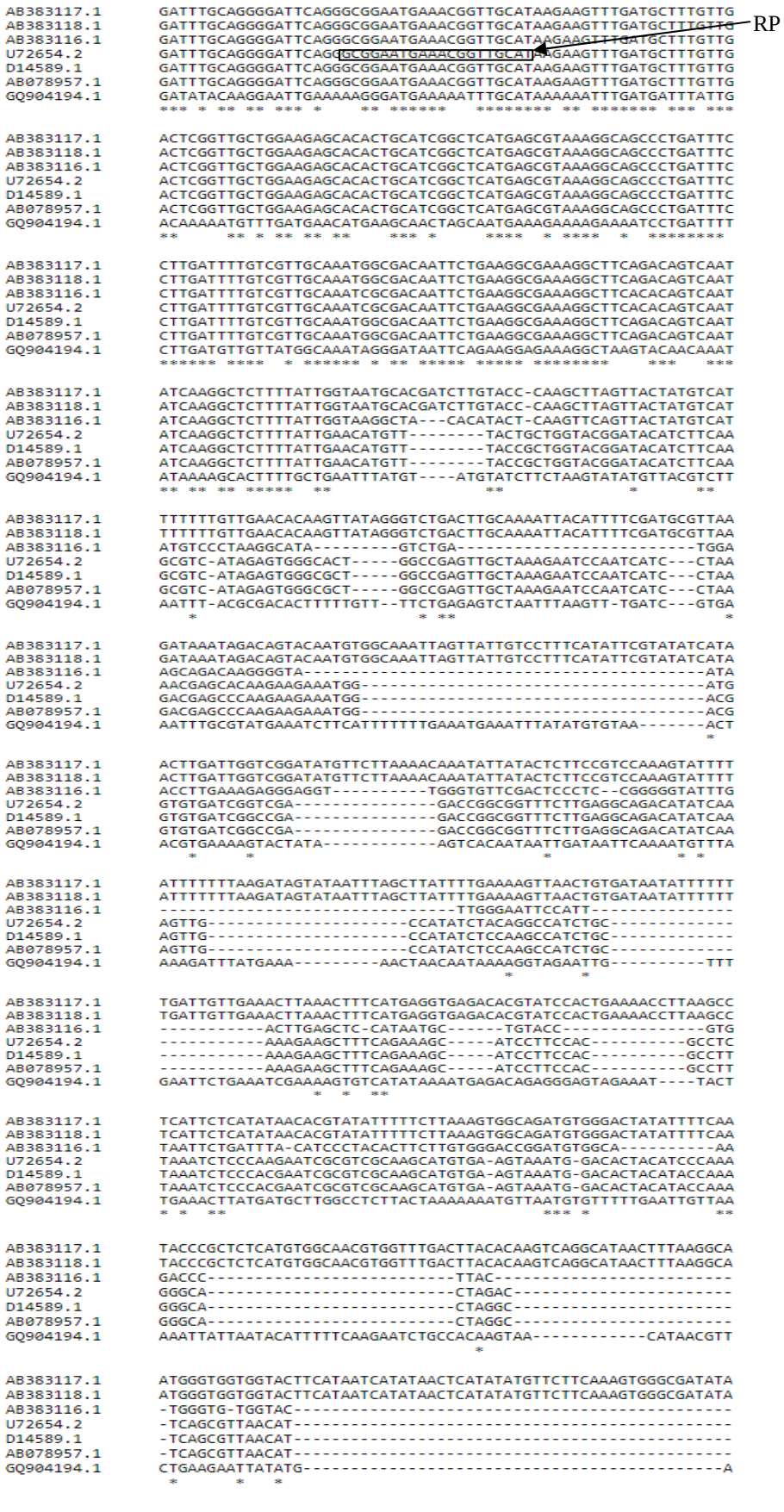
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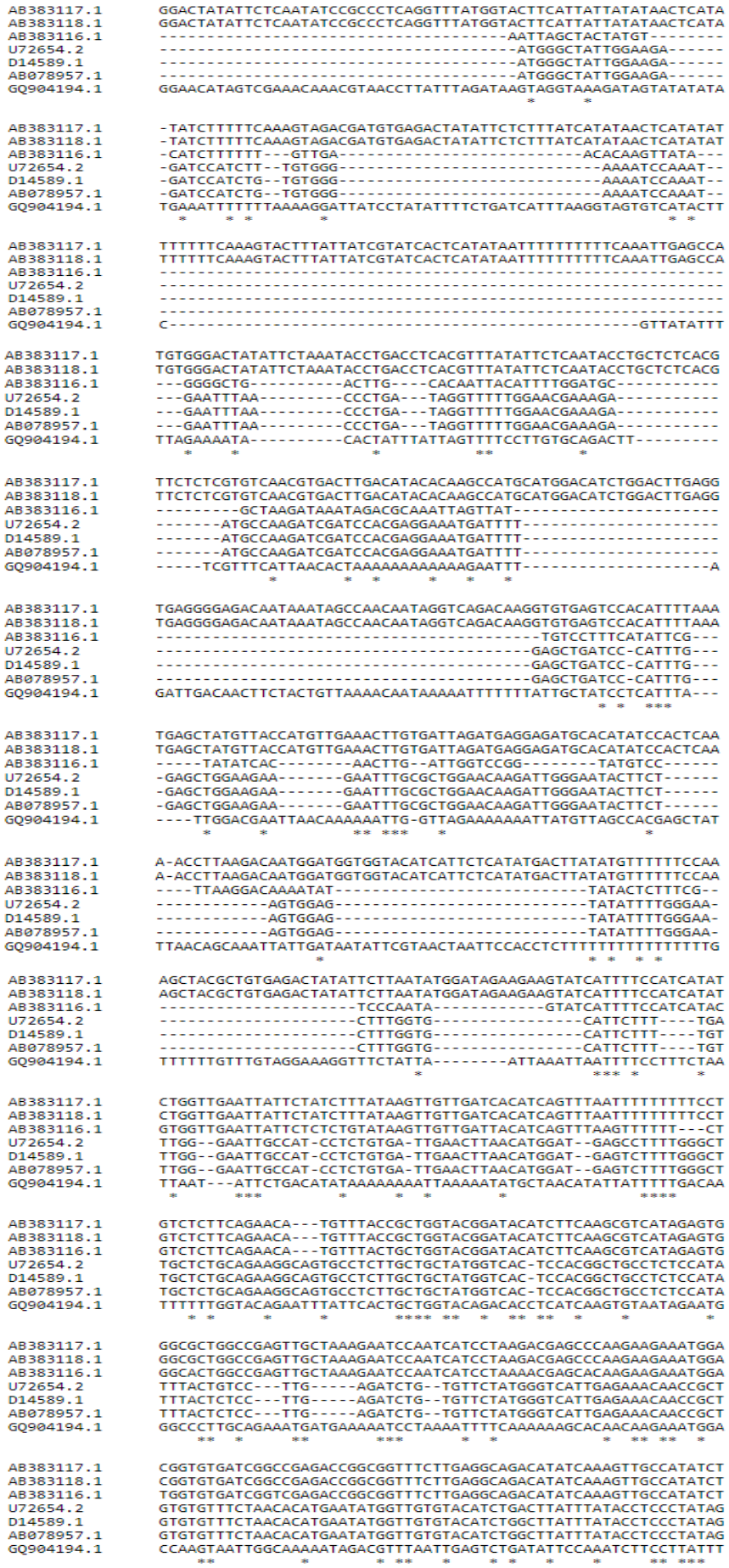
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D14589.1 ----------ATGGGCTATTGGAAGA------
AB078957.1 ----------ATGGGCTATTGGAAGA------
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U72654.2 ----------
D14589.1 ----------
AB078957.1 ----------
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AB078957.1 TTGG--GAATTGCCAT-CCTCTGTGA-TTGAACTTAACATGGAT--GAGTCTTTTGGGCT
GQ904194.1 TTAAT---ATTCTGACATATAAAAAAAAATTAAAAATATGCTAACATATTATTTTTGACAA
AB383117.1 GTCTCTTCAGAACA---TGTTTACCGCTGGTACGGATACATCTTCAAGCGTCATAGAGTG
AB383118.1 GTCTCTTCAGAACA---TGTTTACCGCTGGTACGGATACATCTTCAAGCGTCATAGAGTG
AB383116.1 GTCTCTTCAGAACA---TGTTTACTGCTGGTACGGATACATCTTCAAGCGTCATAGAGTG
U72654.2 TGCTCTGCAGAAGGCAGTGCCTCTTGCTGCTATGGTCAC-TCCACGGCTGCCTCTCCATA
D14589.1 TGCTCTGCAGAAGGCAGTGCCTCTTGCTGCTATGGTCAC-TCCACGGCTGCCTCTCCATA
AB078957.1 TGCTCTGCAGAAGGCAGTGCCTCTTGCTGCTATGGTCAC-TCCACGGCTGCCTCTCCATA
