CHAPTER 2

REVIEW OF LITERATURE

In this chapter, studies related to organogenesis and embryogenesis using leaf explant, axillary shoot proliferation from nodal explants, chemical profiling, quantification of metabolites of *in vitro* cultures and elicitation of metabolites using yeast extract and salicylic acid has been cited.

2.1 Regeneration Studies

The selection of a suitable medium is essential to establish *in vitro* plants and some of the culture media are: Murashige and Skoog's medium (MS, 1962), White's medium (1963), Linsmaier and Skoog (LS, 1965), Gamborg's medium (B5, Gamborg et al., 1968), Nitsch and Nitsch Medium (NN, 1969) and Llyod and McCown's medium woody plant medium (WPM, 1980). There were many reports which states the use of different types of media for cultures and it has been documented that MS media was beneficial for regeneration e.g. optimum shoot regeneration in *Senna sophera* has been reported in presence of MS medium (Parveen and Shahzad, 2014). Later on Sharma et al. (2017) also stated that optimum shoot regeneration in *Bacopa monnieri* was observed on MS media. Similarly, positive effect of MS media was reported in many other studies (Wondyifraw and Surawit, 2004; Wang et al., 2008; Arab et al., 2014). The reason behind superiority of MS media is because it has a high ion concentration, especially nitrogen, potassium, zinc and chlorine (Leifert et al., 1995). Out of these, nitrogen is one of the most important elements for plant growth as it directly affects amino acid and nucleic acid production in the cells (Alvarenga et al., 2015; Grzegorczyk-Karolak et al., 2015; Rahman et al., 2015).

The first aim of present study was to establish cultures using leaf and nodal explants in different media. Under *in vitro* conditions, plant tissues are in heterotrophic mode of nutrition and thus require augmentation of carbohydrates in the media. Amongst different carbohydrate sources, sucrose has been proved superior and widely used one (Thorpe et al., 1986, Pathak et al., 2019). Similarly, plant growth regulators (PGRs) are also important factors which affect the regeneration (Trewavas, 1981). Cytokinins and auxins are two main classes of PGRs as their ratio affects regeneration of *in vitro* culture (Skoog and Miller, 1957).

2.1.1 Shoot Regeneration from Leaf Explant

In vitro organogenesis is quite useful for rapid mass multiplication and production of superior genotypes. This technique has many advantages over other conventional propagation methods, and it is cost effective as it favours for large scale production of desirable medicinal

plants (Seth et al., 2017). Plant regeneration through indirect organogenesis is exploited in basic and applied research across many plant species (Xu and Huang, 2014).

One of the major factors for efficient shoot regeneration is the type and the concentration of cytokinins in the medium (Thorpe et al., 1980; Evans et al., 1983). They are involved in regulation of proteins synthesis which is prerequisite for formation and functioning of mitotic spindles, hence required for cell division and shoot formation (Chawla, 2002; George et al., 2008). BA and Kn are one of the most commonly used cytokinins, and some authors have suggested the use of individual BA or Kn for multiple shoot regeneration from leaf explant. BA (6 µM) was used for optimum shoot regeneration from leaf explant of *Bacopa monnieri* (Joshi et al., 2010), whereas 8.8 µM proved optimum for regeneration in *Solanum xanthocarpum* (Sundar and Jawahar, 2011). Individual BA has also been reported for regeneration of other plant species like *Anthemis xylopoda* (Erdag and Emek, 2009), *Citrus limon* (Kasprzyk-Pawelec et al., 2015), *Anthurium andreanum* (Thokchom and Maitra, 2017), *Inula royleana* (Amin et al., 2017) *Genipa americana* (de Souza et al., 2019) and *Zingiber officinale* (Mehaboob et al., 2019). In few studies, different media were used for shoot induction and elongation e.g. optimum shoot regeneration in *Blepharispermum subsessile* was observed in BA containing medium, however the proliferation and elongation was achieved in medium fortified with BA-GA_3 (Nayak and Kalidass, 2016). Whereas Amudha and Shanthi (2011) have reported that *Acmella calva* leaf explant formed callus in the medium fortified with BAP (3 µM) and NAA (2 µM) which differentiated shoots after subculturing on another medium containing BAP (5 µM). Similarly Lubaina and Murugan (2012) studies on *Plumbago zeylanica* in which callus differentiated from leaf explant in presence of BA and 2,4-D, and it later differentiated shoots after transfer to medium containing BA alone. Whereas in leaf explants of *Chrysanthemum morifolium* direct organogenesis was reported in presence of BA which induced indirect organogenesis by transferring into media containing BA and NAA (Kazeroonian et al., 2018). Another cytokinin Kn is reported in few studies for shoot regeneration like in *Solanum nigrum*, Kn (1.50 mg dm^{-3}) was beneficial for shoot organogenesis as compared to BA (Bhat et al., 2010). Similarly in leaf explant of *Justicia gendarussa* (Agastian et al., 2006) and *Ionidium suffruticosum* (Sonappanavar and Jayaraj, 2011) maximum shoots were formed when inoculated on Kn containing medium.

Like individual cytokinins, synergistic effect of BA and Kn has also been documented in many studies for efficient shoot regeneration. Recently BAP (4.0 mg/l) with Kn (1.0 mg/l)

has been proved to be optimum for regeneration from leaf explant of *Aloe vera* (Singh et al., 2020). Similarly, Rajan et al. (2020) observed indirect organogenesis in *Vernonia anthelmintica* in presence of 4 mg/l BA and 6 mg/l Kn. This PGR combination has been also used for optimum regeneration in *Stevia rebaudiana* (Sreedhar et al., 2008), *Withania somnifera* (Joshi and Padhya, 2010), *Gladiolus grandiflorus* (Shaheenuzzaman et al., 2011) and *Achyranthes aspera* (Sen et al., 2014). Whereas in *Withania coagulans* the combination of BA+Kn was used for shoot formation which further elongated in the medium having BA+Kn+phloroglucinol (Jain et al., 2011). This PGR combination was not only used for regeneration, but is also reported for shoot multiplication and elongation of *Enicostemma hyssopifolium* (Seetharam et al., 2002). Few studies suggested the use of auxins along with BA and Kn for maximum shoot regeneration e.g. combination of BA (2.0 mg/l), Kn (0.5 mg/l) and IAA (1 mg/l) has been documented for *Gynura procumbens* (Banu et al., 2017). Recently Gandhi et al. (2020) also reported that efficient regeneration in *Scoparia dulcis* can be achieved by fortifying media with BA (6.00 µM), Kn (3.59 µM) and IAA (3.93 µM). Whereas use of BA+Kn+NAA has been also well documented in *Punica granatum* (Soni and Kanwar, 2016), *Moringa oleifera* (Jun-Jie et al., 2017), *Brucea mollis* (Das et al., 2018) and *Isodon amethystoides* (Duan et al., 2019).

Cytokinins and auxins are mutually dependent when added together many physiological effects of the cytokinins can be described by their interaction with the auxins (Song et al., 2011). This is because they are known to regulate each other's biosynthesis and when used in synergism they also help in organogenesis as well as branching of shoots (Muller and Leyser, 2011). It is also documented that exogenous application of auxin causes asymmetric distribution of auxin in the explant, which is necessary for de novo initiation of organ primordia under culture conditions (Zhao et al., 2008). Raj et al. (2015) observed that maximum shoots from leaf explants of *Capsicum chinense* can be achieved in presence of BAP (5 mg/l) and IAA (0.1 mg/l). Similarly in *Euphorbia pulcherrima* also, optimum regeneration was reported in presence of 1.0 mg/l BA with 0.2 mg/l IAA (Danial and Ibrahim, 2016). In another Asclepiadaceae member *H. indicus*, optimum regeneration was recorded when MS medium was fortified with combination of BA (20 µM) and IAA (1 µM) (Pathak and Joshi, 2017). The combination of BA with IAA has been also reported for multiple shoot formation in *S. rebaudiana* (Sivaram and Mukundan, 2003), *Spilanthes acmella* (Singh and Chaturvedi, 2012), *Anisochilus carnosus* (Reshi et al., 2017), *Prunus armeniaca* (Mitrofanova et al., 2019), *Andrographis echioides* (Savitikadi et al., 2020) and *B. monnieri* (Tata, 2020).

Recently Sharma et al. (2020) reported that for regeneration in *Populus deltoids*, the media was augmented with BAP (2.5 µM), IAA (2.5 µM) and $AdSO_4$ (15 mg/l) along with ascorbic acid (5 mg/l) and $(NH_4)_2SO_4$ (250 mg/l). Whereas BAP+IAA+GA_3 was reported for organogenesis in *Ziziphus jujuba* (Hou et al., 2017) and *Zanthoxylum armatum* (Purohit et al., 2020). Recently Vanegas-Espinoza et al. (2020) documented that leaf explant of *Tagetes erecta* formed callus when placed on media containing BA (2.0 mg/l) + 2,4-D (2.0 mg/l), which differentiated optimum shoots upon subculture on BA (0.5 mg/l) + IAA (3.0 mg/l).

Another commonly used combination for shoot organogenesis is BA-NAA e.g. leaf explant of *Isatis constricta* differentiated multiple shoots in MS medium supplemented with 1 mg/l of both BA and NAA (Özel, 2018). Similarly, higher shoot induction in *Aechmea ramose* was achieved when equal amount (2 µM) of both BA and NAA has been incorporated in the media (Faria et al., 2018). Media fortified with BA and NAA has been documented in many species like *Begonia* species (Mendi et al., 2009), *Aechmea blanchetiana* and *A. distichantha* (Santa-Rosa et al., 2013), *Cerasus humilis* (Wang et al., 2016), *Begonia homonyma* (Kumari et al., 2017), *Centella asiatica* (Senthil Kumar, 2017), *Ocimum tenuiflorum* (Aggarwal et al., 2020) and *Styrax benzoin* (Nurwahyuni et al., 2020) for shoot regeneration as well as multiplication. Recently Liu et al. (2020) reported that BA with NAA was beneficial for optimum regeneration from leaf explant of *Populus pseudo-simonii* × *P. nigra* var. *italica*. The combination of BA-NAA has been well documented for shoots induction in some Asclepideaceae members like *Ceropegia intermedia* (Karuppusamy et al., 2009), *Wattakaka volubilis* (Vinothkumar et al., 2015) and *Ceropegia bulbosa* (Subbaiyan and Thangapandian, 2017). Whereas supplementation of 11.77 µM $AgNO_3$ was suggested along with 17.76 µM BA and 5.37 µM NAA for optimum regeneration in *Brassica rapa* (Liu et al., 2018). Similarly for optimum indirect organogenesis in *Lepianthes umbellate* the media was added with BA, NAA and GA_3 (Manasa et al., 2019). Recently the report on *Capsicum annuum* suggested that maximum shoots in yellow variety was observed in presence of BA+NAA, whereas in red variety it was observed in BA+NAA+IAA (Akther et al., 2020).

