Mohamed Ramadan Rady

Plant Biotechnology and Medicinal Plants

Periwinkle, Milk Thistle and Foxglove

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Dedicated to my beloved family, Ayah, Achraf, and Salwa

Preface

Recently, secondary metabolite production through plant cell culture and plant biotechnology has attracted interest from scientists. The potential of some plant culture systems for the production of medically important compounds has been demonstrated. Recently, genetic transformation and metabolic engineering are important areas which may provide new ways and efficient systems to increase in vitro production of secondary metabolites in medicinal plants. This system can now provide a commercially realistic alternative to whole plants for the production of some drugs. More recently, the progress on the genetic manipulation of biosynthetic units in microorganisms (synthetic biology) has opened the possibility of in-depth exploration of the large chemical space of natural product derivatives. In spite of several successful reports on the studied plants, there is still a gap in the knowledge for biosynthetic pathways and upscaling of this culture system for commercial utilization.

The purpose of this book is to provide recent information and studies about the induction of cell and organ cultures, establishment of transgenic cultures, and induction of hairy roots from in vitro cultures of periwinkle, milk thistle, and foxglove plants with special emphasis on elicitation strategies by abiotic and biotic elicitors for the production of the bioactive compounds from cultures. This book is expected to serve as a guide for the use of plant biotechnology as alternative source for increasing pharmaceutically important anticancer, flavonolignan, and cardenolide compounds from the studied plants. This book will be valuable to researchers as well as students working in the area of medicinal plant biotechnology. It will also serve as a reference for the pharmaceutical industry.

Giza, Egypt — Mohamed Ramadan Rady

Acknowledgment

Foremost, I would like to thank Allah, without whom, nothing is possible. I dedicate this work to my deceased parents who gave me the best gift of all, higher education and the freedom of choice. Also, I owe a special thanks to my brothers for their love and unwavering support throughout my life and to Dr. Kenneth Teng, the editorial staff of Springer, New York, for his guidance and encouragement and for giving me the opportunity to conduct this book, without him, there would be no book at all. I would also like to acknowledge the Springer team for their help in technical and continuous support and for editing this book. Last, but not least, I would like to gratefully acknowledge the National Research Centre, Egypt, for their constant support and excellent care.

Mohamed Ramadan Rady

Contents

Abbreviations

ABT	1-aminobenzotriazole
ACES	2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid
$AgNO_3$	Silver nitrate
APX1	Ascorbate peroxidase 1
AS	Anthranilate synthase
AVG	Aminoethoxyvinylglycine
B5	Gamborg et al. (1968) medium
BA	Benzyl adenine
CA	Coniferyl alcohol
$CaCI_2$	Calcium chloride
CAD	Cinnamyl alcohol dehydrogenase
$Cd(NO_3)_2$	Cadmium nitrate
CDPK	Calcium-dependent protein kinases
CDs	Cyclodextrins
CH	Casein hydrolase
CHI	Chalcone isomerase
CHS	Chalcone synthase
chsA	Chalcone synthase gene
CLOT	Clotrimazole
CPA	Chlorophenoxyacetic acid
$CuSO_4$	Copper sulfate
D4H	Deacetoxyvindoline 4-hydroxylase
DMAPP	Dimethylallyl diphosphate (DMAPP)
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
$FeSO_4$	Iron sulfate
G(G)PPS	Geranyl (geranyl) diphosphate synthase
G10H	Geraniol 10-hydroxylase
GA_3	Gibberellic acid
GES	Geraniol synthase

GPP	Monoterpene geranyl diphosphate
H_2O_2	Hydrogen peroxide
H_3B0_3	Boric acid
$HgCl_2$	Mercuric chloride
HPLC	High-performance liquid chromatography
IAA	Indole acetic acid
IBA	Indole butyric acid
IPP	Isopentenyl diphosphate (IPP)
JAs	Jasmonates
KCl	Potassium chloride
KI	Potassium iodide
$LaCl_3$	Lanthanum
LiCl	Lithium chloride
LN	Liquid nitrogen
LS	Linsmaier and Skoog (1965) medium
MBPK	Myelin basic protein kinase
MDA	Malondialdehyde
MeJa	Methyl jasmonate
MEP	Methylerythritole phosphate
MES	2-(N-morpholino) ethanesulfonic acid
$MgSO_4$	Magnesium sulfate
mM	Millimolar
$MnSO_4$	Manganese sulfate
MS	Murashige and Skoog (1962) medium
Mst	Mastoparan
Na_2MoO_4	Sodium molybdate
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
nBuOH	n-butanol
Ng	Flavanone naringenin
NmI	Lehmann et al. (1995) medium
NN	Nitsch and Nitsch (1969) medium
NO	Nitric oxide
NTPII	Neomycin phosphotransferase II gene
OD	Optical density
P450scce	Cholesterol side-chain cleavage enzyme
P5βR	4 progesterone-5β-reductase
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PCA	2,5-pyridinedicarboxylic acid
PEG	Polyethylene glycol
PLB	Protocorm-like body
PLD	Phospholipase D
POD	Peroxidase
ppm	Parts per million