GQ904194.1 TTTTTTGGTACAGAATTTATTCACTGCTGGTACAGACACCTCATCAAGTGTAATAGAATG
AB383117.1 GGCGCTGGCCGAGTTGCTAAAGAATCCAATCATCCTAAGACGAGCCCAAGAAGAAATGGA
AB383118.1 GGCGCTGGCCGAGTTGCTAAAGAATCCAATCATCCTAAGACGAGCCCAAGAAGAAATGGA
AB383116.1 GGCACTGGCCGAGTTGCTAAAGAATCCAATCATCCTAAAACGAGCACAAGAAGAAATGGA
U72654.2 TTTACTGTCC---TTG-----AGATCTG--TGTTCTATGGGTCATTGAGAAACCAACCGCT
D14589.1 TTTACTCTCC---TTG-----AGATCTG--TGTTCTATGGGTCATTGAGAAACCAACCGCT
AB078957.1 TTTACTCTCC---TTG-----AGATCTG--TGTTCTATGGGTCATTGAGAAACCAACCGCT
GQ904194.1 GGCCCTTGCAGAAATGATGAAAAATCCTAAAATTTTCAAAAAAGCACAACAAGAAATGGA
AB383117.1 CGGTGTGATCGGCCGAGACCGGCGGTTTCTTGAGGCAGACATATCAAAGTTGCCATATCT
AB383118.1 CGGTGTGATCGGCCGAGACCGGCGGTTTCTTGAGGCAGACATATCAAAGTTGCCATATCT
AB383116.1 TGGTGTGATCGGTCGAGACCGGCGGTTTCTTGAGGCAGACATATCAAAGTTGCCATATCT
U72654.2 GTGTGTTTCTAACACATGAATATGGTTGTGTACATCTGACTTATTTATACCTCCCTATAG
D14589.1 GTGTGTTTCTAACACATGAATATGGTTGTGTACATCTGGCTTATTTATACCTCCCTATAG
AB078957.1 GTGTGTTTCTAACACATGAATATGGTTGTGTACATCTGGCTTATTTATACCTCCCTATAG
GQ904194.1 CCAAGTAATTGGCAAAAATAGACGTTTAATTGAGTCTGATATTCCAAATCTTCCTTATTT