The combination of Kn with different auxins (IAA/NAA) in MS media was also documented for optimum shoot regeneration in many plants. The leaf explant of *Cardiospermum halicacabum* formed callus in presence of 2,4-D (5 µM) which differentiated shoots when subcultured on medium having Kn (8 µM) + IAA (0.5 µM) (Thomas and Maseena, 2006). 2.0 mg/l Kn in combination with 1.0 mg/l NAA formed 11.2 ± 0.2 shoots via indirect organogenesis in *Abutilon indicum* (Rout et al., 2009). Similarly Kn-NAA

combination was also reported for *Peperomia obtusifolia* (Naggar and Osman, 2014) and *Ziziphora tenuior* (Dakah et al., 2014). Whereas Bora et al. (2007) reported high rates of multiplication in carnation cv. 'Lipstick' in the MS medium supplemented with Kn (1.5 mg/l) + NAA (0.5 mg/l) + GA_3 (0.5 mg/l). Leaf explant of *Plectranthus bourneae* formed callus in presence of BA (0.5 mg/l) and NAA (1.0 mg/l) which further induced shoots after subculturing on Kn (1.0 mg/l), NAA (0.7 mg/l) and casein hydrolysate (50 mg/l) (Thaniarasu et al., 2016). Whereas Thakur and Kanwar (2018) observed that maximum shoot induction was achieved in presence of 0.25 mg/l Kn, 0.25 mg/l NAA and 11.5 mg/l TDZ, which was subcultured on media consisting of 2.0 mg/l Kn and 0.25 mg/l NAA for multiplication of shoots in *Dianthus caryophyllus*. Similarly, two different media were reported for optimum regeneration in *Echinops kebericho*, in which shoot induction was done in media containing BA-NAA whereas the multiplication was done in presence of Kn-NAA (Enyew and Feyissa, 2019).

The cytokinin $AdSO_4$ have beneficiary role in organogenesis from leaf explant when added in presence of another cytokinin and/or auxin. Seth and Panigrahi (2019) reported multiple shoots in *Abutilon indicum* in MS media containing 8.88 μM BA, 2.68 μM NAA and 543 μM $AdSO_4$. Similarly, stimulatory effect of this PGR has been also observed for regeneration of *Valeriana jatamansi* (Chen et al., 2014) and *Metabriggsia ovalifolia* (Ouyang et al., 2016). Whereas additive ($AdSO_4$) effects on *in vitro* shoot multiplication was reported in *Simmondsia chinensis* (Bala et al., 2019) and other medicinal plant species (Nandagopal and Kumari, 2006; Bantawa et al., 2009).

2.1.2 Somatic Embryogenesis from Leaf Explant

Somatic embryogenesis is another pathway to regenerate plants in a short period of time. It has several advantages over the other organogenic pathways as SEs are bipolar in nature and thus rooting stage is avoided and it also facilitates the production of synthetic seeds which aids in conservation of threatened medicinal plants (Cheruvathur et al., 2013). *In vitro* development of somatic embryos (SEs) is influenced by many factors of which PGRs play a central role (Van Staden et al., 2008). It has been suggested that cytokinins are known to play important role in this transition as well as in stimulating divisions in pro-embryogenic cells when used alone or in combination with auxins (Feher et al., 2003). There are only few reports which suggest the use of individual PGRs on embryogenesis from leaf explant e.g. MS medium supplemented with BA (1.32 μM) effectively induced embryos in *Curcuma longa*

(Raju et al., 2015). Similarly, individual BA was reported for SE germination in *Phoenix dactylifera* (Sane et al., 2012) and *Scaevola sericea* (Liang et al., 2020).

Whereas reports are scanty for the use of individual Kn, but the synergistic effect of BA with Kn has been documented by some authors. Dhavala et al. (2009) reported that MS medium fortified with combination of 11.1 μM BAP with 13.95 μM Kn induced highest frequency of embryos in *Solanum trilobatum*. Later same combination proved to be beneficial in *Moringa oleifera* (Devendra et al., 2012). Li et al. (2008) observed that in *Picea koraiensis* optimum SEs can be achieved by using combinations of BA (1.10 μM) and Kn (1.16 μM) along with NAA (8.06 μM), whereas SE formation in *Jatropha curcas* was better when media was fortified with combination of several PGRs like BA, Kn, TDZ and IAA (Mweu et al., 2016).

$AdSO_4$ is also known to facilitate somatic embryos under *in vitro* conditions when combined with other cytokinins and auxins (Van Staden et al., 2008). Combination of BA with $AdSO_4$ evoked optimum embryogenesis in *Phaseolus vulgaris* (Cabrera-Ponce et al., 2015). Beneficial effect of $AdSO_4$ for formation of somatic embryogenesis is well documented in *Rosa hybrida* and *Mussaenda erythrophylla* (Das, 2010). In contrast, the combination of BA (13.3 μM), $AdSO_4$ (271.5 μM) and NAA (2.7 μM) was used in *Anacardium occidentale* (Martin, 2003). Whereas Taha et al. (2007) depicted that in *P. dactylifera* maximum SEs were achieved when MS medium was supplemented with BAP+2iP+$AdSO_4$. Similarly many previous studies on *Coffea arabica* and *C. canephora* (Samson et al., 2006), *Carthamus tinctorius* (Walia et al., 2007b), *Bixa orellana* (Parimalan et al., 2011), *Acacia senegal* (Rathore et al., 2012), *Gentiana decumbens* (Tomiczak et al., 2015) and *Carica papaya* (Al-Shara et al., 2020) suggested the use of $AdSO_4$ with cytokinins for embryogenesis.

Majority studies on somatic embryogenesis suggest that the combinations of cytokinins and auxins are known to participate in the cell cycle regulation, cell division and stimulates the embryogenic competence of somatic tissues (Francis and Sorrell, 2001; Gaj, 2004; Lincy et al., 2009). In *Desmodium motorium*, combination of BA (4.44 and 8.88 μM) with IAA (2.9 μM) differentiated embryogenic calli which was further transferred to germinating medium containing BA (8.88 μM) alone (Chitra Devi and Narmathabai, 2011). Midhu et al. (2019) reported that embryogenic callus in *Ophiorrhiza pectinata* developed in presence of IAA (3 mg/l), which was further transferred to medium having combination of BA (0.2 mg/l) and IAA (0.1 mg/l) for maximum SEs formation. The combination of BA+IAA was also used in

plants like *Jatropha curcas* (Sardana et al., 2000) and *Vitis vinifera* (Das et al., 2002; Alavijeh et al., 2016).

Recently Sedaghati et al. (2019) depicted that combination of higher BAP (1.5 mg/l) with lower NAA (0.2 mg/l) induced highest embryos in *Portulaca oleracea*. Whereas lower BAP level (0.5 mg/l) and higher NAA level (2.0 mg/l) induced maximum SEs in *Solanum nigrum* (Sharada et al., 2019). This combination is also reported for somatic embryogenesis in important species like *Phellodendron amurense* (Azad et al., 2009) and *Neolamarckia cadamba* (Li et al., 2019). Whereas embryogenic callus of *Limonium sinense* developed in presence of BA-2,4-D when subcultured in medium supplemented with BA (4.44 μM) and NAA (1.07 μM) proved optimum for germination of SEs (Dam et al., 2017). Similarly, Seth et al. (2017) also reported that medium containing 2,4-D has been used for callus induction from leaf explant of *Abutilon indicum*, which further transferred to medium fortified with BA (13.32 μM) + NAA (2.68 μM) + activated charcoal (200 mg/l) + ascorbic acid (11.54 μM) for efficient embryogenesis. Some studies have reported the use of BA with 2,4-D such as in leaf explants of *Scaevola aemula* (Wang and Bhalla, 2004, 2006). Another cytokinin Kn is also used for embryogenesis, but the reports are less as compared to BA. In *Cichorium intybus* it was reported that combination of Kn (1.5 mg/l) and IAA (0.5 mg/l) along with casein hydrolysate (500 mg/l) induced optimum indirect SEs which matured after subculturing into media containing Kn (1.5 mg/l), IAA (0.1 mg/l), IBA (1.0 mg/l) and casein hydrolysate (500 mg/l) (Abdin and Ilah, 2007).

In *Mucuna pruriens*, the embryogenic callus has been developed in presence of 2,4-D (6.7 μM) and then transferred to SE germination medium supplemented with 2.3 μM Kn + 5.4 μM NAA + 13.6 μM $AdSO_4$ (Vibha et al., 2009). The embryo germination and plantlet formation in *Valeriana officinalis* was achieved in MS medium supplemented with Kn (2 mg/l) and NAA (0.1 mg/l) (Abdi et al., 2019), these combination is also reported in *Valeriana edulis* (Castillo-España et al., 2000). Rathore et al. (2012) depicted that MS medium supplemented with Kn (2.32 μM) + 2,4-D (0.45 μM) + L-glutamine (15 mM) evoked best somatic embryogenesis in *A. senegal*. Whereas Daniel et al. (2018) reported that embryogenic callus in *Abelmoschus esculentus* has been induced in presence of 1.5 mg/l 2,4-D, which facilitated SEs development and maturation by subculturing into medium augmented with 1.0 mg/l Kn, 1.5 mg/l 2,4-D, 300 mg/l L-glutamine and 400 mg/l casein hydrolysate. The combination of Kn-2,4-D is also documented for efficient embryogenesis in *Acacia farnesiana* and *A. schaffneri* (Ortiz et al., 2000), *C. arabica* (Gatica-Arias et al., 2008), *Vigna*

aconitifolia (Choudhary et al., 2009) and *Paspalum scrobiculatum* (Ceasar and Ignacimuthu, 2010).