PUF	Polyurethane foam
ROS	Reactive oxygen species
SAAT	Sonication-assisted Agrobacterium-mediated transformation
SAR	Systemic acquired resistance
SCCE	Sterol side chain cleaving enzyme
SGD	Strictosidine-β-dglucosidase
SH	Schenk and Hildebrandt (1972) medium
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
$SrCl_2$	Strontium
STB	Stirred-tank bioreactors
STR	Strictosidine synthase
STS	Stilbene synthase
TAB	Tetramethylammonium bromide
TDC	Tryptophan decarboxylase
TDZ	Thidiazuron
TIA	Terpenoid indole alkaloid
TRIA	Triacontanol
TTC	Triphenyltetrazolium chloride
Tx	Taxifolin
UV	Ultraviolet
$VaSO_4$	Vanadyl sulfate
WPM	Loyd and McCowan (1980) medium
YE	Yeast extract
$ZnSO_4$	Zinc sulfate
µM	Micromolar
µg	Microgram
2,4-D	2,4-Dichlorophenoxyacetic acid
3-KSI	3 Δ4,5-3-ketosteroid-isomerase
3β-HSD	3β-hydroxysteroid dehydrogenase

About the Author

Mohamed Ramadan Rady is a Professor Emeritus at Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Egypt. He has obtained his doctoral degree in plant biotechnology from Agronomy Department, College of Agriculture, Al-Azhar University, Cairo, Egypt. His basic area of research is plant biotechnology (plant secondary metabolites and genetic manipulation of medicinal plants), and he did work on several medicinal and aromatic plants. To date, he has successfully run bilateral research project as Principal Investigator on periwinkle plant with The Hungarian Academy of Sciences. He has also participated in various national and international conferences. He is an Associate Editor of *Journal of Genetic Engineering and Biotechnology* (Springer Nature), and reviewer of many national and international scientific journals. He is involved in research, teaching, training, and supervising research students at the department and other universities. Based on his research contributions, he has been awarded a prize of the National Research Centre for scientific encouragement in the field of agriculture and biological sciences. Dr. Rady has published more than 45 research articles on medicinal plants in reputed journals. He has authored one book chapter and edited this book.

Chapter 1
Plant Biotechnology and Periwinkle

Contents

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Abstract *Catharanthus roseus* is one of the most extensively investigated medicinal plants, which can produce more than 130 alkaloids, including the powerful antitumor drugs vinblastine and vincristine which are used in the treatment of cancer. Alkaloids are one of the most important secondary metabolites known to play a vital role in various pharmaceutical applications leading to an increased commercial importance in recent years. An overview of recent studies which have been used using various approaches of plant tissue, organ culture, regeneration, cryopreservation, and transformation of *C. roseus* is presented in this chapter. One of the most effective strategies for enhancing the biotechnological production of alkaloid compounds is elicitation. This chapter summarizes the recent research work of various in vitro cultures, abiotic, biotic elicitors, and precursor feeding applied to *C. roseus* cultural system and their stimulating effects on the accumulation of TIAs.

Keywords *Catharanthus roseus* · Biosynthesis · Cell and callus culture · Regeneration · Cryopreservation · Transformation · Hairy root cultures · Elicitation · Alkaloids

1 Introduction

Catharanthus roseus is an important medicinal plant, belonging to the family Apocynaceae, and is a rich source of alkaloids, which are distributed in all parts of the plant. *C. roseus* is a perennial, evergreen herb that was originally native to the island of Madagascar. It has been widely cultivated for hundreds of year and has been widely cultivated in all warm and pantropical regions of the world (Aslam et al. 2010). The subshrub grows about 30–100 cm high with glossy and dark green leaves of 2–5 cm long and 1–3 cm broad. The wild *C. roseus* plant has a pale pink phlox-like flower with a purple eye in the center, but various cultivars have been developed with flower colors ranging from purple, violet, red, pink, and white (Fig. 1.1).

