CHAPTER 2

REVIEW OF LITERATURE

This chapter cites the important works related to regeneration utilizing leaf and nodal explants, HPTLC fingerprinting of shoots and elicitation of metabolites using yeast extract, salicylic acid and methyl jasmonate.

2.1 REGENERATION STUDIES

In this experiment, leaves and nodes were utilized to establish shoot cultures in MS medium containing sucrose as a source of carbohydrates as they require continuous supply of exogenous carbon source. Different types of carbohydrates have been used but sucrose is reported to be prominent which acts as energy source and provide optimum osmotic requirement of organogenesis (Verma and Dougall, 1977; Thorpe et al., 1986; Feng et al., 2010; Silva, 2010). Another important factor is plant growth regulators (PGRs) which have important role in cell growth as well as differentiation, hence becoming an important factor which affects the regeneration under *in vitro* condition (Trewavas, 1981). Cytokinins and auxins are two main classes of PGRs and their ratio influences the organogenesis as well as the mode of regeneration i.e. direct or indirect (Skoog and Miller, 1957; Christianson and Warnick, 1985; Bhojwani and Razdan, 1996).

2.1.1 Effect of PGRs on Shoot Regeneration from Leaf Explant

Leaf is a good source for regeneration as this explant is easily available and the chances of genetic variation is reduced due to maturity of the explant which may arise when immature explants are used (Singh et al., 2013). Another advantage of this explant is its usefulness in genetic transformation studies (Kumar et al., 2010). It has been used to regenerate large number of shoots in many important plants of Asclepiadaceae family like *Tylophora indica* (Faisal and Anis, 2003; Thomas and Phillip, 2005), *Leptadenia reticulata* (Arya et al., 2003; Patel et al., 2014), *Ceropegia spiralis* (Murthy et al., 2010) and *Ceropegia bulbosa* (Phulwaria et al., 2013b).

Thorpe et al. (1980) and Evans et al. (1983) reported that *in vitro* shoot regeneration and its proliferation mainly dependents on type and the concentration of cytokinins. They are involved in regulation of proteins synthesis which are prerequisite for formation and functioning of mitotic spindles and hence required for cell division and shoot formation (Chawla, 2002; George et al., 2008). Cell expansion, division and regulation of shoot formation as well as multiplication are the main functions of cytokinins (Davies, 1995; Mok and Mok, 2001). *Bacopa monnieri* leaf explants when placed on MS medium fortified with different concentrations of BA, optimum shoot regeneration was

observed at 6 µM (Joshi et al., 2010). Similarly in *Solanum xanthocarpum* optimum number of shoots regenerated when the medium was fortified with 8.8 µM BA (Sundar and Jawahar, 2011). In another study on *Tylophora indica*, 2.5 mg/l BAP proved to be beneficial for organogenesis (Kalimuthu and Jeyaraman, 2012). Kn is an another commonly used cytokinin for shoot regeneration and 1 µM induced maximum shoots from *T. indica* leaves as reported by Rathinavel and Sellathurai (2010). Leaf explant of *Justicia gendarussa* (Agastian et al., 2006) and *Ionidium suffruticosum* (Sonappanavar and Jayaraj, 2011) also formed maximum shoots when inoculated on Kn fortified medium. In *Solanum nigrum*, Kn proved to be better for regeneration as compared to BA (Bhat et al., 2010).

There are many reports which document that shoot regeneration can be achieved utilizing two different media. In *T. indica*, leaf explants differentiated callus on medium having 2,4,5-T (10 µM) and when it was transferred to medium containing 5 µM Kn it induced 64.8±0.74 shoots in 85% cultures (Faisal and Anis, 2003). Later Thomas and Philip (2005) have also reported in *T. indica* that medium containing BA (1.5 µM) and 2,4-D (7 µM) induced callus, and shoots regenerated from it when it was subcultured to a medium fortified with TDZ (8 µM). Whereas Sahai et al. (2010) reported shoot induction in presence of TDZ and multiplication in BA containing medium for the same plant. Similarly in *Plumbago zeylanica* callus was formed in medium fortified with BA and 2,4-D, and differentiated shoots when transferred to medium supplemented with only BA (Lubaina and Murugan, 2012). Amudha and Shanthi (2011) have also reported in *Acmella calva* that the leaf explant formed callus on BAP (3 µM) and NAA (2 µM) containing medium which differentiated shoots in presence BAP (5 µM).

Combination of cytokinins has been reported to induce optimum number of shoots in many studies like in *Stevia rebaudiana* leaf explant formed maximum shoots in presence of BA (8.88 µM) with Kn (4.65 µM) (Sreedhar et al., 2008). However the growth of shoots were stunted and they were transferred to medium fortified with IBA (4.92 µM) for elongation. Similarly Jain et al. (2011) reported that leaves of *Withania coagulans* induced optimum number of shoots in medium having BA (22.2 µM) and Kn (2.3 µM), but were transferred to medium having BA (2.2 µM), Kn (2.3 µM) and phloroglucinol (3.9 µM) for further elongation of shoots. On the contrary in *Withania somnifera* optimum shoot regeneration was reported in medium containing BA at 4 µM with Kn at 4 µM (Joshi and Padhya, 2010) and synergistic effect of these cytokinins for shoot regeneration is also reported for *Achyranthes aspera* and *Gladiolus grandiflorus* (Shaheenuzzaman et al., 2011; Sen et al., 2014). Whereas Seetharam et al. (2002) reported multiplication and elongation

of *Enicostemma hyssopifolium* shoots in medium supplemented with BA and Kn. Whereas Soniya and Das (2002) used combination of BA with another cytokinin picloram for regeneration in *Piper longum*.

Similarly the combination of cytokinin with auxin is also used as they are known to regulate each other's biosynthesis, helps in organogenesis, as well as in branching of shoots (Muller and Leyser, 2011; Su et al., 2011). *Stevia rebaudiana* leaf explant formed 7.9±0.6 shoots in medium supplemented with combination of BA (8.87 μM) and IAA (5.71 μM) (Sivaram and Mukundan, 2003). Young and old leaves of *Brunfelsia calycina* differentiated shoots in different concentrations of BA and IAA (Liberman et al., 2010). Haque and Ghosh (2013) have reported similar results in *Tylophora indica* in which only young leaves differentiated shoots via indirect organogenesis on medium containing 2.0 mg/l BAP and 0.2 mg/l IAA whereas mature leaves directly differentiated shoots in presence of only 2.0 mg/l BAP. A combination of BA and IAA also has been reported to be beneficial for *Hypericum frondosum* (Touchell et al., 2008) and *Hypericum* hybrid variety H2003-004-016 (Meyer et al., 2009). Similarly in many other plant species like *Cichorium intybus* (Velayutham et al., 2006), *Pityopsis ruthii* (Wadl et al., 2011), *Spilanthes acmella* (Singh and Chaturvedi, 2012), *Streptocarpus rexii* (North and Ndakidemi, 2012), *Ajuga bracteosa* (Kaul et al., 2013) and *Bacopa monnieri* (Kumari et al., 2015) this combination of PGRs was favourable for shoot regeneration.