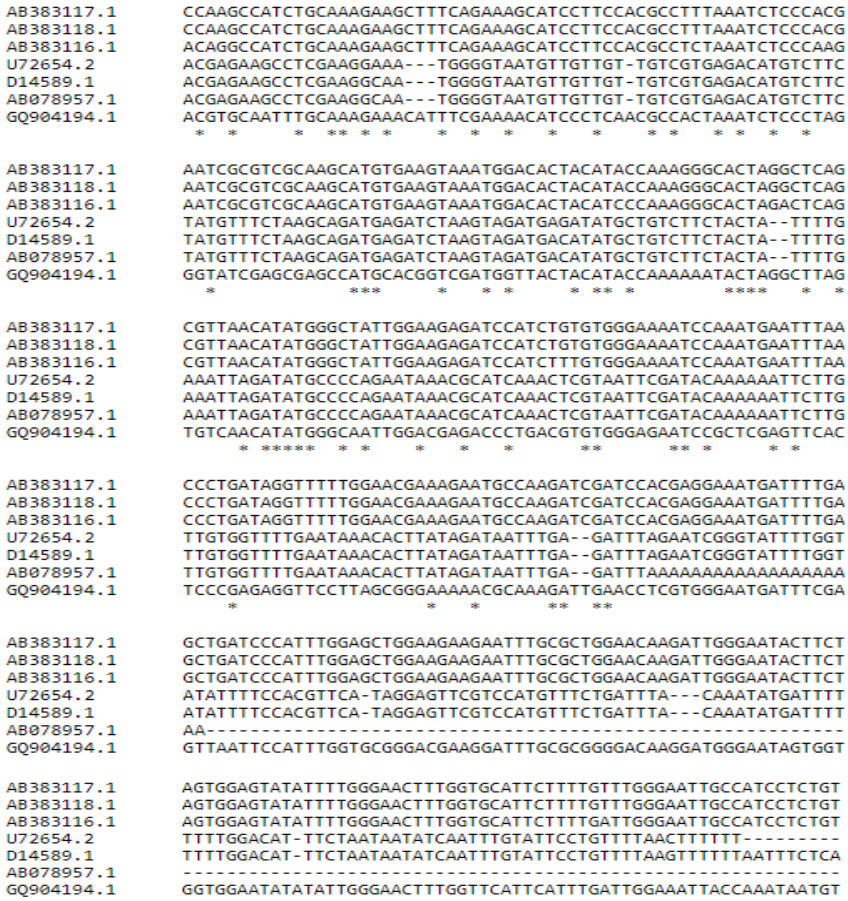
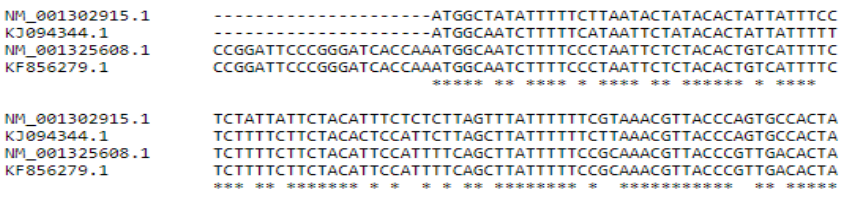