Catharanthus roseus contain important alkaloids, viz., vincristine and vinblastine, which are used in the treatment of cancer. These alkaloids interfere with the mitotic cell division process of the cancerous cells. They stop formation of microtubules, and thus chromosomes are unable to arrange on metaphase plate (Negi 2011). The vinblastine and vincristine can lower the number of white cells in blood. A high number of white cells in the blood indicate leukemia. So they act as anticancer drug. These alkaloids prevent mitosis in metaphase, and they bind to tubulin and thus prevent the cell from making the spindles it needs to divide (Kalidass et al. 2010). However, ajmalicine, serpentine, vindoline, and catharanthine are major alkaloids. In these alkaloids, ajmalicine and serpentine are useful for treatment of hypertension. Vindoline and catharanthine are the obvious precursors in the biosynthetic pathways of dimeric indole alkaloids such as vinblastine and vincristine. But their isolation from intact *C. roseus* plants is very costly because of their extremely low concentrations. Alkaloids from the *C. roseus* are

Fig. 1.1 *C. roseus* plant

normally obtained from the field-grown plants. It requires lots of space and infrastructure; in addition the raw material is season dependent and is affected by various fluctuating environmental risk factors. The antitumor alkaloids are produced in trace amounts (0.0003% dry weight) (Negi 2011). The high prices of these anticancer products, ranging from $1 million to $3.5 million per kilogram, have led to a widespread research interest over the past 25 years in the development of alternative sources for the production of these compounds (Verpoorte et al. 1991). The low yield and high market price of the pharmaceutically important alkaloids of *C. roseus* have created interest in improved alternative routes for their production (Verma et al. 2012). However, most TIAs are present in very small amounts, especially the dimeric/bisindole alkaloids. Thus large quantities of raw material are needed for compound isolation. For example, to isolate 1 g of vinblastine, about 500 kg of *C. roseus* leaves are required (Van der Heijden et al. 2004). In addition, it is also difficult to synthesize TIAs by chemical methods due to their complicated structures (Yang and Stöckigt 2010). However, the yields of these TIAs are low in wild-type plants, and the total chemical synthesis is impractical in large scale due to high cost and their complicated structures (Wang et al. 2012).

As the demand for medicinal plants is growing at a very fast pace, consequently some of them are increasingly being threatened even in their natural habitats (Muthukumar et al. 2004). For these reasons, a biotechnological approach using plant cell or tissue cultures is being explored as alternative production method of the valuable bioactive metabolites from plants. Plant tissue culture might be a source of these monomeric and dimeric alkaloids, and therefore, many attempts have been made to establish a culture that produces them in large amounts. Ajmalicine, serpentine, and catharanthine are produced by some cell culture lines in amounts several times those obtained from intact plant (Kurz et al. 1981). Researchers are focusing their attention to enhance the alkaloids yield by various ways, chemically, enzymatically, and synthetically, or by cell culture method.

In this chapter, recent studies for establishment of cell, callus, organ, and transgenic cultures were reviewed. Optimization of the culture medium, plant growth regulators, and culture conditions were extensively studied to improve the cell biomass accumulation and the TIA production. However, abiotic, biotic elicitors and precursor feeding to enhance accumulation of TIAs in different cultures of *C. roseus* are highlighted.

2 Biosynthesis of TIA in *C. roseus*

Alkaloids of *C. roseus* comprise a group of more than 130 terpenoid indole alkaloids (TIAs) which represent one of the largest and most diverse groups of alkaloids in this plant. It is the sole resource of vinblastine and vincristine, which are two of the biggest concerns of TIAs because of their powerful anticancer activities (Van Der Heijden et al. 2004). A complete knowledge of the biosynthetic pathway of the targeted compounds and its regulation is essential to increase the metabolic flux toward the desired products. The biosynthesis of *Catharanthus*' alkaloids has been studied extensively. The biosynthetic pathway of TIAs in *C. roseus* and characterization of the related genes encoding the enzymes involved in this pathway are summarized in Fig. 1.2.