Another commonly used auxin along with cytokinin is NAA and in two *Echinacea* sp., the combination of BAP (4.44 μM) with NAA (0.054 μM) proved to be optimum for shoot regeneration in *E. purpurea* whereas BAP (26.6 μM) and NAA (0.11 μM) was favourable for *E. pallida* (Koroch et al., 2002, 2003). Leaf explant of *Vernonia cinerea* formed shoots in presence of BA (3.0 mg/l) but multiplication occurred when subcultured on medium fortified with BA and NAA (Seetharam et al., 2007). Whereas Gopalakrishnan et al. (2009) reported that leaf explants of *Plumbago rosea* differentiated shoots in medium fortified with BA-NAA, which elongated when transferred to a medium with BAP and GA_3. Leaves of *Artemisia vulgaris* differentiated callus on medium supplemented with BA and NAA, and this callus formed shoots when transferred to medium with BA and GA_3 (Borzabad et al., 2010). Leaf explant of *Ruta graveolens* formed callus in medium fortified with 2,4,5-T, which was able to form shoots when transfered to BA and NAA containing medium (Ahmad et al., 2010). Whereas callus formed from leaf explant of *Leptadenia reticulata* in medium having BAP (0.5 mg/l) and 2,4-D (0.5 mg/l) differentiated 30.70±1.70 shoots when subcultured on medium with BAP (0.5 mg/l) and NAA (0.1 mg/l)

(Patel et al., 2014). Combination of BA and NAA has also been reported for shoot regeneration in *Pogostemon cablin* (Paul et al., 2010) and *Catharanthus roseus* (Verma and Mathur, 2011).

Another cytokinin i.e. Kn is also used along with auxin for shoot regeneration from leaf explant. Leaves of *Cardiospermum halicacabum* induced callus in medium fortified with 5 μM 2,4-D which differentiated 28±1.9 shoots when subcultured on medium having Kn (8 μM) and IAA (0.5 μM) (Thomas and Maseena, 2006). Reddy et al. (2001) reported that leaves of *Coleus forskohlii* formed callus when placed on medium fortified with Kn (2.4 μM) and induced shoots when subcultured on medium supplemented with Kn (4.6 μM) and NAA (0.54 μM). Whereas leaf explant of *Ziziphora tenuior* induced callus in medium fortified with 0.5 mg/l Kn and 1.5 mg/l NAA under dark condition. This callus was subcultured to medium having 2 mg/l BA for shoot induction and multiplication was achieved again in presence of Kn and NAA (Dakah et al., 2014).

2.1.2 Effect of PGRs on Shoot Regeneration from Nodal Explant

Plants contain quiescent axillary buds which have the potential to develop into shoots under favourable conditions. In conventional method of vegetative propagation, the axillary buds takes over the function of the main shoot when the terminal bud is decapitated and this is the basis of *in vitro* regeneration using node as an explant (Faisal et al., 2007). This method is relatively easy and it also yields plants that are genetically identical with the mother plants (Hu and Wang, 1983). Regeneration by this method has been proved to be an effective and reliable method of mass multiplication for many plants (Stefaan et al., 1994). Important medicinal plants of Asclepiadaceae family like *Tylophora indica* (Faisal et al., 2007), *Leptadenia reticulata* (Arya et al., 2003; Sudipta et al., 2011; Rathore et al., 2013), *Ceropegia candelabrum* (Beena et al., 2003) and *Ceropegia hirsute* (Nikam et al., 2008) have been regenerated through nodal explant.

Stefaan et al. (1994) suggested that BA is an efficient and a promising cytokinin for regeneration from nodal explant. Sahoo and Chand (1998) also reported that axillary bud break and subsequent development into shoots under *in vitro* conditions is a cytokinin function. *Cunila galioides* nodal explant regenerated maximum shoots when they were placed in medium containing 8.8 μM BA (Fracaro and Echeverrigaray, 2001), whereas 5.0 μM concentration of BAP has been reported for *Vitex trifolia* nodal explant (Hiregoudar et al., 2006). *Ceropegia hirsute* and *C. spiralis* nodes formed optimum shoots in medium fortified with BAP (Nikam et al., 2008; Murthy et al., 2010). Formation of 15.28±0.96

shoots were reported in *Phyllanthum amarus* when cultured in medium supplemented with 0.5 mg/l BAP (Sen et al., 2009). Thomas and Yoichiro (2010) have reported that higher level of BA (17.7 µM) induced maximum shoots in *Justicia gendarussa*. Although shoot regeneration is genotype dependent, many medicinal plants like *Wrightia tinctoria*, *Bacopa monnieri*, *Munronia pinnata* and *Aegle armelos* regenerated maximum shoots in medium containing BAP (Purohit and Kukda, 2004; Mohapatra and Rath, 2005; Gunathilake et al., 2008; Yadav and Singh, 2011). In another report, *Naringi crenulata* nodes regenerated maximum shoots on MS medium containing BAP (2.0 mg/l) along with $AdSO_4$ (25.0 mg/l) and glutamine (150 mg/l) (Singh et al., 2011b). Nodal explant of *Gynura procumbens*, *Lawsonia inermis* and *Veronica anagallis-aquatica* also required BA for optimum shoot regeneration (Shahzad et al., 2011; Alizah and Nurulaishah, 2015; Moharana et al., 2018). There are many reports which suggested the use of BAP for proliferation of shoots in *Acmella calva* (Amudha and Shanthi, 2011), *Stevia rebaudiana* (Soliman et al., 2014) and *Allamanda cathartica* (Khanam and Anis, 2018).

Kn and TDZ are other two cytokinins which are used, nodes of *C. juncea* formed 8.5±0.3 shoots in medium supplemented with 7.5 µM Kn (Nikam and Savant, 2009). Sivakumar et al. (2014) have used Kn (4.36 µM) for shoot regeneration from nodes of *Tinospora cordifolia*, but it was replaced by 8.82 µM BA for further multiplication of shoots. In a recent report on *Rotala rotundifolia* the combination of Kn (0.25 mg/l) and GA_3 (0.25 mg/l) proved better for shoot regeneration (Dogan, 2017). Incorporation of TDZ in the medium facilitated optimum shoot regeneration from nodal explant of *Cineraria maritima* and *Ceropegia pusilla* (Banerjee et al., 2004; Kondamudi et al., 2010), whereas Jana et al. (2013) used 2-iP for nodes of *Sophora tonkinensis*.