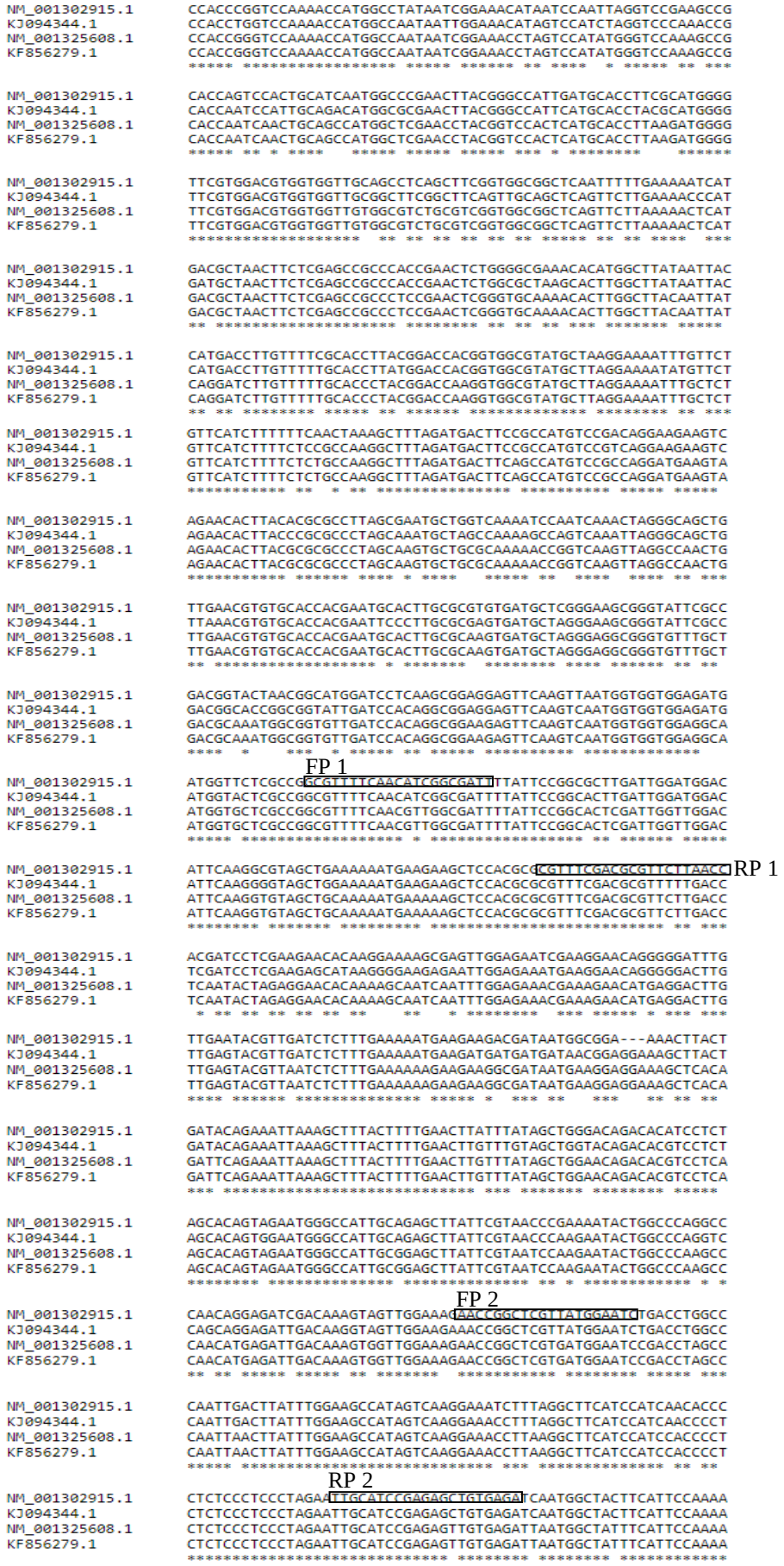