2.1.3 Development of Somatic Embryos

Somatic embryo germination and its development is critical process because most of the times the development of SEs into plantlet requires different growth regulators (Pasternak et al., 2002). SEs of *Carthamus tinctorius* and *Haworthia retusa* germinated and matured in presence of GA_3 (Kumar et al., 2008; Kim et al., 2019). Similarly addition of 2 mg/l GA_3 in the medium increased the conversion rate in *J. curcas* embryos to plantlets (Cai et al., 2011). Whereas Raju et al. (2014) reported that SEs of *Curcuma amada* germinated well upon subculturing on half strength MS medium fortified with 1.44 μM GA_3 after being placed in dark condition. Previously GA_3 has been documented to be responsible for development of SEs in different plant species (Cangahuala-Inocenteet al., 2007; Siddiqui et al., 2011; Baskaran and Van Staden, 2012; Raomai et al., 2014; Yücesan et al., 2014; Raju et al., 2015; Syombua et al., 2019). Whereas in *Cryptomeria japonica* (Igasaki et al., 2003) and *Panax quinquefolius* (Zhou and Brown, 2006) this PGR was used for elongation of shoots in SE derived plantlets.

Some studies states the use of cytokinins and GA_3 for development of SEs as reported in *Wedelia calendulacea* where SEs germinated into plantlets after transfering to MS medium containing BAP and GA_3 (Sharmin et al., 2014). Beneficial effect of GA_3 along with BA for conversion of SEs into plantlets has been well documented in *Gossypium hirsutum* (Ganesan and Jayabalan, 2004), *V. aconitifolia* (Choudhary et al., 2009) and *Passiflora edulis* (Huh et al., 2017). Similarly, GA_3 with other cytokinins also promoted the embryo maturation, germination and it's conversion into plantlets in many species like *Daucus carota* (Jimenez et al., 2005), *Rhinacanthus nasutus* (Cheruvathur et al., 2013a) and *Abutilon indicum* (Seth et al., 2017). Whereas combination of GA_3 and phloroglucinol has been used for maximum conversion of SEs into plantlets in *Lachenalia viridiflora* (Kumar et al., 2016) and *Ledebouria ovatifolia* (Baskaran et al., 2016). On the contrary, Daniel et al. (2018) observed that SEs of *Abelmoschus esculentus* were developed better when placed in medium supplemented with BAP (1.0 mg/l) + GA_3 (0.5 mg/l) + L-Glutamine (300 mg/l) + casein hydrolysate (400 mg/l). Recently Bertero et al. (2020) depicted the positive effect of coconut water on conversion of SEs into plantlets in *Minthostachys verticillata*.

During somatic embryogenesis, many times direct as well as indirect secondary somatic embryos (SSEs) are simultaneously formed. This could be due to the fact that embryogenic cells can either undergo development of embryos or continue to differentiate into secondary embryos (Raemakers et al., 1995). SSEs were reported in many important plant species such as *Anacardium occidentale* (Martin, 2003), *Piper nigrum* (Ramakrishnan Nair and Dutta Gupta, 2006), *Carthamus tinctorius* (Kumar et al., 2008), *Acrocomia aculeate* (Moura et al., 2009), *Zingiber officinale* (Lincy et al., 2009), *Bixa orellana* (Parimalan et al., 2011), *Desmodium motorium* (Chitra Devi and Narmathabai, 2011), *Primulina tabacum* (Yang et al., 2012), *Curcuma longa* (Raju et al., 2015), *Medicago sativa* (Sangra et al., 2019), *Scaevola sericea* (Liang et al., 2020) and *Camellia assamica* (Bajpai and Chaturvedi, 2021) and other species (Karami et al., 2009; Bao et al., 2012; Cheruvathur et al., 2013a).

2.1.4 Axillary Shoot Proliferation from Nodal Explant

In conventional vegetative propagation, the axillary buds take over the function of the main shoot when the terminal bud is removed. This phenomenon is the basis for *in vitro* regeneration using nodal explant as under suitable PGR conditions the quiescent axillary bud elongate and develop into shoots (Faisal et al., 2007). Organized meristems are less prone to spontaneous genetic changes than disorganized tissues (Rani and Raina, 2000; Ngezahayo and Liu, 2014). Thus, this method is considered as one of the reliable way to produce clonal plants, which are genetically identical to the mother plant.

Generally cytokinins are reported to have profound effect on regeneration through nodal explant as they are known to release bud dormancy and promote axillary bud outgrowth (Shimizu-Sato et al., 2009; Yaish et al., 2010). Among different PGRs, stimulatory effect of BA on axillary shoot regeneration has been well documented in many important plant species (Fracaro and Echeverrigaray, 2001; Hiregoudar et al., 2006). It was noted that nodes of *Exacum travancoricum* formed 21.4 ± 1.8 shoots in presence of BA (13.32 μM), which increased to 26.9 ± 1.79 shoots after third subculture in the same medium (Elangomathavan et al., 2006). Similarly, large number of shoots (34.1 ± 0.22) were induced from nodes of important medicinal plant *Andrographis paniculata* at 10 μM BA (Purkayastha et al., 2008). In addition, many Asclepiadaceae members like *Ceropegia hirsute* (Nikam et al., 2008), *C. spiralis* (Murthy et al., 2010) and *Caralluma lasiantha* (Aruna et al., 2012) also evoked optimum axillary shoots regeneration in presence of BA. Recently Mandal et al. (2021) depicted that among different cytokinins, BAP at 2.0 mg/l evoked best response from nodal

explant of *Aegle marmelos*. Superiority of BA over other PGRs has also been observed in *Wrightia tinctoria* (Purohit and Kukda, 2004), *Bacopa monnieri* (Mohapatra and Rath, 2005), *Munronia pinnata* (Gunathilake et al., 2008), *Phyllanthum amarus* (Sen et al., 2009), *Aegle armelos* (Yadav and Singh, 2011), *Gynura procumbens* (Alizah and Nurulaishah, 2015), *Coccinia abyssinica* (Kahia et al., 2016), *Lawsonia inermis* (Moharana et al., 2018) and *Helianthus verticillatus* (Nowakowska et al., 2020).

Another commonly used cytokinin Kn is reported in few studies for axillary shoot proliferation. The nodes of *C. juncea* formed 8.5 ± 0.3 shoots in medium supplemented with 7.5 µM Kn (Nikam and Savant, 2009) and similarly Padmapriya et al. (2011) documented large number of shoots i.e. 49 ± 1.32 when nodes of *Solanum nigrum* were cultured in 15 µM Kn. Whereas Kn evoked shoots in *Tinospora cordifolia* but for multiplication the explants were subcultured on BA (Sivakumar et al., 2014). Kn has been reported for optimum regeneration from nodal explants of *Ginkgo biloba* (Mantovani et al., 2013) and *Cucumis sativus* (Abu-Romman et al., 2015). But in *Rotala rotundifolia* the combination of Kn (0.25 mg/l) with GA_3 (0.25 mg/l) proved to be better for axillary shoot regeneration (Dogan, 2017).

Synergistic effect of cytokinins on axillary shoot proliferation has been well reported as they facilitate multiple shoot regeneration from nodal explant. Ahmed et al. (2005) stated that nodal explants of *Phyla nodiflora* evoked better response when placed in PGR combination of BA (2.5 mg/l) and Kn (0.5 mg/l). In Asclepiadaceae member *Hemidesmus indicus*, 8-10 shoots with 80% response was reported in BAP (1.0 mg/l) with Kn (2.0 mg/l) (Rama Devi et al., 2014). Later on individually BA and Kn evoked less shoots, but their combination at BA (10 µM) and Kn (5 µM) regenerated optimum 11.00 ± 0.24 shoots in the same plant as reported by Pathak et al. (2017). Similar results are documneted in nodal explant where BA and Kn positively affected shoot formation e.g. in *Enicostemma hyssopifolium* (Seetharam et al., 2002), *Piper longum* (Soniya and Das, 2002), *Eclipta alba* (Baskaran and Jayabalan, 2005), *Artemisia vulgaris* (Sujatha and Kumari, 2007), *Crataeva nurvala* (Walia et al., 2007a), *Swertia chirata* (Balaraju et al., 2009), *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).

Nevertheless there are some reports which suggest that BA-Kn helped in multiplication and elongation of shoots as well. Like in *Enicostemma hyssopifolium* where nodal explant induced shoots in presence of BA-IAA which was further transferred to combination of BA-Kn for elongation and multiplication. *In vitro* shoots of *Passiflora edulis* which developed in

presence of BAP (2.0 mg/l) were subcultured for multiplication of shoots on medium fortified with 1.0 mg/l of both BAP and Kn (Shekhawat et al., 2015b). In the same way *Morinda citrifolia* shoots were grown in presence of BAP and multiplied on BAP and Kn augmented medium (Shekhawat et al., 2015a). Whereas multiple shoot formation was reported in *Hedyotis biflora* when MS medium fortified with PGRs like BAP (1.0 mg/l), Kn (0.5 mg/l), $AdSO_4$ (25 mg/l) and IAA (0.1 mg/l) along with additives like ascorbic acid (50 mg/l), arginine (25 mg/l) and citric acid (25 mg/l) (Revathi et al., 2019).