There are many reports which suggest that a combination of two cytokinins is essential for shoot regeneration. *Phyla nodiflora* nodal explants regenerated maximum shoots when cultured in medium fortified with combination of BA (2.5 mg/l) and Kn (0.5 mg/l) (Ahmed et al., 2005). Similarly Sudipta et al. (2011) have reported the use of BA and Kn for regeneration in *Leptadenia reticulata* nodal explant. The same combination has been reported for shoot regeneration in a number of important plants like *Andrographis paniculata* (Dandin and Murthy, 2012), *Catharanthus roseus* (Sain and Sharma, 2013), *Chlorophytum borivilianum* (Ashraf et al., 2014) and *Piper betle* (Elahi et al., 2017). The report of Seetharam et al. (2002) document that the combination of BA (1 mg/l) with IAA (0.5 mg/l) induced 11.00±0.44 shoots from nodal explant of *Enicostemma hyssopifolium*. These shoots further elongated and multiplied in medium supplemented with BA (0.5

mg/l) and Kn (1 mg/l). Similarly in *Adhatoda vasica* (Abhyankar and Reddy, 2007), *Passiflora edulis* and *P. foetida* (Shekhawat et al., 2015a, 2015b), BA-Kn combination has been used for multiplication of shoots. Many workers have reported addition of third PGR along with BA and Kn for optimum regeneration for e.g. in Asclepiadaceae member *L. reticulata*, medium fortified with BA, Kn and 2-ip regenerated maximum shoots (Rathore et al., 2013).

A combination of cytokinin with low concentration of auxin results in high frequency of shoot formation from axillary bud which indicates the synergistic effect of these two groups of PGRs (Skirvin, 1984). Sivaram and Mukundan (2003) have reported that nodal explant of *Stevia rebaudiana* formed optimum shoots in medium fortified with combination of BA (1 mg/l) and IAA (0.5 mg/l). Similarly *Zehneria scabra* nodes regenerated shoots in presence of BAP (5 mg/l) with IAA (0.5 mg/l) (Anand and Jeyachandran, 2004). Gopi et al. (2006) have reported maximum shoots (14.3±1.5) in *Ocimum gratissimum* nodes in medium supplemented with 0.5 mg/l BAP and 0.25 mg/l IAA. Similarly high BA and low IAA concentration proved to be optimum for shoot regeneration in *Ruta graveolens* (Bohidar et al., 2008), *Prosopis cineraria* (Kumar and Singh, 2010), *S. rebaudiana* (Anbazhagan et al., 2010), *Celastrus paniculatus* (Phulwaria et al., 2013a) and *Toddalia asiatica* (Anand et al., 2015). Mousa and Bakhashwain (2014) observed that in *Simmondsia chinensis*, genotype "Hada Al-Sham" regenerated maximum shoots in the culture medium containing low BAP (5 μM) and high level of IAA (10 μM).

BA and NAA together are also used for nodal regeneration, as Martin et al. (2006) reported formation of multiple shoots in *C. paniculatus* when medium was supplemented with BA (0.05 mg/l) and NAA (0.01 mg/l). In nodes of other species like *Centella asiatica* (Mohapatra et al., 2008), *Boerhaavia diffusa* (Biswas et al., 2009), *Stemona tuberose* (Biswas et al., 2011) and *Rumex vesicarius* (Nandini et al., 2013) also regenerated optimum shoots in presence of BA and NAA. Nodes of *Aristolochia indica* formed callus in presence of BAP (0.8 mg/l) and it differentiated shoots when subcultured in medium containing BAP (0.8 mg/l) and NAA (0.5 mg/l) (Pattar and Jayaraj, 2012). Whereas in *Vernonia cinerea* nodal explant, medium fortified with combination of BA and NAA have been used for multiplication of shoots (Seetharam et al., 2007). Whereas nodes of *Holarrhena antidysenterica* regenerated maximum shoots in medium supplemented with BA, NAA along with addition of urea (100 mg/l) (Ahmed et al., 2001).

Kn with IAA had synergistic effect for regeneration in *Cardiospermum halicacabum* as it formed 23±1.0 shoots from nodal explant (Thomas and Maseena, 2006).

Whereas combination of Kn (10.0 mg/l), IAA (1.0 mg/l) and $AdSO_4$ (30.0 mg/l) was required to induce optimum shoots from *S. rebaudiana* (Mitra and Pal, 2007). On the other hand Mehta and Subramanian (2005) reported maximum shoots in *Asparagus adscendens* when placed in medium containing 0.46 μM Kn with 0.27 μM NAA.

2.1.3 Regeneration using *In Vitro* Nodes

When plants are not available, alternative to *in vivo* nodes are *in vitro* nodes, which have been used for induction of multiple shoots in medicinal plants (Anand and Jeyachandran, 2004) and Hayta et al. (2011) reported that presence of BA formed multiple shoot. BA at 2 mg/l induced maximum regeneration in *Lippia alba* cv. Kavach (Gupta et al., 2001), whereas in *Vitis vinifera* cv. Napoleón medium fortified with BA (8.9 μM) formed maximum shoot formation (Ibáñez et al., 2003). Similarly in species like *Lavandula pedunculata* (Zuzarte et al., 2010), *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012), *Pogostemon cablin* (Jin et al., 2014), *Paederia foetida* (Behera et al., 2018) and *Lawsonia inermis* (Moharana et al., 2018) also optimum regeneration was achieved in medium fortified with BA. Combination of both BA and Kn together in medium facilitated optimum shoot regeneration for e.g. BA (1 mg/l) and Kn (1.5 mg/l) formed maximum shoots in *Vanilla planifolia* (Abebe et al., 2009). Similarly 0.5 mg/l concentration of both BA and Kn formed multiple shoots (62.45±0.6) in *P. cablin* (Swamy et al., 2010). Whereas medium supplemented with BA (2 mg/l), Kn (1 mg/l) and NAA (1 mg/l) has been reported for *Vanilla plantifolia* (Renuga and Saravana, 2014) and BAP (2.0 mg/l), Kn (0.5 mg/l) and GA_3 (2.0 mg/l) in *Enicostema axillare* ssp. *littoralis* (Loganathan and Bai, 2014).