The biosynthesis of TIA in *C. roseus* is a complex metabolic pathway involving different subcellular compartments including plastids, cytosol, nucleus, endoplasmic reticulum (ER), and vacuole, in which biosynthetic machinery lies within membranes for alkaloid metabolism (Pomahacova et al. 2009). More than 50 biosynthetic events are composed of the involved genes, enzymes, regulatory genes, and intra-/intercellular compartments (Zhao et al. 2013). TIA biosynthesis requires two precursors from two different biosynthetic routes, i.e., tryptamine from the shikimate/tryptophan pathway and secologanin from the iridoid/methylerythritol phosphate (MEP) pathway (Pan et al. 2016).

Among the two precursor pathways, the iridoid pathway is considered a major rate-limiting factor for TIA production in *C. roseus* cell cultures (Zhao and Verpoorte 2007; Pan et al. 2016). The iridoid precursors of the TIA derive from 8-hydroxygeraniol (also known as 10-hydroxygeraniol) which is formed upon hydroxylation of geraniol generated from monoterpene geranyl diphosphate (GPP). GPP is a condensation product of the basic isoprene units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The other enzymes such as geraniol 10-hydroxylase (G10H), NADPH-cytochrome P-450 reductase, and anthranilate synthetase (AS) have the similar TDC activities which are involved in the biosynthesis of indole alkaloids.

Formation of Tryptamine and Secologanin

Tryptamine is derived from indole biosynthetic pathway. Tryptamine is derived from a single enzymatic conversion of the amino acid L-tryptophan (product of