Another cytokinin $AdSO_4$ is also reported to stimulate axillary bud regeneration in many plant species when combined with cytokinin or combinations of cytokinin and auxin. Nodal explant of *Bacopa monnieri* formed total 18.00 ± 0.35 shoots when they were allowed to grow on MS media containing BAP (1.5 mg/l) + $AdSO_4$ (60 mg/l) + IAA (0.2 mg/l) (Ramesh et al., 2006). Similar PGR combination at different concentrations i.e. BA (1.5 mg/dm^3), $AdSO_4$ (50 mg/dm^3) and IAA (0.1 mg/dm^3) have been reported for maximum shoot proliferation in *Nyctanthes arbor-tristis* (Rout et al., 2008). Another auxin NAA is also used along with BA+$AdSO_4$ for efficient regeneration in *Sida cordifolia* (Sivanesan and Jeong, 2007) and *Decalepis salicifolia* (Ahmad et al., 2018). Whereas in *Naringi crenulata* the nodes regenerated maximum shoots when cultured in BAP (2.0 mg/l), $AdSO_4$ (25.0 mg/l) and glutamine (150 mg/l) supplemented medium (Singh et al., 2011d). Similarly BAP (0.5 mg/l), $AdSO_4$ (50.0 mg/l) and glutamine (150 mg/l) has been reported for efficient regeneration of *Cocculus hirsutus* (Meena et al., 2012). Moreover, the combination of BA and $AdSO_4$ was documented for multiplication in *Dendrocalamus strictus* (Pandey and Singh, 2012) and shoot elongation in *Bambusa arundinacea* (Venkatachalam et al., 2015). Recently, Choudhary et al. (2020) documented that nodes of *Farsetia macrantha* regenerated shoots in presence of BA (0.5 mg/l) + $AdSO_4$ (25 mg/l) along with additives like ascorbic acid (50 mg/l), citric acid (25 mg/l) and L-arginine (25 mg/l). Further regenerated shoot clumps were subcultured on medium augmented with BA (0.5 mg/l) + Kn (0.25 mg/l) for multiplication. Whereas reports on association of Kn with $AdSO_4$ for nodal regeneration was scanty. In antidiabetic plant *Stevia rebaudiana*, presence of Kn (9.3 μM) and $AdSO_4$ 40 mg/l influenced maximum shoot generation (Khan et al., 2014). Similarly promotive effect of $AdSO_4$ for enhancement of axillary shoot regeneration was also documented in *Holarrhena antidysenterica* (Raha and Roy, 2001), *Melia azedarach* (Husain and Anis, 2004), *Curcuma angustifolia* (Shukla et al., 2007), *Petrocarpus marsupium* (Husain et al., 2008), *Ficus religiosa* (Siwach and Gill, 2011) and *Decalepis arayalpathra* (Ahmad et al., 2018).

2.1.5 Regeneration through Nodal Derived Callus

The PGRs are known to induce axillary buds into shoots but occasionally it form callus at the base of nodes. When this callus is morphogenic in nature, it has the potency to differentiate shoots or somatic embryos as reported in many previous experimental studies.

2.1.5.1 Indirect Organogenesis

In medicinal plant *Aegle marmelos*, Islam et al. (2007) observed that the nodes formed callus when placed in BA (0.3 mg/l) and 2,4-D (2.0 mg/l) and it differentiated shoots upon transfer to a medium containing BA (2.0 mg/l) and NAA (0.1 mg/l). Similarly nodal explant of *L. reticulata* also induced morphogenic callus in presence of Kn (10 mg/l) + NAA (1.5 mg/l) and it was transferred to medium containing Kn (2 mg/l) + IBA (1 and 1.5 mg/l) for shoot formation (Parabia et al., 2007). Whereas 1.5 mg/l 2,4-D induced callus from nodes of *Cassia alata* which required hormone combination of 0.5 mg/l Kn and 1.5 mg/l 2,4-D for caulogenesis (Hasan et al., 2008). Similarly role of 2,4-D in inducing callus from nodal explant and cytokinin on shoot regeneration was also reported in *Rauvolfia serpentine*. In this plant optimum callus was documented in presence of Kn+2,4-D which further differentiated shoots in BAP containing medium (Gupta et al., 2014). On the contrary *Gypsophila paniculata* nodes induced callus in presence of BA (44.3 μM) which then differentiated shoots after subculturing on reduced level of BA (13.3 μM) (Kanchanapoom et al., 2011). Later on Saha et al. (2013) recorded that TDZ (0.5 μM) formed callus at base of *Albizia lucida* nodes which differentiated shoots with 61.67% frequency after transferring to media fortified with BA (8.88 μM) + NAA (0.54 μM) + $AgNO_3$ (5.89 μM). Similarly nodal derived callus of *Lycium barbarum* efficiently regenerated 23.33 ± 1.86 shoots after transferring to BA (0.5 mg/l) (Karakas, 2020). Recent study in *Morus alba* stated that 2,4-D (1 μM) and NAA (2 μM) formed callus and further differentiated multiple shoots in presence of BAP (7.5 μM) + TDZ (2.0 μM) (Rohela et al., 2020).

In the same way nodal derived callus was utilized for indirect organogenesis in plants like *Aristolochia indica* (Siddique et al., 2002; Pattar and Jayaraj, 2012), *Kigelia pinnata* (Thomas and Puthur, 2004), *Fragaria* sp. (Biswas et al., 2010), *Vanilla planifolia* (Tan et al., 2011), *Ananas comosus* (Ibrahim et al., 2013), *Withania somnifera* (Udayakumar et al., 2014), *Solanum tuberosum* (Kumlay and Ercisli, 2015) and *Alhagi maurorum* (Malik et al., 2020).

2.1.5.2 Somatic Embryogenesis

Similarly, few studies have reported that nodes were able to form embryogenic callus which differentiated somatic embryos under suitable PGR combinations e.g. callus formed from nodes of *Dioscorea alata* in presence of picloram (1.0 mg/l) which differentiated embryos after transferring to medium having BAP (0.5 mg/l) and 2,4-D (1.0 mg/l) (Belarmino and Gonzales, 2008). Whereas Cheruvathur et al. (2013b) documented the role of IBA in embryogenesis from nodal derived callus of *Hemidesmus indicus*. Likewise in plants like *L. reticulata* (Martin, 2004), *Dioscorea rotundata* (Manoharan et al., 2016) and *Mirabilis jalapa* (Rohela et al., 2016), nodal explant differentiated callus which was then utilized for plant regeneration through somatic embryogenesis.

2.1.6 Rooting of *In vitro* Shoots

Rooting of *in vitro* shoots is a critical step in success of any regeneration protocol because the final aim is to transfer the plantlets to field. Rooting in microshoots can be achieved via fortifying the media with commonly used auxins such as IBA and NAA which is reported to have profound effect on induction of roots (George and Sherrington, 1984). As the auxin increases cell division which differentiate adventitious roots (Abdul, 1987; Saleh, 1991). There are many reports which states the use of full strength MS media fortified with IBA for rooting in shoots of *Echinacea purpurea* (Koroch et al., 2002), *Munronia pinnata* (Gunathilake et al., 2008), *Brunfelsia calycina* (Liberman et al., 2010), *Solanum nigrum* (Bhat et al., 2010), *Ajuga bracteosa* (Kaul et al., 2013), *Gynura procumbens* (Banu et al., 2017), *Genipa americana* (de Souza et al., 2019) and *Zingiber officinale* (Mehaboob et al., 2019). Recently Savitikadi et al. (2020) documented that combination of IAA and IBA is beneficial for rooting in *Andrographis echioides* shoots.

Another factor which affects the rooting response is strength of MS medium (Murashige, 1979) and for majority of the species it has been observed that half and quarter strengths are more beneficial (Skirvin and Chu, 1979; Garland and Stoltz, 1981; Zimmerman and Broome, 1981). Sivaram and Mukundan (2003) suggested that *in vitro* shoots of *Stevia rebaudiana* induced 12-13 roots when placed in half strength MS medium fortified with IBA. Similarly, optimum rooting in ½MS medium supplemented with IBA has been reported for *Bacopa monnieri* (Joshi et al., 2010), *Cannabis sativa* (Lata et al., 2010), *Catharanthus roseus* (Verma and Mathur, 2011), *Anisochilus carnosus* (Reshi et al., 2017), *Ziziphus jujuba* (Hou et al., 2017), *Brucea mollis* (Das et al., 2018), *Abutilon indicum* (Seth and Panigrahi,

2019) and *Portulaca quadrifida* (Pathak et al., 2019). Further reduction in strength of MS medium to quarter has been well documented in *B. monnieri* (Mehta et al., 2012a), *Terminalia bellerica* (Mehta et al., 2012b), *C. roseus* (Sain and Sharma, 2013) and *Hemidesmus indicus* (Shekhawat and Manokari, 2016; Pathak and Joshi, 2017). Similarly, IBA has been used for rooting in *Cichorium intybus* (Velayutham et al., 2006), *H. indicus* (Sreekumar et al., 2002; Nagahatenna and Peiris, 2007; Sundarmani and Hasina, 2015), *Camellia sinensis* (Bidarigh and Azarpuor, 2013), *Rubia cordifolia* (Khadke et al., 2013) and *Allamanda cathartica* (Khanam and Anis, 2018).

Likewise, there were some plants in which optimum rooting was observed in presence of NAA e.g. in shoots of *Pulsatilla koreana* reported to formed 14.37 roots in ½MS medium fortified with NAA (3 mg/l) (Lin et al., 2011). Whereas Thokchom and Maitra (2017) documented that ½MS with NAA (1.0 mg/l) induced optimum roots in *Anthurium andreanum*. Similarly, NAA has been reported for rooting in *Decalepis hamiltonii* (Anitha and Pullaiah, 2002), *Vitex trifolia* (Hiregoudar et al., 2006), *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012), *Moringa oleifera* (Jun-Jie et al., 2017), *Styrax benzoin* (Nurwahyuni et al., 2020) and *Aegle marmelos* (Mandal et al., 2021). Whereas combination of both auxins is reported for optimum rooting in *Begonia homonyma* (Kumari et al., 2017) and *Isodon amethystoides* (Duan et al., 2019).

2.1.7 *In vitro* Studies on Selected Plants

2.1.7.1 Leptadenia reticulata

Previously many studies were carried out for regeneration using leaf and node explant of *L. reticulata* and they are discussed below.

- **Leaf Explant**

Although leaf is one of the most important explant for induction of large number of shoota, in *L. reticulata* the reports are scanty. Hariharan et al. (2002) documented that indirect SEs were developed on MS medium supplemented with BA (2.0 mg/l) and NAA (0.5 mg/l) which were then germinated on medium having Kn (1.0 mg/l). Similarly somatic embryogenesis was induced in presence of BA (8.87 μM) and IBA (2.46 μM) and were converted to plantlets on ½MS medium supplemented with BA (0.22 or 0.44 μM) and GA_3 (1.44 μM) (Martin, 2004). Later on Patel et al. (2014) developed an efficient shoot regeneration protocol in callus proliferation was achieved on BAP (0.5 mg/l) and 2,4-D (0.5

mg/l) and after subculturing to shoot differentiation medium containing BAP (0.5 mg/l) and NAA (0.1 mg/l) formed 30.70 ± 1.70 shoots.