Combinations of cytokinin with auxin have also been reported to regenerate optimum shoots for e.g. combination of BA with IAA was optimum for *Stevia rebaudiana* (Laribi et al., 2012) and *Rosmarinus officinalis* (Leelavathi et al., 2013). Whereas combination of BA and IBA has been reported for shoot regeneration in *Wedelia chinensis* (Martin et al., 2003), *Gentiana cruciata* (Hayta et al., 2011) and *Solanum viarum* (Mahadev et al., 2014). Whereas *in vitro* nodes of *Salvia fruticosa* regenerated optimum response (3.88 shoots, 100% response) when placed in medium containing combinations of BA (0.75 μM), NAA (0.1 μM) and GA_3 (0.1 μM) (Arikat et al., 2004).

2.1.4 Rooting of Shoots

The *in vitro* shoots lack roots and have to be transferred to a medium with rooting hormone and generally auxins have been used for root induction in the microshoots. The concentration of auxin is critical to stimulate rooting as excess of the same may result in callusing. Earlier studies reported that the auxins increased cell division or convert the cells of shoot base into merestimatic cells; which follows formation of adventitious root meristem and in turn roots (Abdul, 1987; Saleh, 1991). Another important factor is the concentration of MS medium salts (Murashige, 1979) and especially half and quarter strengths of MS medium are beneficial (Skirvin and Chu, 1979; Garland and Stoltz, 1981; Zimmerman and Broome, 1981). Half strength MS medium fortified with IBA induced 12-13 roots in *Stevia rebaudiana* (Sivaram and Mukundan, 2003), whereas in *Tylophora indica* also shoots formed optimum roots for the same combination with IBA (Faisal and Anis, 2003; Thomas and Philip, 2005). Shoots of *Bacopa monnieri* (Joshi et al., 2010), *Cannabis sativa* (Lata et al., 2010), *T. indica* (Verma et al., 2010; Sharma et al., 2014) and *Catharanthus roseus* (Verma and Mathur, 2011) have induced roots in ½MS medium supplemented with IBA. Further reduction in strength of the medium to quarter is also reported to be beneficial for rooting. Shoots of *Acmella calva* when placed in ¼MS medium fortified with 4 µM of IBA induced 55.4 roots/shoot (Amudha and Shanthi, 2011). This media combination has been reported for rooting in *Wrightia tinctoria* (Purohit and Kukda, 2004), *B. monnieri* (Mehta et al., 2012b), *Terminalia bellerica* (Mehta et al., 2012c), *C. roseus* (Sain and Sharma, 2013) and *Leptadenia reticulata* (Rathore et al., 2013). Whereas full strength MS media fortified with IBA was used for rooting in shoots of *Echinacea purpurea* (Koroch et al., 2002), *Munronia pinnata* (Gunathilake et al., 2008), *Brunfelsia calycina* (Liberman et al., 2010) and *Ajuga bracteosa* (Kaul et al., 2013). Whereas ¼MS medium fortified with IBA (1.5 mg/l) and activated charcoal (100 mg/l) induced optimum rooting in microshoots of *L. reticulata* (Patel et al., 2014).

Some of the plants rooted in medium fortified with NAA like shoots of *Pulsatilla koreana* reported to formed 14.37 roots in ½MS medium fortified with NAA (3 mg/l) (Lin et al., 2011). Similarly NAA has been reported for rooting in *Decalepis hamiltonii* (Anitha and Pullaiah, 2002), *Vitex trifolia* (Hiregoudar et al., 2006) and *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012). However IBA has been proved to be better as compared to NAA for rooting in *Tylophora indica* (Thomas and Philip, 2005), *Cichorium intybus* (Velayutham et al., 2006), *Munronia pinnata* (Gunathilake et al., 2008) and *Rubia cordifolia* (Khadke et al., 2013). Whereas *in vitro* rooting in *Astragalus cariensis* (Erisen et

al., 2010) and *T. indica* (Sahai et al., 2010) have been reported in medium fortified either IBA or NAA.

2.1.5 *In Vitro* Studies in *Hemidesmus indicus*

H. indicus is an important medicinal plant and number of regeneration studies have been carried out utilizing nodal explant but there is only one report which states the use of leaf explant for shoot regeneration. Sreekumar et al. (2000) have reported the formation of 2.75 shoots in 70% cultures in ½MS medium supplemented with combination of BA (2.22 µM) and NAA (1.07 µM). The use of nodal explant for shoot regeneration is documented by several workers and formation of 8.2±0.4 shoots with 95% frequency has been reported by Patnaik and Debata (1996) in medium fortified with Kn (1.15 µM) and NAA (0.054 µM). Later on report of Sreekumar et al. (2000) used ½MS medium with BA (2.22 µM) to regenerate 6.6 shoots (84% response). Saha et al. (2003) observed that in medium supplemented with BAP (2.0 mg/1) and NAA (0.1 mg/l) evoked an optimum response for shoot regeneration and the same has been documented by Nagahatenna and Peiris (2007). Whereas the combination of BAP (1.0 mg/l), NAA (0.5 mg/l) and $AdSO_4$ (15 mg/l) along with elevated sucrose concentration (4%) formed 6.13 shoots as reported by Misra et al. (2003). Siddique et al. (2006) reported callus induction from nodes when placed in medium fortified with Kn (2.0 mg/l), NAA (0.5 mg/l) and PG (1 mg/l) which induced shoots upon transferring to a medium fortified with Kn (2.5 mg/l) and NAA (1.0 mg/l). Siddique and Bari (2006) as well as Sundarmani and Hasina (2015) have reported that Kn and NAA proved better for shoot regeneration. Individual BA has been reported by Shanmugapriya and Sivakumar (2011) and Singh et al. (2015). Combination of BAP and Kn has been reported by Rama Devi et al. (2014) whereas Shekhawat and Manokari (2016) reported media fortified with BAP, Kn and IAA for optimum regeneration from nodal explant.

Multiplication using *in vitro* nodes have been reported by Sreekumar et al. (2000) and reported that ½MS medium fortified with BA (2.22 µM) and NAA (1.07 µM) induced optimum 9.37 shoots. Later on Nagahatenna and Peiris (2007) observed formation of 4.47±1.64 shoots from *in vitro* nodes when placed in medium supplemented with BAP (5 mg/l).

Patnaik and Debata (1996) reported that combination of Kn (1.15 µM) and IBA (7.35 µM) induced 4.4±0.5 roots in microshoots of *H. indicus*. Similarly Kn and IBA has been reported by Siddique et al. (2006) and Siddique and Bari (2006) for rooting of shoots.

However many reports documented the use of IBA as a rooting hormone in different strengths of MS media. Sreekumar et al. (2002) reported that ¼MS medium supplemented with IBA (9.80 µM) induced 12 roots (98% frequency) and similarly report of Shekhawat and Manokari (2016) also stated that same media strength with IBA induced optimum rooting. Whereas ½MS medium fortified with IBA have been documented in earlier reports (Saha et al., 2003; Nagahatenna and Peiris, 2007; Rama Devi et al., 2014; Sundarmani and Hasina, 2015). Whereas ½MS medium supplemented with sucrose (0.75%) along with IBA (2.0 mg/l), NAA (1.0 mg/l) and activated charcoal (100 mg/l) induced optimum roots (5.20±0.84, 98% response) (Misra et al., 2003). Later on Singh et al. (2015) reported rooting in medium fortified with IAA (2.0 mg/l).