Figure 44: Multiple sequence alignment of *F3'5'H* using CLUSTALW analysis for G1P2 primer. FP- Forward primer, RP- reverse primer and primer sequence is in the box.

Similarly the primers for gene *F3'H* were synthesized using sequence of *Solanum lycopersicum* (NCBI accession no. NM_001302915.1) and sequence alignments after CLUSTALW analysis is given below along with selected primer sequences.

- **G2P1 and G2P2 primers**




FP 1
RP 1
FP 2
RP 2

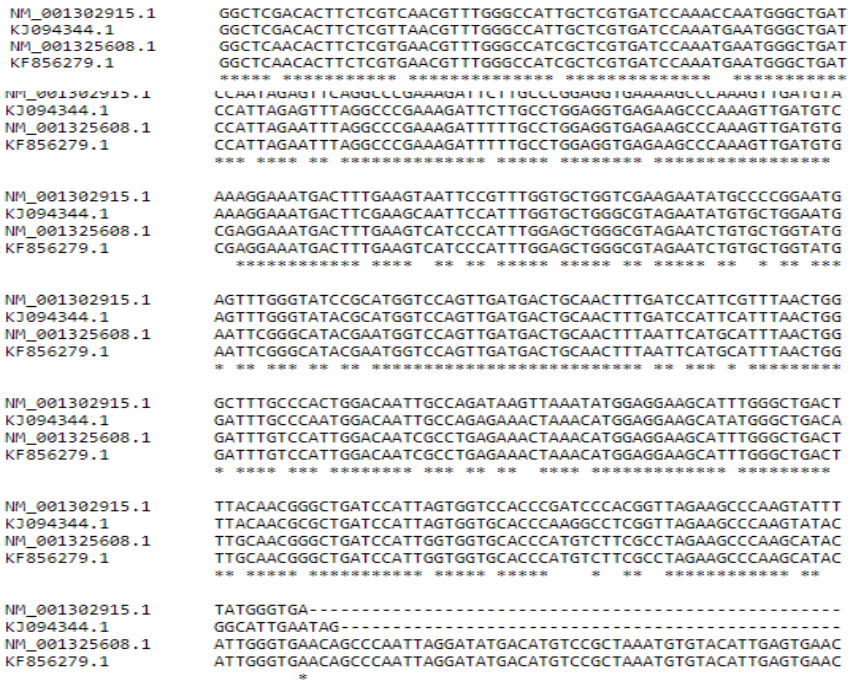


Figure 45: Multiple sequence alignment of *F3'H* using CLUSTALW analysis for G2P1 and G2P2 primers. FP- Forward primer, RP- reverse primer and primer sequence is in the box.

From the sequence alignment, gene sequences having maximum similarities and also matching the parameters of desired characteristics were selected and the sequence of the same is given in Table 18 along with amplicon size.