- **Nodal Explant**

Another commonly used explant for shoot regeneration in *L. reticulata* is node, which was reported by several authors. Arya et al. (2003) observed formation of 4.93 ± 0.77 shoots in presence of BA (18 μM) and IAA (0.6 μM). Whereas in another study shoot induction was recorded in BAP (5.0 mg/l) which were transferred to combination of BAP (1.5 mg/l) + Kn (0.5 mg/l) for multiplication (Shekhawat et al., 2006). Later on Parabia et al. (2007) optimized medium having Kn (10 mg/l) and IBA (1 mg/l) for multiple shoots induction (6.8 ± 0.12). They have also observed formation of nodular callus in presence of Kn (10 mg/l) and NAA (1.5 mg/l) and it differentiated shoots after subculuring on Kn (2 mg/l) + IBA (1 and 1.5 mg/l) fortified medium. Similarly combination of Kn (10 mg/l) and IBA (1 mg/l) was also reported for optimum axillary shoot proliferation (Bharat et al., 2011). Sudipta et al. (2011) reported medium with combinations of BA (0.25 mg/l) and Kn (0.25 mg/l) which formed 5.70 ± 0.23 shoots. Further, this number increased to 6.20 ± 0.10 by addition of 10% CW (Sudipta et al., 2013). An efficient regeneration protocol was then developed in which initially 6.5 ± 0.70 shoots emerged from nodes of *L. reticuata* in presence of BAP (5.0 mg/l) along with additives (50 mg/l ascorbic acid, 25 mg/l each of $AdSO_4$, L-arginine and citric acid). Further the shoot clumps were transferred to multiplication medium augmented with BAP (1.0 mg/l) + Kn (0.5 mg/l) + 2-iP (0.5 mg/l) + NAA (0.1 mg/l) + additives which formed 23.9 ± 2.60 shoots. They have also reported formation of nodular callus at base of node which differentiated 8.4 ± 0.69 shoots when subcultured on MS medium + BAP (1.0 mg/l) + NAA (0.1 mg/l) (Rathore et al., 2013). Dhawan and Damor (2013) also reported shoot regeneration through nodal explants in presence of NAA (1.0 mg/l) with BAP (4.0 mg/l). On the contrary, Martin (2004) reported that nodal explants failed to induce shoots through axillary bud, and instead the nodes induced embryogenic callus in presence of BA (8.87 μM) and IBA (0.45 μM) which differentiated 17.3 SEs upon subculturing on BA (8.87 μM) with IBA (2.46 μM).

- ***In vitro* Rooting**

In vitro rooting of *L. reticulata* shoots, have been tried in different MS media in previous studies e.g. Arya et al. (2003) reported that rooting can be achieved in PGR free ½MS medium containing 100 mg/1 activated charcoal. Later Bharat et al., 2011 reported that

full MS medium containing IBA (0.25 mg/l) was optimum for rooting of shoots and similarly the same has been documented in many other studies (Parabia et al., 2007; Dhawan and Damor, 2013). Sudipta et al. (2011) rooted *in vitro* regenerated shoots in full strength MS media containing IBA (2 mg/l) and 200 mg/l activated charcoal. A combination of IBA (1.5 mg/l) and activated charcoal (100 mg/l) in quarter strength of medium in *L. reticulata* was given by Patel et al. (2014). Similarly ¼MS with IBA (3.0 mg/l) has been used for rooting of shoots by Rathore et al. (2013).

2.1.7.2 Tylophora indica

T. indica is one of the important medicinal plant of India which has many medicinal properties, but due to less availability in wild, many reports attempted *in vitro* regeneration of this species.

- **Leaf Explant**

Previous studies carried out on leaf explant reported both organogenesis as well as embryogenesis. Indirect organogenesis through leaf explants has been achieved in *T. indica* in which callus was induced on 2,4,5-T (10 μM) and it differentiated 64.8 ± 0.74 shoots after 10 weeks of culture period on shoot induction media having Kn (5 μM) (Faisal and Anis, 2003). Later on Thomas et al. (2005) also reported two different media for indirect organogenesis, callus inducing media contained BA+2,4-D and transfer to TDZ (8 μM) supplemented medium induced a total of 66.7 shoots with 100% response. Similarly callus was formed on medium augmented with TDZ (2.5 μM) and it was subcultured on medium fortified with another cytokinin i.e. BA (5 μM) for differetiation of 26.80 ± 0.97 shoots at the end of 12 weeks (Sahai et al., 2010a). Role of cytokinin was also reported by Rathinavel and Sellathurai (2010) in the same plant where 4.6 ±1.1 shoots were induced in presence of Kn (1 μM). On the contrary combination of cytokinin and auxin was reported to be suitable for shoot formation in report of Verma et al. (2010), in which MS medium supplemented with BAP (2.0 mg/l) and IBA (0.5 mg/l) differentiated organogenic callus with shoot buds which further elongated on TDZ (0.1 mg/l) and formed shoots. Sadguna et al. (2013) developed callus in BA+2,4-D combination and it was transferred to BAP (2.0 mg/l) + L- glutamic acid (2.0 mg/l) for multiple shoot (18.0 ± 0.4) formation. Haque and Ghosh (2013) studied the effect on age of leaf explant for regeneration potency and observed that 18.6 ± 0.36 shoots induced directly on BAP (2.0 mg/l) from ‘mature leaves’ explants. Whereas 25.2 ± 0.57 shoots were produced

from 'young leaf' explant via indirect organogenesis on BAP (2.0 mg/l) and IAA (0.2 mg/l). In line with this report, combination of BA (1 mg/l) with IAA (0.5 mg/l) has been documented for indirect shoot regeneration in another study also (Soni et al., 2015). Moreover two different media were also developed by Sharma et al. (2014) in which BAP (2.0 mg/l) with IBA (0.5 mg/l) formed callus whereas multiple shoots (12.00 ± 1.50) emerged and elongated on MS medium fortified with TDZ (0.1 mg/l).

Indirect somatic embryogenesis formed 27.0 SEs (90.3% response) after 150 days from leaf derived callus in *T. indica* in presence of BA (2 mg/l) (Manjula et al., 2000). Whereas in another report, combination of Kn (0.05 μM) with 2,4-D (9.04 μM) induced embryogenic calli and it differentiated 25.0 SEs after 10 weeks upon subculture on 2-ip (9.84 μM) with IBA (0.05 μM), further conversion of mature embryos to plantlets was done on basal medium (Jayanthi and Mandal, 2001). Another procedure for induction of somatic embryoids through mature leaves of *T. indica* was developed by Chandrasekhar et al. (2006). They have reported that combination of 0.5 μM TDZ with 1.5 μM 2,4-D which facilitated highest SEs induction and were successfully converted into plantlets after transferring onto semisolid MS medium without PGRs. Sahai et al. (2010a) documented that maximum callus differentiated was observed in presence of either BA (5 μM) or TDZ (2.5 μM) which induced 10.20 ± 0.37 SEs (90% response) after 12 weeks in presence of BA (5 μM). They also reported that for maturation and germination into complete plantlets medium devoid of any PGRs.

- **Nodal Explant**

As compared to leaf explant, only few studies reported the developmental protocol for shoot formation from nodal explant. However Faisal and Anis (2005) developed an efficient high-frequency shoot regeneration protocol from node derived callus. They have reported callus formation in presence of 10 μM 2,4,5-T followed by induction of 45 shoots after transferring of callus onto the Kn (5 μM) containing medium. Later on 8.6 ± 0.71 shoots were reported in 93% cultures via axillary shoot proliferation in MS media fortified with BA (2.5 μM) + NAA (0.5 μM) + ascorbic acid (100 mg/l) (Faisal et al., 2007). Similarly combination of cytokinin and auxins were reported for optimum shoot production in other reports e.g. Kn (2 mg/l) and IAA (0.5 mg/l) regenerated 3.59 shoots (Soni et al., 2015) and TDZ (1.5 mg/l) + IBA (0.1 mg/l) induced 5.84 ± 0.72 shoots (Najar et al., 2018).

- **Rooting**

Faisal and Anis (2003) documented that ½MS medium supplemented with 0.5 µM IBA was beneficial to induce maximum 4.30± 0.47 roots. Similarly ½MS medium supplemented with 3 µM IBA was used for optimum rooting of shoots by Thomas et al. (2005). Later on 0.5 µM of IBA dosage ½MS medium was observed to form optimum 5.8 ± 0.37 roots and 13.20 ± 0.80 cm length (Sahai et al., 2010). Many other authors also suggested the use of ½MS medium with IBA for rooting of *T. indica* shoots (Verma et al., 2010; Haque and Ghosh, 2013; Sadguna et al., 2013; Sharma et al., 2014). Whereas full MS with IBA (1 mg/l) (Soni et al., 2015) and IBA (0.5 mg/l) (Najar et al., 2018) has been reported for optimum rooting from shoots.

2.2 Phytochemical Analysis

In vitro regenerated cultures must be analyzed qualitatively and quantitatively to assess their biosynthetic potential. This can be done by various methods such as qualitative analysis using HPTLC fingerprinting, quantification of selected metabolites and also the metabolites content can be enhanced via elicitor application. Many previous reports documented these aspects and some of them are summarized below.