2.2 SECONDARY METABOLITE STUDIES

The shoot cultures which developed from *in vivo* nodal were utilized to study their secondary metabolites. First the shoots were analysed for their chemical integrity in comparison to *in vivo* shoots which was done by HPTLC fingerprinting. The important metabolites like lupeol and rutin were quantified in the shoots along with elicitation using yeast extract, salicylic acid and methyl jasmonate.

2.2.1 Qualitative Analysis of Shoots

Chemical fingerprint of plants can be developed using different chromatographic techniques which are valid method for identification as well as authentication of medicinal plants (Valentao et al., 1999; Xie, 2001). Such chemical identities of a particular plant can become a biochemical marker and can be used in differentiating the species from its adulterant (Johnson et al., 2011). Various techniques can be adopted to analyze samples but HPTLC is being widely used as it offers better resolution of metabolites with accuracy (Sethi, 1996; Pawar et al., 2011). Comparative faster analysis, easy, low costing and simultaneous detection of more samples by this technique is advantageous (Wagner and Bladt, 2001; Birk et al. 2005). It has been proved that HPTLC is precise and liner for identification, authentication and characterization and in turn helps in quality control and standardization of herbal formulations (Patil et al., 2014). As analysis of large number of sample can be done at a time, this method has been used to identify the metabolite content in medicinal plants like *Adhatoda vasica* (Varma et al., 2011), *Oroxylum indicum* (Saraf and Srinivas, 2014) and *Crocus sativus* (Mangal et al., 2018).

This method has been used to evaluate the biosynthetic potential of *in vitro* regenerated plants. *In vitro* shoots of *Passiflora caerulea* showed similar HPTLC fingerprint as wild shoots and reported to be chemically true to type in nature (Busilacchi et al., 2008). Similarly Gunathilake et al. (2008) analyzed three different extracts (hexane, dichloromethane and ethyl acetate) of wild and *in vitro* plants of *Munronia pinnata* and observed similar chemical constituents within the two. Similarly identical chemical profiling between wild and *in vitro* derived plants have been reported in *Bacopa monnieri* (Patni et al., 2010), *Sarcandra glabra* (Zhu et al., 2011), *Withania somnifera* (Shetty and Chandra, 2012) and *Curculigo orchioides* (Alagar et al., 2014). Sometimes variation in HPTLC fingerprint has been reported as in *B. monnieri* the methanol and petroleum ether extract shoots showed a variation between wild and *in vitro* shoots (Srivastava and Shrivastava, 2008). Similarly Kharade et al. (2014) have also reported difference in peak areas for methanolic extract of *Curcuma longa* plant. Variation between wild and *in vitro* plant have been also reported in *Arnicae folium*, *Arnicae caulis* and *Celastrus paniculatus* (Stefanache et al., 2014; Anusha et al., 2016) and recently Gantait and Kundu (2017) reported that different storage temperature of synthetic seeds affected HPTLC profile of *Rauvolfia serpentina*. Whereas Rojsanga et al. (2017) used extracts of different plant parts of *in vivo* plant and when compared with *in vitro* grown callus, shoots and roots of *Oroxylum indicum*. These findings documented that *in vitro* cultures synthesized metabolites but variation in fingerprint was observed. HPTLC fingerprint is also used for analysis of biosynthetic potential of *in vitro* grown rhizomes of *Asparagus adscendens* (Mehta and Subramanian, 2005) and *C. angustifolia* (Jena et al., 2018) as well as roots of *W. coagulans* and *W. somnifera* (Preethi et al., 2014).

2.2.2 Quantification and Elicitation of Metabolites in Shoots

2.2.2.1 Medicinal properties of lupeol

Terpenoids are the largest group of plant secondary metabolites and more than 25,000 structures have been identified (Verpoorte and Memelink, 2002; Lewinsohn, 2004). They are important structural components of plant membranes, and free triterpenes serve to stabilize phospholipid bilayers in plant cell membranes (Liby et al., 2007). Terpenoids are C30 compounds arising from the cyclization of 3S-2,3-epoxy,2,3-squalene (Gurib-Fakim, 2006) which is biosynthesized through isopentenoid pathway and depending upon the type of terpenoid it includes two pathways: mevalonic acid (MVA) pathway (e.g. ses- and tri-terpenoids) and methylerythritolphosphate (MEP) pathway (e.g. mono- and di-terpenoids)

(Taiz and Zeiger, 2006). Lupeol ($C_{30}H_{50}O$, Fig. 3), a naturally occurring pentacylcic triterpene is present in fruits, vegetables and medicinal plants including *H. indicus* (Moreau et al., 2002; Chaturvedi et al., 2008; Saleem, 2009). It synthesized via MEP and/or MVA pathway (Fig. 4).

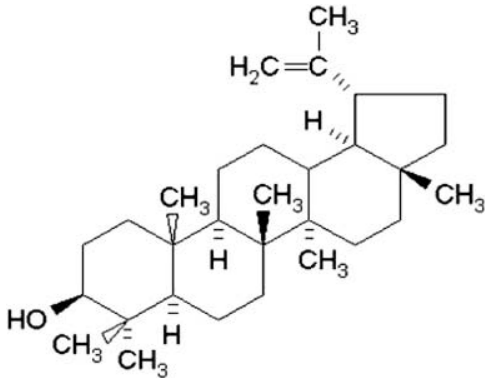

Figure 3: Chemical structure of lupeol

It has analgesic, anti-inflammatory (Fernandez et al., 2001; Lima et al., 2007), antioxidant (Nagaraj et al., 2000; Prasad et al., 2008) and cytoprotective properties (Geetha and Varalakshmi, 2001). Along with this it has different activities such as antimutagenic (Nigam et al., 2007), hypotensive (Saleem et al., 2003), antitumor (Hata et al., 2002; Aratanechemuge et al., 2004), anti-asthmatic (Vasconcelos et al., 2008) and anti-arthritic (Blain et al., 2009). Lupeol has beneficial effect on oxidative and inflammatory abnormalities in the hypercholesterolemic conditions (Sudhahar et al., 2007). Saleem et al. (2004) observed that it has antitumor effects in the 7,12-dimethylbenz[a]anthracene (DMBA) induced mouse skin tumorigenesis. Later on Nigam et al. (2007) reported that lupeol has anti-proliferative and apoptotic activity which prevent skin carcinomas.