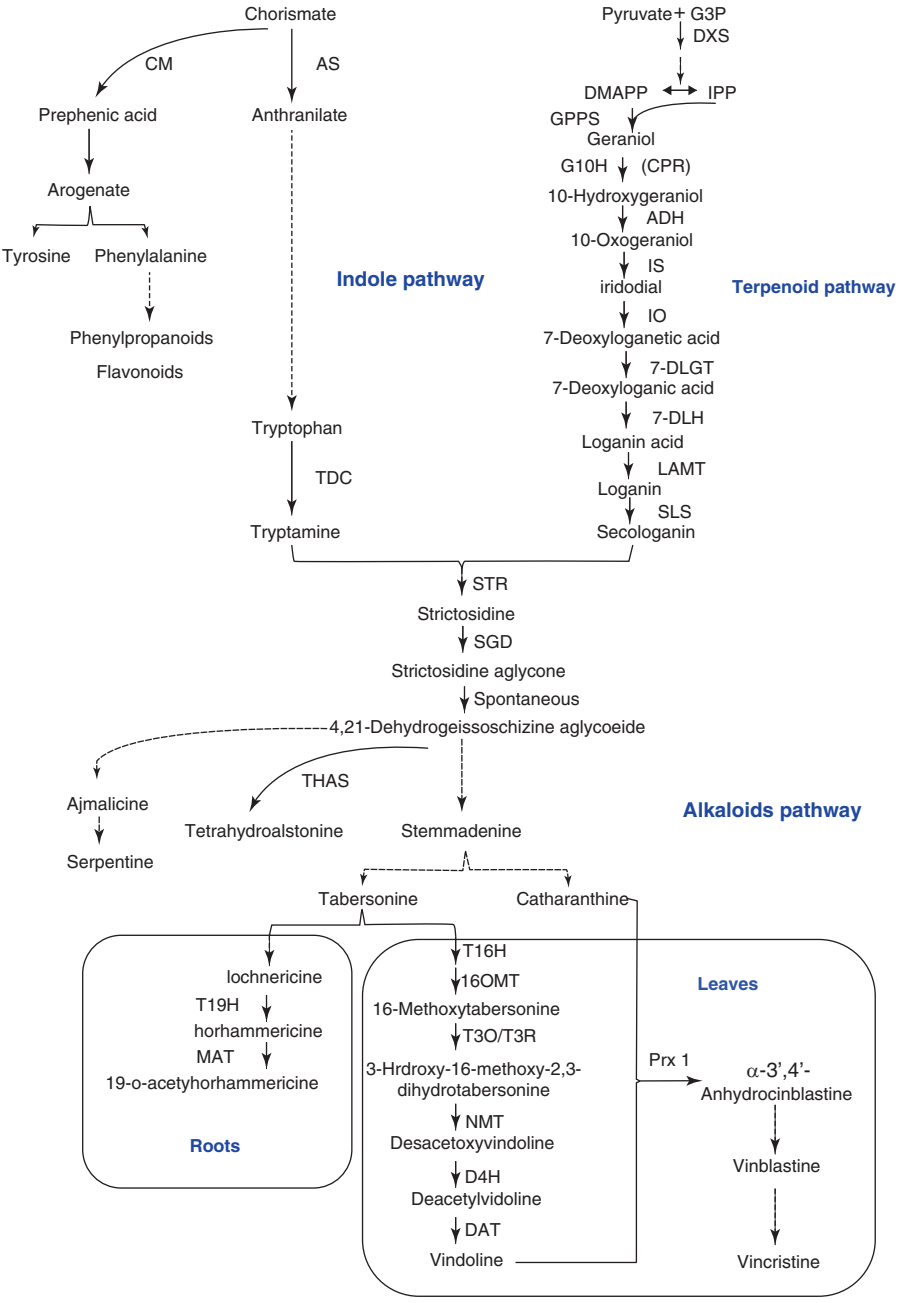

Fig. 1.2 Terpenoid indole alkaloid pathway. (Dashed lines represent the unknown steps) (Adapted from Sun et al. 2016)

the plastidial shikimate pathway) by the enzyme tryptophan decarboxylase (TDC) (De Luca et al. 1989), while secologanin is derived from the monoterpene geranyl diphosphate (GPP) in the plastidial methylerythritol phosphate (MEP) pathway (Contin et al. 1998b; Hong et al. 2003). Strictosidine synthetase (STR) catalyzes the coupling of tryptamine and secologanin to produce strictosidine. These two compounds are the universal precursors of all TIAs in plants.

Biosynthesis of Strictosidine

Strictosidine is the central intermediate in the biosynthesis of many alkaloids in *C. roseus*, which is derived from the condensation of secologanin and tryptamine by strictosidine synthase (STR) (Fig. 1.2). Strictosidine synthase (STR) was shown to be localized in the vacuole; thus tryptamine and secologanin from the MEP pathway need to be transported to the vacuole to produce strictosidine. Subsequently, strictosidine is transported out of the vacuole to be deglucosylated by strictosidine-*β*-dglucosidase (SGD) which is associated with the nucleus (Guirimand et al. 2010). The reversible intermediate 4,21-dehydrogeissoschizine can also be converted into stemmadenine which leads to the production of vindoline and catharanthine, the monomeric precursors of bisindole alkaloids vinblastine and vincristine (El-Sayed and Verpoorte 2007).

Biosynthesis of Vinblastine and Vincristine

Biosynthesis of vinblastine and vincristine involves a series of enzymatic reaction localized in the endoplasmic reticulum (ER) (tabersonine 16-hydoxylase [T16]), cytosol (16-hydroxytabersonine 16-*O*-methyltransferase [OMT], desacetoxyvindoline 4-hydroxylase [D4H], and deacetylvindoline 4-*O*-acetyltransferase [DAT]), thylakoid membrane of chloroplasts (*N*-methyltransferase [NMT]), and vacuole (peroxidase [PRX1]) (Costa et al. 2008; Guirimand et al. 2011). The bisindole alkaloids vinblastine and vincristine are derived from the coupling of the monomeric alkaloids catharanthine and vindoline; the process is catalyzed by the major class of vacuolar III peroxidase (CrPrx1) (Costa et al. 2008). These monomeric alkaloids produced anhydrovinblastine by a peroxidase which is a reduction product. The product α-3′,4′-anhydrovinblastine catalyzed by CrPrx1 is the common precursor of all dimeric alkaloids, which can be future converted into vinblastine and vincristine through several steps (Zhu et al. 2015).

In general, the TIAs are condensation products of two biosynthetic routes, requiring coordination of the amount of intermediates supplied by both pathways. Starting from the amino acid tryptophan and the monoterpenoid geraniol, the biosynthesis of vinblastine requires the participation of at least 35 intermediates, 30 enzymes, 30 biosynthetic, 2 regulatory genes, and 7 intra- and intercellular compartments. Although many genes and corresponding enzymes have been characterized in this pathway, our knowledge on the whole TIA biosynthetic pathway still remains largely unknown up to date. Full elucidation of TIA biosynthetic pathway is an important prerequisite to understand the regulation of the TIA biosynthesis in the medicinal plant and to produce valuable TIAs by synthetic biological technology (Zhu et al. 2014).