2.2.1 Chemical Profiling of *In vitro* Cultures

Chemical fingerprint is a method based on the chromatogram pattern of compounds that provide special characteristics. This can be developed using different chromatographic techniques which are valid methods for identification of chemical constituents (Valentao et al., 1999; Xie, 2001; Fiehn, 2002; Wolfender et al., 2015). Among different chromatographic methods, HPTLC is the simplest and versatile technique which has potential to detect even microgram quantities of metabolites within a short time period. These advantages bring about the possibility of screening the secondary metabolites present in the plant extracts (Itankar et al., 2015). It also offers better resolution of metabolites with accuracy (Sethi, 1996; Pawar and Guru, 2011), has low running cost and simultaneously able to detect many compounds makes it beneficial for medicinal plant studies (Wagner and Bladt, 2001; Birk et al., 2005). HPTLC fingerprint is also accepted by the World Health Organization (WHO), the Food and Drug Administration (FDA), the European Medicines Agency (EMEA), the German Commission E, the British Herbal Medicine Association, and the Indian Drug Manufacturers' Association (Cieśla et al., 2008). In this technique, visible pattern of bands provides data of compounds

present in the sample and further densitometry scanning gives information of each band which is represented as peaks and generates characteristic chromatograms with defined parameters including Rf, peak height and area (Moffat, 2001; Selvaskanthan et al., 2020). These fingerprints can be compared on the basis of number, sequence, position and colour of the bands and it is used for identification of plants especially in absence of standard compound (Mammen et al., 2011b; Kamboj and Saluja, 2017). The advantage of HPTLC fingerprint is that it helps in identification of marker compounds in the extract and also used for the isolation, purification and characterization of the useful phytoconstituents (Narayanan and Marimuthu alias Antonysamy, 2016).

The prerequisite for HPTLC fingerprint is to take solvents of different polarities so that they can extract all groups of metabolites (Harborne, 1984). Generally hexane extract contains terpenoids, aglycones, saponins, waxes and fats; ethyl acetate extract contains flavonoids, alkaloids, tannins, phenols, glycosides and chlorophylls; and methanol extract contains polyphenols, phenones, saponins, tannins, flavonoids, anthocyanins and xanthoxyllines (Houghton and Raman, 1998). Study on wild and *in vitro* plants of *Munronia pinnata* was done using extracts prepared from three different solvents (hexane, dichloromethane and ethyl acetate) and observed similar chemical constituents between them (Gunathilake et al., 2008). Recently, Selvaskanthan et al. (2020) also used hexane, ethyl acetate and methanol extract for development of fingerprint of callus and *in vivo* samples of *Gyrinops walla*. As different solvents extract different groups of metabolites, variation in number of bands can be observed and the same has been well documented in *Tabernaemontana catharinensis* (Boligon et al., 2013) and *Amsonia orientalis* (Acemi et al., 2020; Selvaskanthan et al., 2020).

This technique has also been used to assess the biosynthetic potential of *in vitro* samples of other Asclepiadaceae members like *Ceropegia juncea* (Nikam and Savant, 2009), *Hemidesmus indicus* (Pathak et al., 2017) and *T. indica* (Patel et al., 2020). Similarly, Busilacchi et al. (2008) assessed *in vitro* shoots of *Passiflora caerulea* using HPTLC fingerprint and reported it to be chemically similar to wild shoots. Later on comparative fingerprints were also depicted for wild and *in vitro* cultures of *P. caerulea* and *P. incarnata* (Ozarowski et al., 2013). 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), *Chlorophytum borivilianum* (Basu and Jha, 2013) and *Curculigo orchioides* (Alagar et al., 2014). Sometimes the PGRs affect the synthesis of certain compounds under *in vitro* conditions, it can be

detected by HPTLC and fingerprints can be compared with *in vivo* samples, as reported by Srivastava and Shrivastava (2008) in *B. monnieri*. Similarly, Kharade et al. (2014) have also reported difference in peak areas due to PGRs effect on *Curcuma longa*. 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 *in vivo* plant and compared it with *in vitro* grown callus, shoots and roots of *Oroxylum indicum* and reported slight variation in their profile. Recently nodal derived *in vitro* cultures of *Eclipta alba* also showed slight change in fingerprint profile of hexane, ethyl acetate and methanol extract due to influence of PGRs in the medium (Pathak et al., 2021). Similar results are also observed in *Primula veris* (Morozowska and Wesołowskam, 2004), *Arnicae folium* and *A. caulis* (Stefanache et al., 2014), *Celastrus paniculatus* (Anusha et al., 2016) and *Crataeva tapia* (Sharma et al., 2016).

The successful regeneration protocol also depends on hardening and acclimatization of *in vitro* plants and in many studies the hardened plants were also evaluated for their ability to synthesize metabolites similar to source plants. De Silva and Senarath (2009) documented that hardened plants and seed raised plants of *W. somnifera* showed similar HPTLC profile. Likewise, analysis in *Curcuma angustifolia* revealed that chromatogram of the conventionally propagated plant had similar fingerprint pattern to that of micropropagated plants (Jena et al., 2018). Tissue culture derived and field grown plants have similar HPTLC profile in *Asparagus adscendens* (Mehta and Subramanian 2005), *Celastrus paniculatus* (Martin et al. 2006), *Curcuma longa* (Nayak et al., 2011; Singh et al., 2011b), *Kaempferia galanga* (Mohanty et al., 2011a, b), *Piper nigrum* (Ahmad et al. 2013), *Nothapodytes nimmoniana* (Prakash et al. 2016) and *Centella asiatica* (Buranasudja et al., 2021). Whereas report in *Erigeron breviscapus* stated that the profile changed when the cultures are under *in vitro* conditions, but after hardening plant showed similar profile in comparison with mother plants (Liu et al., 2008). In many studies, 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 Selection of Secondary Metabolites

Quantitative analysis was carried out for *p*-coumaric acid in *L. reticulata* and lupeol in *T. indica*. Though the reports are scanty for these plants, other plants studies for these metabolites are summarized below.

2.2.2.1 Medicinal Properties of p-Coumaric Acid

Phenolic compounds are common constituents of vascular plants and it divided into three classes: soluble, ester-bound and lignin-derived compounds (Wilson et al., 1986; Opsahl and Benner, 1993). These are nutritional elements that naturally occur in small quantities in foods. These componds are beneficial to human health but are not essential for the human body. Phenolic compounds are an important class of secondary metabolites widely found in higher plants especially in cereal grains and in beverages, such as teas, wines, and coffee (Teodoro et al., 2015). Phenolic acids are divided into two groups as hydroxybenzoic acid and hydroxycinnamic acid derivatives (Karamac et al., 2005; Mattila et al., 2005). Whole-grain cereals are primary sources of hydroxycinnamic acids such as ferulic, vanillic, sinapic and p-coumaric acid (Sosulski et al., 1982; Fardet et al., 2008).

p-Coumaric acid, a phenolic acid of the hydroxycinnamic acid family, is biologically synthesized through the shikimate pathway with phenylalanine and tyrosine as precursors (Fig. 5). It plays a central role in secondary metabolism because it can be consequently transformed to phenolic acids (e.g. caffeic acid, ferulic acid, chlorogenic acid and sinapic acid), flavonoids, lignin precursors and other metabolites (El-Seedi et al., 2012). It has been well known that *p*-coumaric acid plays a key role as an antioxidant and an anti-inflammatory (Rice-Evans et al., 1997; Svobodová et al., 2003). The oral administration of *p*-coumaric acid is known to be also helpful for stomach cancer by reducing the formation of carcinogenic nitrosamines (Biswick et al., 2010). Wide range of investigations have shown that it also exhibit various bioactivities, including antimutagenic, antiulcer, antiplatelet and anti-cancer activities, in addition to mitigating atherosclerosis, oxidative cardiac damage, neuronal injury, anxiety, gout and diabetes (Pragasam et al., 2013). *p*-Coumaric acid can be found in many fruits (apples, pears, grapes, oranges, tomatoes and berries), vegetables (beans, potatoes and onions) and cereals (maize, oats and wheat) in the form of free acid or ester (Clifford, 2000; Garrait et al., 2006; Mateos et al., 2006; Luceri et al., 2007; Lou et al., 2012; Reveron et al., 2012). In plants, it is found as a component of lignins and tannins (Barros et al., 2009; Naga Vamsi Krishina et al., 2014).

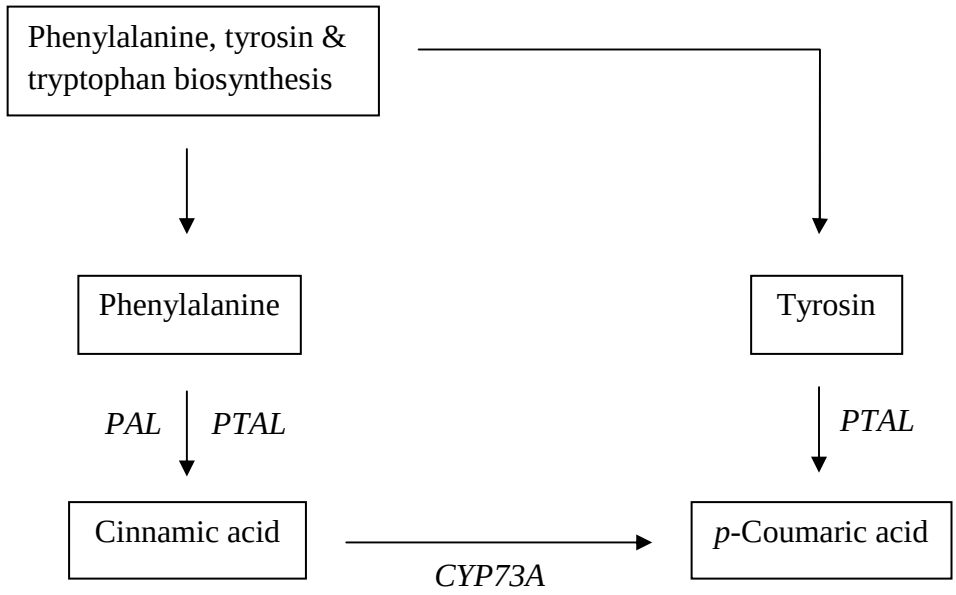

Figure 5. *p*-coumaric biosynthesis via phenylpropanoid pathway.
PAL: Phenylalanine ammonia lyase, *PTAL*: Phenylalanine/tyrosine ammonia lyase, *CYP73A*: trans-cinnamate 4-monooxygenase