Many studies have also reported that it has anticancerous properties against various types of cancer (Saleem et al., 2001, 2005a, 2005b, 2009; Preetha et al., 2006; Manoharan et al., 2012) and it may be due to role of lupeol in activation of different pathways which prevents/arrest the cancer cells (Chaturvedi et al., 2008). This compound is reported to be non-toxic to normal cells and hence can become a potential chemoprotective agent (Patocka, 2003; Saleem, 2009). Apart from this it has been reported that administration of lupeol can be beneficial for human as it normalizes the serum lipid profile, triglycerides and cholesterol levels (Sudhahar et al., 2006; Sasaki et al., 2008; Itoh et al., 2009).

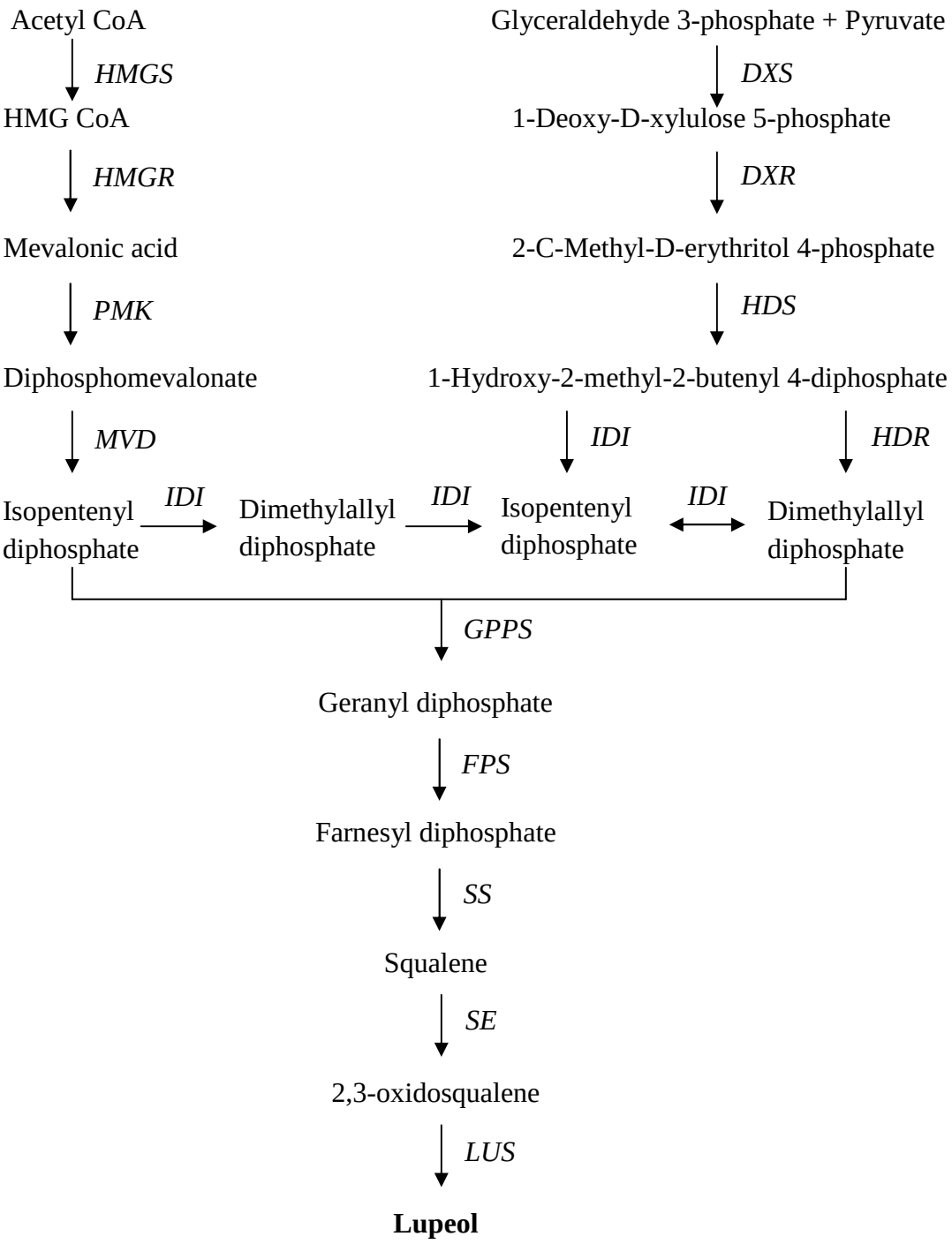

Figure 4: Biosynthetic pathway of lupeol (adapted from KEGG)

[*HMGS*: hydroxymethylglutaryl-CoA synthase, *HMGR*: hydroxymethylglutaryl-CoA reductase, *PMK*: phosphomevalonate kinase, *MVD*: diphosphomevalonate decarboxylase, *DXS*: 1-deoxy-D-xylulose-5-phosphate synthase, *DXR*: 1-deoxy-D-xylulose-5-phosphate reductoisomerase, *IDI*: isopentenyl diphosphate isomerase, *HDS*: 4-hydroxy-3-methylbut-2-(*E*)-enyl diphosphate, *GPPS*: geranylgeranyl pyrophosphate synthase, *FPS*: farnesyl phosphate synthase, *SS*: squalene synthase, *SE*: squalene epoxidase, *LUS*: lupeol synthase]

2.2.2.2 Medicinal properties of rutin

Flavonoids are C15 compounds which are distributed throughout the plant kingdom and more than 2000 different types have been identified till date (Taiz and Zeiger, 2006). The main function of flavonoids in plants is to protect it from UV rays of Sun and they are present in flowers which aids in pollination (Gurib-Fakim, 2006). The basic structure of flavonoids is 2-phenyl chromane or an Ar-C3-Ar skeleton and biosynthetically they are derived from a combination of the Shikimic acid and the acetate pathways. In plants, flavonoids can either occur as aglycones or as O- or C-glycosides. Rutin ($C_{27}H_{30}O_{16}$, Fig. 5), a flavonoid is also called as vitamin P, which is present in many plant species (Harborne, 1986) is synthesized via pathway represent in fig. 6.