Table 18: Primers used for gene expression analysis

Primer	Sequence (5'-3')	Amplicon size (bp)
G1P1FP	TGCCCAAGACATGGTTTTTGC	135
G1P1RP	AGCATGTGGCCTAGCTCAACA	
G1P2FP	GGTGGTTGAGTTGATGACTGTTG	109
G1P2RP	ATGCAACCGTTTCATTCCGC	
G2P1FP	GCGTTTTCAACATCGGCGATT	107
G2P1RP	GGTTAAGAACGCGTCGAAACG	
G2P2FP	AACCGGCTCGTTATGGAATC	127
G2P2RP	TCTCACAGCTCTCGGATGCAA	

(2) RNA isolation, cDNA preparation and amplification

Isolation of RNA was done using Bio-Rad kit and all the RNA were checked on agarose gel (1%) and it was observed that the quality and yield of RNA was higher in Qiagen kit (Fig. 46).

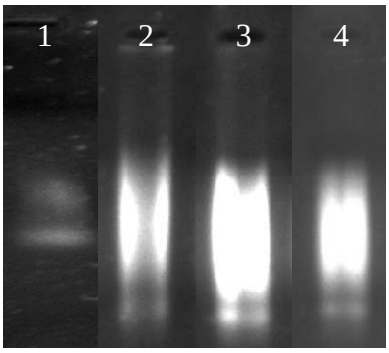
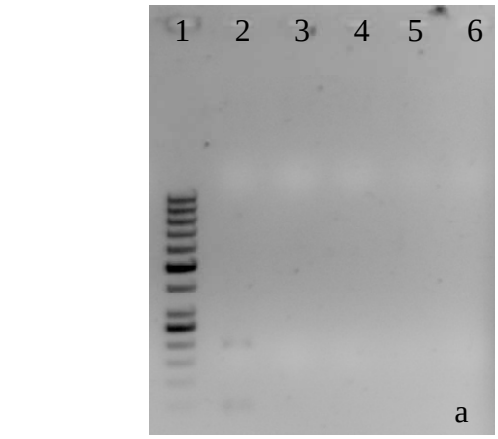


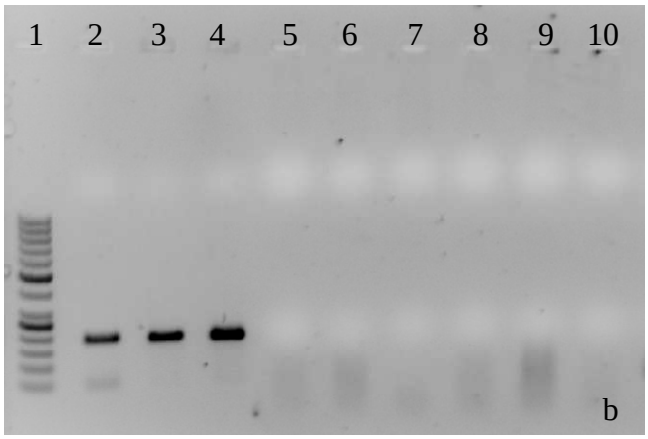
Figure 46: Isolated RNA from shoots of different samples- (1) *in vivo* (2) *in vitro* (MS-1), (3) *in vitro* treated with YE (50 mg/l) and (4) *in vitro* treated with SA (50 µM).

All the RNAs were checked for its purity and yield in UV spectrophotometer and the yields were 1 µg/ml and purities were between 1.9-2.1. cDNA was synthesized from the RNA and gene expression was done with the primers. β-actin was used as a housekeeping gene and its amplification was observed in all samples which confirmed cDNA synthesis. However the primer sets for both the genes in all the samples were unable to amplify the bands (Fig. 47a, b, c).

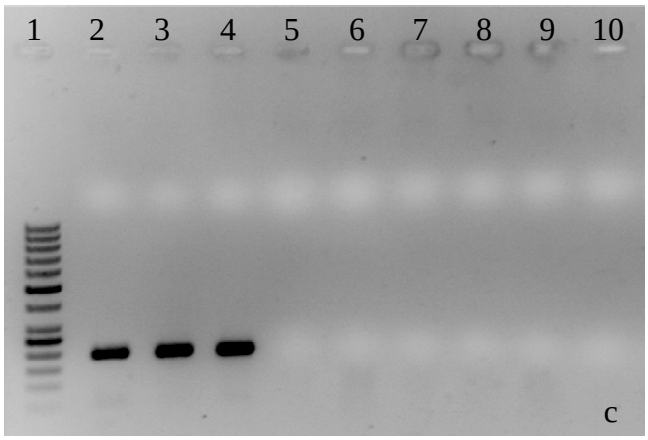
The observations of this study revealed that β-actin specific amplification was observed in all the samples, whereas no amplification was observed in any primer sets for both the genes. Gradient PCR was also tried in temperature range from 54-58 ºC, however amplification of only β-actin gene was observed but amplification for any of the two genes was absent. Thus it was confirmed that cDNA synthesis was proper and β-actin was present in the plant, but the sequences of selected genes either changed or absent in *H. indicus*.