2.2.2.2 Quantification of p-Coumaric Acid

Due to its medicinal properties, *p*-coumaric acid has been selected for quantification in different *in vitro* cultures. Aerial and underground parts of wild, *in vitro* and acclimatized plants of *Pelargonium sidoides* were assessed for *p*-coumaric acid and it was observed that the content was less in *in vitro* culture as compared to wild and acclimatized plants (Moyo et al., 2013). Similarly, Karakas and Turker (2013) also compared the content between wild and *in vitro* grown leaves of *Bellis perennis* and their findings stated that the *in vitro* leaves had slightly less content than wild plant. In another study it was observed that the content of this metabolite is significantly higher in cell suspension cultures than in non-organogenic callus cultures of *Larrea divaricata* (Palacioa et al., 2012). *In vitro* culture of *Merwilla plumbea* had more content of phenolics including *p*-coumaric acid, especially in the aerial part, and it was reported because of the presence of cytokinins in the medium (Aremu et al., 2013). Some studies have documented the effect of combination of BA+NAA at different concentrations on accumulation of metabolite e.g. in shoot cultures of *Ruta graveolens* ssp. *divaricata* (Ekiert et al., 2014) and *Hypericum perforatum* (Kwiecie et al., 2015). Ahmad et al. (2019) reported positive effect of TDZ on synthesis of *p*-coumaric acid in leaf derived callus of *Ipomoea*

turbinate. In corroboration with this study, TDZ along with NAA in presence of blue light facilitated maximum accumulation of compound in stem derived callus of *Lepidium sativum* (Ullah et al., 2019). Whereas MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l NAA was used for callus induction in *Rosmarinus officinalis* it affected accumulation of different phenolics including *p*-coumaric acid (Coskun et al., 2019). In addition, content of this metabolite was also higher in callus of *Schisandra chinensis* (Szopa and Ekiert, 2012) and *Pyrostegia venusta* (Coimbra et al., 2017). Few studies had also reported that not only PGRs, but the type of medium also affects the synthesis of metabolites e.g. when shoot and callus cultures of *Aronia melanocarpa* were grown in Linsmaier and Skoog medium, *p*-coumaric acid was produced only in callus culture (Szopa et al., 2013). Whereas when MS medium was used, shoot culture synthesized more *p*-coumaric acid than callus culture for the same plant (Szopa and Ekiert, 2014). Recently, Rattan et al. (2021) assessed callus derived from leaf and root of *Rhodiola imbricate* and observed that the content of this metabolite is higher in former. Similarly higher level reported in suspension culture of *L. sativum* (El-Haggar et al., 2021).

2.2.2.3 Medicinal Properties of Lupeol

Triterpenoids represent a large group of plant isoprenoids synthesized from the C30 precursor squalene, a linear hydrocarbon, which is oxidized to 2,3-oxidosqualene and then rearranged by special enzymes, oxidosqualene cyclases, to various cyclic structures (Kumari et al, 2013; Moses et al., 2013). Two main groups can be distinguished among these derived structures: steroids and pentacyclic triterpenoids. Both groups are distinctly differing by their functions in the plants, and therefore they are commonly known as primary and secondary metabolites, respectively (Hartmann et al., 1998; Moreau et al., 2002). In plants, it is synthesized via two pathways: mevalonic acid (MVA) pathway (e.g. ses- and triterpenoids) and methylerythritolphosphate (MEP) pathway (e.g. mono- and di-terpenoids) (Taiz and Zeiger, 2006). These molecules have important ecological and agronomic functions, contributing in pest and pathogen resistance. They also have a wide range of commercial applications in the food, cosmetics, pharmaceutical, and industrial biotechnology sectors (Thimmappa et al., 2014; Pérez-González et al., 2019).

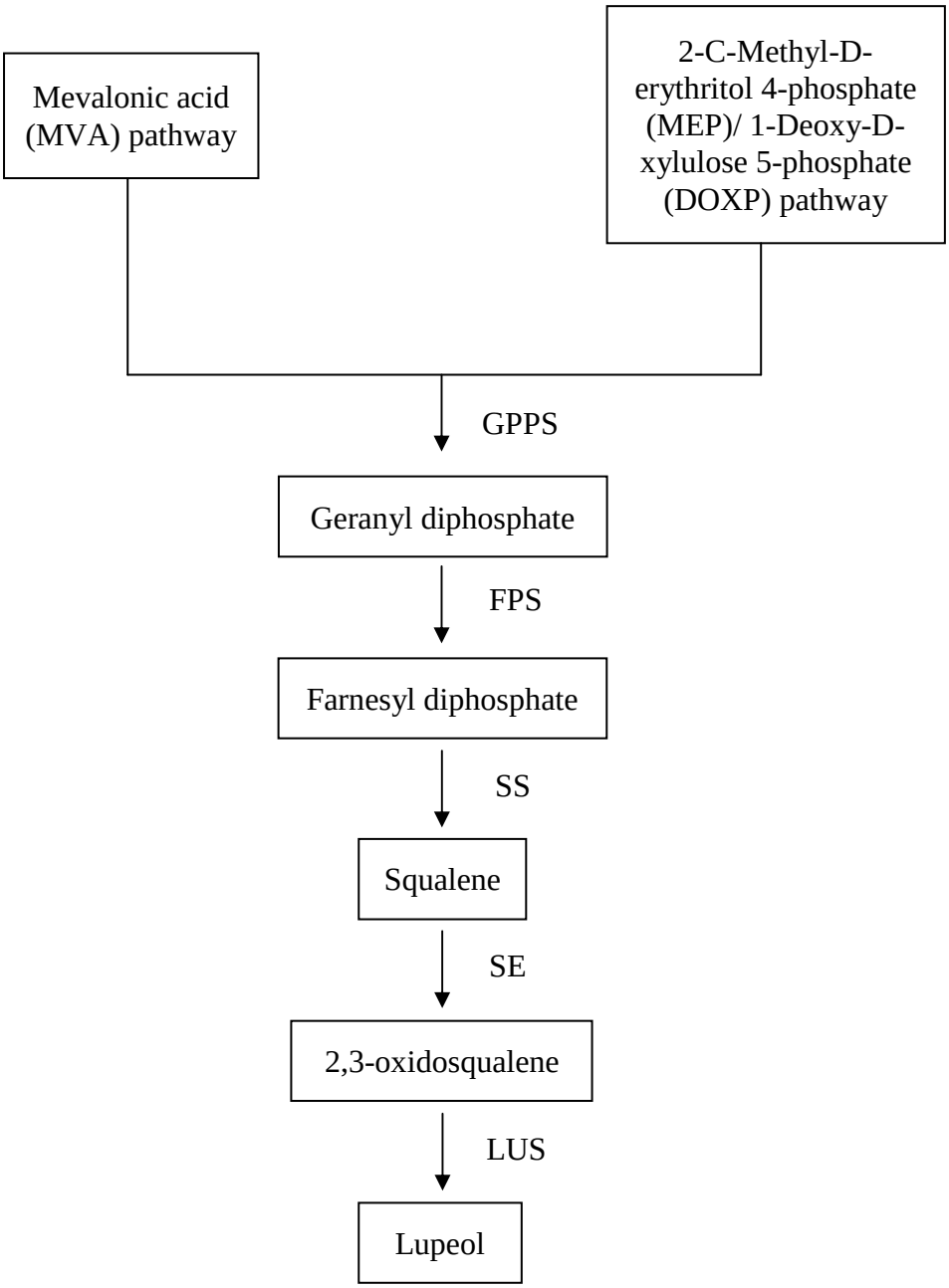

Figure 6. Lupeol biosynthesis via MVA/MEP pathways.
GPPS: geranylgeranyl pyrophosphate synthase, *FPS*: farnesyl phosphate synthase, *SS*: squalene synthase, *SE*: squalene epoxidase, *LUS*: lupeol synthase

Lupeol ($C_{30}H_{50}O$) is a well-known triterpene (Fig. 6) and the melting point is 215–216 °C with a molecular weight of 426.7174 (g/mol). It is broadly distributed in the plant kingdom and is found in edible fruits (olive, mango, strawberry and grapes), vegetables and several

medicinal plants. It is been reported for its potent anti-cancer activity in different cell lines as human prostate, breast, skin, liver, and blood cancer (Gallo and Sarachine, 2009; Rauth et al., 2016; Bhattacharyya et al., 2017b). It has great potential to act as an anti-microbial, anti-protozoal, anti-proliferative, anti-invasive and anti-angiogenic. According to studies of *in vitro* and *in vivo* models, it has also been tested for its biological activities against conditions including wound healing, diabetes, cardiovascular disease, kidney and arthritis disease (Siddique and Saleem, 2011). 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).

2.2.2.4 Quantification of Lupeol

Previous studies have reported quantification of lupeol in Asclepiadaceae member *Hemidesmus indicus.* Misra et al. (2003) documented that the synthesis of lupeol in *in vitro* shoots and roots is comparable with parent plant. Whereas leaf and root derived callus as well as shoot cultures in PGRs combinations BA+NAA, BA+2,4-D and Kn+NAA respectively affected its synthesis (Misra et al., 2005). Among all these, the maximum lupeol was recorded in shoot cultures grown in presence of BA+NAA combination. Later on Pathak et al. (2017) assessed lupeol content in nodal derived shoots grown in presence of BA+Kn and IAA and observed that cytokinin facilitated its synthesis. In the same plant root derived callus also synthesized lupeol and the content enhanced under *in vitro* conditions as compared to wild roots (Purohit et al., 2015). Recently, Chóez-Guaranda et al. (2021) observed that callus of *Vernonanthura patens* were able to synthesize lupeol, similar studies on other plants *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) also reported the same. Whereas in *Glycyrrhiza uralensis in vitro* stolon was also reported to synthesize lupeol (Kojoma et al., 2010).

2.2.3 Elicitation of Targeted Metabolites

Biosynthetic pathways responsible for the synthesis of secondary metabolites are often inducible by exogenous addition of elicitor and precursor feeding leading to stimulation of secondary products accumulation (Barber et al., 2000; Vats, 2018). There are some reports

suggesting the enhancement of *p*-coumaric acid in *in vitro* cultures using different elicitors. Ishikawa et al. (2007) suggested that enhancement of *p*-coumaric acid and furanocoumarins in *Glehnia littoralis* can be observed after treatment of *in vitro* cultures with YE. Similarly, the production of different phenolic acids including *p*-coumaric acid significantly enhanced in cell suspension cultures of *Malus* × *domestica* (Cai et al., 2014). Later on, the report documented that addition of 100 µg/ml of YE to the medium resulted in its maximum production in *Althaea officinalis* (Younesikelaki et al., 2018).