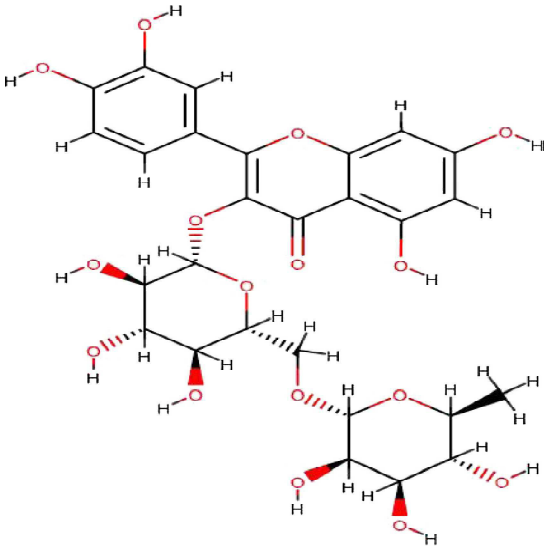

Figure 5: Chemical structure of rutin

Rutin has UV-B protective (Choquenet et al., 2008), antioxidant (Sharma et al., 2013b), anti-cancerous (Lin et al., 2012; Perk et al., 2014), anti-inflammatory, antidiabetic (Lee and Jeune, 2013), anti-hypercholesterolemic (da Silva et al., 2001; Kanashiro et al., 2009) and anti-asthamatic (Jung et al., 2007) activities. Rutin is proved to have beneficial effect on diseases related to central nervous system and prevents neuroinflammation (Khan et al., 2009; Javed et al., 2012), is an anticonvulsant (Nieoczym et al., 2014) and shows an anti-alzheimer activity (Javed et al., 2012; Wang et al., 2012). It has organ protective activities (Yeh et al., 2014) like retinoprotective (Chiou and Xu, 2004), neuroprotective (Azevedo et al., 2013), cardioprotective (Annapurna et al., 2009) and nephroprotective (Kamel et al., 2014).

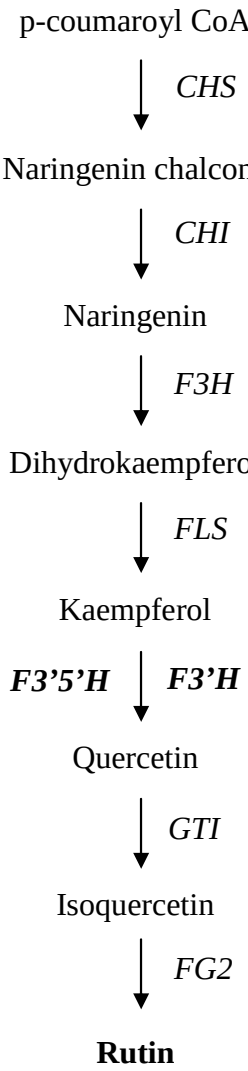

Figure 6: Biosynthetic pathway of rutin (adapted from KEGG).

[*CHS*: Chalcone synthase, *CHI*: chalcone isomerise, *F3H:* naringenin 3-dioxygenase, *FLS*: flavonol synthase, *F3'5'H*: flavonoid 3',5'-hydroxylase, *F3'H*: flavonoid 3'-monooxygenase, *GTI*: flavonol 3-O-glucosyltransferase, *FG2*: flavonol-3-O-glucoside L-rhamnosyltransferase]

2.2.2.3 Lupeol and rutin quantification

There are few reports on quantification of lupeol in nodal derived shoots of *H. indicus* and its synthesis in leaf derived callus is also affected by PGRs of the medium (Misra et al., 2003, 2005). Similarly Purohit et al. (2015) reported lupeol in callus induced from roots of the same plant, and the content enhanced under *in vitro* conditions as compared to wild roots. In other studies, callus cultures of *Verbesina encelioides* (Jain et al., 2008), *Cryptostegia grandiflora* (Singh et al., 2011a), *Crataeva tapia* (Sharma et al.,

2016) and *Solanum melongena* (Vanitha et al., 2016) are known to synthesize lupeol, whereas in *Glycyrrhiza uralensis in vitro* stolon was reported to synthesize it (Kojoma et al., 2010).

There are many reports on rutin quantification in *in vitro* shoots of different plants and *in vitro* cultures have been reported to be a source for rutin extraction as an alternative *to in vivo* source in *Vaccinium corymbosum* (Contreras et al., 2015) and *Schisandra chinensis* (Szopa et al., 2017). Earlier studies in *H. indicus* have reported rutin synthesis in shoot as well as in callus cultures, and the content varied according to PGRs of the medium (Misra et al., 2003, 2005). Similarly *in vitro* plantlets of *Hypericum perforatum* cv. Topas, *Fabiana imbricate*, *Alpinia purpurata* and *Thymus pseudopulegioides* (Pasqua et al., 2003; Schmeda-Hirschmann et al., 2004; Victório et al., 2009; Günaydin et al., 2017) synthesized rutin. In many other studies, shoot cultures were utilized to quantify rutin e.g. Shilpashree and Rai (2009) quantified rutin in shoot cultures of *H. mysorense*. Whereas in other studies in *Alpinia purpurata* (Kale and Namdeo, 2015), *Ruta graveolens* (Al-Ajlouni et al., 2015) and *Saussurea involucrata* (Kuo et al., 2015) synthesis of rutin has been reported in both callus as well as shoots.

Shoot cultures are reported to be an alternative to callus or cell suspension culture for the production of valuable metabolites (Mehrotra et al., 2007). They have been used for extraction of metabolites in medicinal plants like *Erigeron breviscapus* (Liu et al., 2008), *Bacopa monnieri* (Parale and Nikam, 2009; Praveen et al., 2009), *Huernia hystrix* (Amoo and Van Staden, 2013) and *Scutellaria alpine* (Grzegorczyk-Karolak et al., 2017).

2.2.2.4 Elicitation of lupeol and rutin

Elicitors are external stimuli which are capable of inducing changes in the plant cell and in turn lead to a series of reactions which at last increases secondary metabolites production. It was noted that they bind to a receptor in the plasma membrane, which then activates specific gene(s) through signal transduction pathways (Sudha and Ravishankar, 2002). Earlier study by Misra and Mehrotra (2006) reported that enhancement of lupeol and rutin using γ-rays in callus culture of *H. indicus*. Later on Singh et al. (2011a) enhanced lupeol quantity using ergosterol in *Cryptostegia grandiflora.* Whereas lupeol was increased after UV-B radiation in *Vitis vinifera* (Gil et al., 2012) and after jasmonic acid in *Jatropha curcas* (Zaragoza-Martinez et al., 2016).