Lane 1: Molecular weight marker
Lane 2: β-actin
Lane 3: Primer G1P1
Lane 4: Primer G2P1
Lane 5: Primer G1P2
Lane 6: Primer G2P2



Lane 1: Molecular weight marker
Lane 2: β-actin in MS-1
Lane 3: β-actin in YE (50 mg/l)
Lane 4: β-actin in SA (50 µM)
Lane 5: MS-1 using G1P1
Lane 6: YE (50 mg/l) using G1P1
Lane 7: SA (50 µM) using G1P1
Lane 8: MS-1 using G2P1
Lane 9: YE (50 mg/l) using G2P1
Lane 10: SA (50 µM) using G2P1



Lane 1: Molecular weight marker
Lane 2: β-actin in MS-1
Lane 3: β-actin in YE (50 mg/l)
Lane 4: β-actin in SA (50 µM)
Lane 5: MS-1 using G1P2
Lane 6: YE (50 mg/l) using G1P2
Lane 7: SA (50 µM) using G1P2
Lane 8: MS-1 using G2P2
Lane 9: YE (50 mg/l) using G2P2
Lane 10: SA (50 µM) using G2P2

Figure 47: cDNA amplification (a) *in vivo* shoots using all primers (b) *in vitro* shoots using G1P1 and G2P1 primers and (c) *in vitro* shoots using G1P2 and G2P2 primers.

In the study rutin and quercetin were not synthesized in any of the samples including *in vivo* shoots which can be linked with expression of these genes (Li et al., 2017). Earlier studies have suggested that *F3'H* gene plays an important role in the regulation of rutin and quercetin biosynthesis and absence of these metabolites might suppress the gene expression (Holton, 1996; Liu et al., 2014). The absence of quercetin but

presence of kaempferol in the present study is in line with the study of Lillo et al. (2008), and they have reported that the later appears constitutively but accumulation of former metabolite depends on several factors.

The genes selected for the study are responsible for flower colour and suppression of these genes changes the colour to orange/red by producing anthocyanins based on pelargonidin (Tanaka and Brugliera, 2013). The ancestral angiosperms produced violet/blue flowers containing delphinidin based anthocyanins (Rausher, 2006) but in the course of evolution the colour changed from blue to red owing to their loss of functions (Rausher, 2008). Change in the flower colour of *Ipomoea* sp. from blue to red is due to mutation in *F3'H* gene (Hoshino et al., 2003), whereas in *Gentiana scabra* flowers the change is from blue to pink which is due to insertion of transposable elements in *F3'5'H* (Nakatsuka et al., 2006). Flowers of *H. indicus* are reddish yellow in colour and these genes are absent or less expressive. However there is no report on these genes in *H. indicus* but study by Bak et al. (2011) reported that *F3'5'H* gene is absent in *Arabidopsis* whereas expression of *F3'H* is regulated independently from other genes of the pathway. Whereas Ma et al. (2014) reported that in mulberry the expression of *F3'H* is tissue specific and is absent in leaf. The loss in function may also be due to the environmental factors (Li et al., 2017).

Both these genes were first studied in *Petunia* plant (Holton et al., 1993; Brugliera et al., 1999) but they are reported to have diverged before the speciation of higher plants in *Torenia* (Ueyama et al., 2002). Other study on Asteraceae family also documents that these species lost *F3'5'H* gene and later on reacquired by duplication and neofunctionalization of a *F3'H* gene (Seitz et al., 2006). Similarly Ishiguro et al. (2012) reported that *F3'5'H* gene is lost in *Antirrhinum majus* after speciation from *A. kelloggi* or other ancestors. These genes are also reported to contain plural loci which can be evidenced from vast number of isoforms (Tanaka and Brugliera, 2013) e.g. in grapes copy number of both the genes has increased by duplication (Falginella et al., 2010).

Therefore it can be concluded that the absence of rutin is coupled with absence of both *F3'5'H* and *F3'H* gene expression which may be due to change in gene sequence or loss of its function. This also could be due to epigenetic changes and it needs to be further investigated.