Many reports have documented the use of other elicitors like methyl jasmonate (MJ) for enhanced production of *p*-coumaric acid in *Leucojum aestivum* shoot cultures (Ivanov et al., 2013). The higher concentration of MJ (100 µM) was beneficial for enhancement of same metabolite in cell suspension culture of *Scrophularia striata* (Sadeghnezhad et al., 2016). Sanchez-Pujante et al. (2020) also observed enhancement after MJ treatment in broccoli cell suspension culture. On the other hand, in cell suspension culture of *Cocos nucifera* (Chakraborty et al., 2009) and *S. striata* (Kamalipourazad et al., 2016) the accumulation of *p*-coumaric acid increased upon exogenous application of chitosan. Moreover the *p*-coumaric acid content increased in cell suspension cultures of *Cucumis melo* after treatment with jasmonic acid (Nafie et al., 2011) and after SA treatment in *Polygonum multiflorum* (Thiruvengadam et al., 2016). Whereas El-Ashry et al. (2019) observed that its content increased after addition of phenyl alanine and salicylic acid in callus culture of *Gardenia jasmonides*. Duran et al. (2019) suggested that application of metanonin induced biosynthesis of this metabolite in callus culture of *Ocimum basilicum*. Studies were also carried out for enhancement of this metabolite in hairy roots with addition of elicitors e.g. jasmonic acid has been used in *Momordica charantia* (Chung et al., 2016), however in *Linum album* it was observed that fungal elicitor (Tashackori et al., 2016) and chitosan (Samari et al., 2020) were beneficial.

Whereas only few studies have been carried out for elicitation of lupeol in *in vitro* cultures, for e.g. Misra and Mehrotra (2006) reported that enhancement of lupeol using γ-rays in callus culture of *Hemidesmus indicus*. Later on Singh et al. (2011a) enhanced lupeol quantity using SA in *Cryptostegia grandiflora*. Whereas lupeol increased after UV-B radiation in *Vitis vinifera* (Gil et al., 2012) and after jasmonic acid in *Jatropha curcas* (Zaragoza-Martinez et al., 2016).

2.2.4 Elicitation of Metabolites using YE and SA

Elicitation of *in vitro* cultures is an excellent strategy for enhanced accumulation of bioactive secondary metabolites in relatively short time (Hussain et al., 2012). Biotic elicitor yeast extract (YE) and abiotic elicitor salicylic acid (SA) are commonly used for elicitation of medicinally important secondary metabolites in *in vitro* cultures. Earlier report by Suzuki et al. (2002) depicted that treating cell suspension cultures of *Medicago truncatula* with YE increased the triterpenoid biosynthesis by up-regulating the expression of genes related to its biosynthesis. Similar effect of YE was observed for enhanced expression of terpenoid genes and in turn its production which has been well studied in *Scutellaria baicalensis* (Yoon et al., 2000) and *Salvia miltiorrhiza* (Zhao et al., 2010). Variation in YE level affected the production of pseudojujubogenin content in *Bacopa monnieri* and maximum content was recorded when shoots were treated with 2 mg/ml YE (Kamonwannasit et al., 2008). Likewise, reports on *Astragalus chrysochlorus* (Ozgur and Sule, 2009) and *Iphiona mucronata* (Al-Gendy et al., 2015) documented that YE treatment increased amount of different phenolic compounds after treatment of callus cells with YE. Singh et al. (2015b) documented more than 2 fold enhancement in echitamine production when callus of *Alstonia scholaris* was treated with YE. Positive effect of YE on production level of metabolites is also reported in silymarin in *Silybum marianum* (Sampedro and Jorge, 2005), rosmarinic acid in *Lithospermum erythrorhizon* (Ogata et al., 2004) and *Orthosiphon aristatus* (Hunaefi and Smetanska, 2013), xanthone in *Hypericum perforatum* (Conceicao et al., 2006), psoralen in *Psoralea corylifolia* (Parast et al., 2011), vasicine in *Adhatoda vasica* (Bhambhani et al., 2012), biphenyl in *Sorbus aucuparia* (Qiu et al., 2012), carotenoid in *Cleome rosea* (Silva da Rocha et al., 2015), echitamine in *Alstonia scholaris* (Singh et al., 2015b), chicoric acid and rosmarinic acid in *Ocimum basilicum* (Açıkgöz et al., 2020) and mulberroside A, oxyresveratrol and resveratrol in *Morus alba* (Inyai et al., 2021).

Ginsenoside biosynthesis in *Panax ginseng* increased when farnesyl diphosphate synthase (*FPS*) and isopentenyl pyrophosphate isomerase (*IPPI*) genes were up-regulated after addition of YE in the medium (Rahimi et al., 2014). Similarly, tanshinone enhancement in *Salvia miltiorrhiza* has been correlated with increased expression of pathway genes e.g. 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*), isopentenyl diphosphate isomerise (*IPPI*) and geranylgeranyl diphosphate synthase (*GGPPS*) after YE treatment (Li et al., 2016). Other reports documented that lower concentration of YE was more beneficial for increased production of ginsenoside

and phenolics in *Panax quinquefolium* and *Impatiens balsamina* (Kochan et al., 2017; Kasem, 2018) respectively. Recently Açıkgöz et al. (2020) stated that different concentrations of YE enhanced different metabolite in *O. basilicum* as maximum accumulation of chicoric acid was recorded at 50 mg/l treatment whereas 200 mg/l concentration was optimum for rosmarinic acid. Addition of YE has been proved to be advantageous for production of other important metabolites such as plumbagin and rosmarinic acid in *Drosera burmanii* (Putalun et al., 2010), plumbagin in *D. indica* (Thaweesak et al., 2011); furanocoumarin in *Ruta graveleons* (Diwan and Malpathak, 2011), flavonoid in *Merwilla plumbea* (Baskaran et al., 2012), rosmarinic acid in *Melissa officinalis* (Nasiri-Bezenjani et al., 2014) and phenol, 2, 4-bis (1, 1-dimethylethyl) in *Orthosiphon stamineus* (Razali et al., 2017) has been enhanced using YE as an elicitor. Maqsood and Abdul (2017) observed that incorporation of YE enhanced vinblastine and vincristine in protoplast derived different stages of embryo and leaves of *Catharanthus roseus*.

Abiotic elicitor salicylic acid (SA) is a potent signalling molecule in plants and it has role in growth, metabolism, development, interaction, biotic and abiotic stresses (Senaratna et al., 2000). It is known to activate plant defence response and thus used as an elicitor to enhance the secondary metabolite production under *in vitro* conditions (Kang et al., 2004; Goyal and Ramawat, 2008). Exogenous SA increased the expression of 3-hydroxy-3-methylglutaryl CoA synthase (*HMGS*) and HMG CoA reductase (*HMGR*) genes of terpenoid biosynthesis in *Brassica juncea* (Alex et al., 2000). Whereas Hayashi et al. (2004) reported that SA treatment accumulated precursors of terpenoids biosynthesis in *Glycyrrhiza glabra*. Similarly, in *Taxus chinensis* (Wang et al., 2004), *P. ginseng* (Ali et al., 2006), *T. baccata* (Khosroushahi et al., 2006) and *T. chinensis* var. *Mairei* (Fan et al., 2006) it also enhanced terpenoid biosynthesis. Later on Kovacik et al. (2009) observed that the content of different phenolic acids increased after elicitation using SA in leaf rosettes of *Matricaria chamomilla*. Many pharmaceutically important metabolites were also enhanced after SA treatment such as caffeic acid and rosmarinic acid content in *Thymus membranaceus* (Perez-Tortosa et al., 2012), flavanoids in *Cistus heterophyllus* (Lopez-Orenes et al., 2013), hypericin and pseudohypericin in *Hypericum perforatum* (Gadzovska et al., 2013), rosmarinic acid in *Solenostemon scutellarioides* (Sahu et al., 2013), withanolides in *Withania somnifera* (Sivanandhan et al., 2013), bacoside A in *Bacopa monnieri* (Largia et al., 2015; Sharma et al., 2015), bacoside and corotenoid content in *Ruta angustifolia* (Othman et al., 2015), essential oils and phenolics in *Achillea millefolium* (Gorni and Pacheco, 2016), phenolic compounds in

Ajuga integrifolia (Abbasi et al., 2020) and reserpine and ajmalicine in *Rauvolfia serpentina* (Dey et al., 2020).

The elicitor effect depends on type of metabolite as well as on plant species e.g. increase in hypericin and pseudohypericin was recorded at 50 μM of SA in *Hypericum hirsutum* shoots, whereas in *H. maculatum* the same metabolites were increased at higher concentration i.e. 200 μM (Coste et al., 2011). Later on Patil et al. (2013) also reported that different concentrations of SA showed difference in optimum production of two metabolites as 50 μM proved beneficial for digitoxin and 200 μM for digoxin in shoots of *Digitalis purpurea* (Patil et al., 2013). Many studies depicted that lower concentrations of SA was beneficial for e.g. in cell cultures of *Ginkgo biloba*, SA (0.1 mM) improved the production of ginkgolides A and B with 3.1 and 6.1 times respectively (Kang et al., 2009). Similarly lower concentrations of SA has been used for enhancement of metabolites in cell culture of different plants e.g. production of psoralen was enhanced when 100 μM were added to the suspension culture of *Psoralea corylifolia* (Hari et al., 2018). Likewise, lower concentrations of SA up-regulated the accumulation of metabolites in *Orostachys cartilaginous* (Wen et al., 2019) and *Anethum graveolens* (Bulchandani and Shekhawat, 2020). In accordance, *in vitro* shoot cultures of *Swertia paniculata* also synthesized amarogentin, swertiamarin and mangiferin at lower SA levels (9 mM) (Kaur et al., 2020). Whereas recent study by Nazir et al. (2021) depicted that higher concentration of SA (200 μM) showed highest diosgenin production in shoot cultures of *Dioscorea deltoidea*.