Study on *Deschampsia antarctica* shoots reported that rutin quantity enhanced after UV-B treatment (Sequeida et al., 2012). Whereas rutin in shoots of *Rauvolfia serpentine*

enhanced when they were treated with methyl salicylate (Nair et al., 2013). Other studies were reported for callus and cell suspension cultures and *Citrus hystrix* callus synthesize more rutin after elicitation using agarose (Suri et al., 2002). Cell culture of *Vitis vinifera* enhanced rutin synthesis after treatment with *Phaeomoniella chlamydospora* (Sak et al., 2014). *In vitro* plantlets of *Fagopyrum tataricum* synthesize maximum rutin after treatment with SA (Hou et al., 2014) whereas phenylalanine increased rutin in callus of *Saussurea involucrata* (Kuo et al., 2015). Tůmová et al. (2016) reported that pyrazine carboxamide derivatives increase rutin production in callus cultures of *Fagopyrum esculentum*. Recently Sarkate et al. (2017) also reported that enhancement of rutin after YE elicitation in cell culture of *Malus domestica* 'florina', whereas the content was increased after treatment with biosynthesized silver nanoparticles in cell suspension culture of bitter ground (Chung et al., 2018).

2.2.2.5 Elicitation of metabolite using YE, SA and MJ

In the present study biotic i.e. yeast extract (YE) and abiotic i.e. salicylic acid (SA) and methyl jasmonate (MJ) were used for elicitation of compounds. Earlier report by Suzuki et al. (2002) depicted that treating cell suspension cultures of *Medicago truncatula* with YE increased the expression of genes related to triterpenoid biosynthesis. Another report suggested that contents of p-coumaric acid and furanocoumarins increased in *in vitro* leaves of *Glehnia littoralis* after treatment with YE (Ishikawa et al., 2007). Kamonwannasit et al. (2008) observed that pseudojujubogenin content in *Bacopa monnieri* shoots varied according to change in concentration. YE has been reported to enhance furanocoumarin in *Ruta graveleons* (Diwan and Malpathak, 2011), flavonoid in *Merwilla plumbea* (Baskaran et al., 2012) and phenol, 2, 4-bis (1, 1-dimethylethyl) in *Orthosiphon stamineus* (Razali et al., 2017) shoots. Whereas in other studies it increased the metabolite contents in plants, like plumbagin in *Drosera indica* (Thaweesak et al., 2011) and rosmarinic acid in *Melissa officinalis* (Nasiri-Bezenjani et al., 2014). Recently Maqsood and Abdul (2017) observed that that incorporation of YE enhanced vinblastine and vincristine content in protoplast derived different stages of embryo and leaves of *Catharanthus roseus*.

Commonly used abiotic elicitors like SA and MJ are plant signaling molecules which are involved in the defence response and are used as chemical inducers under *in vitro* conditions for secondary metabolite production (Goyal and Ramawat, 2008). In last decade, SA has emerged as a key signalling compound which is involved in the activation

of plant defence responses (Kang et al., 2004). Alex et al. (2000) reported that SA increased the expression 3-hydroxy-3-methylglutaryl CoA synthase (HMGS) and HMG CoA reductase (HMGR) genes of mevalonate pathway (terpenoid biosynthesis) in *Brassica juncea*. Kovacik et al. (2009) reported that phenolic acids content increased after elicitation using SA in leaf rosettes of *Matricaria chamomilla*. In shoots of three *Hypericum spp.* (*H. hirsutum*, *H. maculatum* and *H. perforatum*) SA enhanced the quantity of hypericin and pseudohypericin (Coste et al., 2011; Gadzovska et al., 2013). Enhancement of caffeic acid and rosmarinic acid content in *Thymus membranaceus* (Perez-Tortosa et al., 2012), flavanoids in *Cistus heterophyllus* (Lopez-Orenes et al., 2013), withanolides in *Withania somnifera* (Sivanandhan et al., 2013) and bacoside and corotenoid content in *Ruta angustifolia* (Othman et al., 2015) shoot cultures after SA treatment. However Zaheer and Giri (2015) reported that lower concentrations of SA were beneficial for andrographolides production in *Andrographis paniculata* shoot cultures. Whereas Patil et al. (2013) observed that YE enhanced digoxin whereas SA enhanced both digitoxin and digoxin in shoot cultures of *Digitalis purpurea*.

Whereas MJ act as the intracellular signal compounds (Creelman and Mullet, 1997) and activates genes which induces the enzymes responsible for the synthesis of secondary metabolites (Kumar and Sopory, 2010). Zhao et al. (2013) reported that genes of triterpenoid biosynthesis were up-regulated by elicitation using MJ in *Gentiana straminea*. Whereas study on *H. perforatum* reported that it increased flavonoid content (Wang et al., 2015). MJ increased asiaticoside production in *Centella asiatica* plantlets (Kim et al., 2004), galanthamine content in shoots of *Narcissus confusus* (Colque et al., 2004) and *Leucojum aestivum* (Schumann et al., 2013). It has also been reported to enhance carnosic acid, carnosol and rosmarinic acid in *Salvia officinalis* (Grzegorczyk and Wysokinska, 2009), mitragynine in *Mitragyna speciosa* (Wungsintaweekul et al., 2012), phenolic acids in *Leucojum aestivum* (Ivanov et al., 2013), bacoside A in *Bacopa monnieri* (Sharma et al., 2013a; Largia et al., 2015), rosmarinic, chlorogenic and caffeic acids in *Eryngium planum* (Kikowska et al., 2015) shoots.

2.2.3 Gene Expression Studies for Rutin Biosynthetic Pathway

Flavonoid 3',5'-hydroxylase (*F3'5'H*) and flavonoid 3'-monooxygenase (*F3'H*) are classified into cytochrome P450 family but have different subfamilies i.e. CYP75A and CYP75B, respectively (Ueyama et al., 2002). These genes are known to catalyse hydroxylation of flavonol B-ring and converts kaempferol to quercetin in flavonoid

biosynthesis (Fig. 6). *F3'5'H* covets B ring into 3',4',5'-trihydroxylated B-ring whereas *F3'H* converts into 3',4'-dioxygenated B-ring (Ayabe and Akashi, 2006). Both these genes were first studied in *Petunia* sp. (Holton et al., 1993; Brugliera et al., 1999), however there are meagre studies carried out at the molecular level on P450s of flavonoid pathway especially on CYP75 family (Ayabe and Akashi, 2006). Expression of both genes in *Arabidopsis* seedlings was observed in correlation between sucrose and PGRs (Loreti et al., 2008). Similarly Wang et al. (2014) have reported that expression of *F3'5'H* gene is induced by light and sucrose in *Camellia sinensis* seedlings. Expression of another gene i.e. *F3'H* was reported to increase after UV-B exposure in hairy roots of *Fagopyrum tataricum* (Huang et al., 2016). Callus cultures of *Salvia miltiorrhiza* up-regulates *F3'H* gene after elicitation with SA (Zhang et al., 2016). Whereas recently expression of this gene in hairy roots of buckwheat and *Isatis tinctoria* is reported to increase after it was treated with ethephon and chitosan (Li et al., 2017; Jiao et al., 2018).