3 In Vitro Culture of *C. roseus*

3.1 Cell and Callus Cultures of C. roseus

Various explants (stem, leaf, hypocotyl shoot tips, roots, and petioles) were used from in vitro germinated seedlings or from in vivo plants of *C. roseus* which have been tested as primary explant sources on several basal media with plant growth regulators for obtaining callus cultures. It has been noted that the plant cell's growth is fast in agitated suspension compared to solid medium because of easier uptake of nutrients by the cells. Establishment of callus and cell suspension cultures in *C. roseus* is considered the first step to study biosynthetic capacity and improve the production of TIAs. The procedures of standardizing friable calli or suspension culture development become a necessary step to manufacture valuable plant metabolites.

Several studies have investigated the effects of plant growth regulators, culture media, and cultural conditions for establishment of *C. roseus* cultures. In vitro studies of cell and callus culture induction from *C. roseus* were presented in Table 1.1.

Miura and Hirata (1987) obtained callus cultures of greenhouse-grown *C. roseus* plants. They found that MS medium supplemented with 1.0 mg/l NAA and 0.1 mg/l kinetin induced callus tissues from young leaf explants. Initiated calli were brown and tight and differentiated many hairy white roots and maintained high antimitotic activity for several passages. However, isolation of vinblastine in callus culture with differentiated roots was detected. Zhao et al. (2001a) observed that there was no obvious difference between the compact callus cluster cultures derived from leaf explants and stem explants from *C. roseus* grown on MS liquid induction medium supplemented with 5.37 μM NAA and 4.65 μM kinetin. They also postulated that the level of cellular/tissue differentiation might be responsible for these different alkaloid synthesis capabilities. Sucrose regime affected some properties (the size, degree of compaction, differentiation level) of the compact callus cluster cultures and therefore influenced alkaloid production. The optimal sucrose concentration for alkaloid and biomass production by that cultures was 50 or 60 g/l. Junaid et al. (2006) found that MS medium supplemented with 1.0 mg/l 2,4-D was the best for callus induction from hypocotyl explants of *C. roseus*. The maximum callusing response percentage was 85. The hypocotyl callus was friable, light yellow, and fast growing. In another study, callus cultures were initiated from single-node explants of field-grown *Vinca minor* when cultured on MS medium supplemented with the combination of 7.21 mg/l BAP and 2 mg/l NAA (Raouf Fard et al. 2008). Also, callus cultures were initiated from petiole explants of *C. roseus* greenhouse-grown plants, when cultured on MS medium containing 0.1 mg/l NAA + 0.1 mg/l Kin after 6-week incubation (Ataei-Azimi et al. 2008).

Four aseptically explants (shoot tip, leaf, stem, and root) were excised from the plantlets as well as sterilized seeds of *C. roseus* and used for callus initiation (Taha et al. 2008). They found that response of shoot tip explant was the best between the different explants on MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l

Table 1.1 Studies on cell and callus cultures induction of *C. roseus*

Explant used	Medium used	Culture condition	Growth regulators (mg/l)					Reference
			2,4-D	NAA	Kin	BA	TRIA	
Young leaf segments of greenhouse-grown plants	MS	Cultures were incubated at 25 °C in the dark		1.0	0.1			Miura and Hirata (1987)
Stem and leaf explants from cultivated plants in a greenhouse	MS	Cultures were incubated on a rotary shaker (120 rpm) at 23–28 °C in darkness		5.37 μM	4.65 μM			Zhao et al. (2001a)
Hypocotyl of 5–7 days old in vitro germinated seedlings	MS	Cultures were incubated under a 16 h photoperiod at 25 °C light/20 °C dark	1.0					Junaid et al. (2006)
Single node	MS	All cultures were incubated at 25 ± 2 °C under a 16 h photoperiod		2		7.21		Raouf Fard et al. (2008)
Petiole segments from leaves of 6-week-old seedlings, germinated in greenhouse	MS	Cultures were placed in a growth chamber at dark period for first of 2 weeks and 24 h light period for second of 4 weeks at 35 °C		0.1	0.1			Ataei-Azimi et al. (2008)
Shoot tip from in vitro germinated seedlings	MS	Cultures were incubated in a growth chamber at 25 ± 2 °C under a 16/8 h light dark photoperiod	1.0		1.0			Taha et al. (2008)
Shoot tips collected from the field-grown plants	MS	Cultures were maintained in dark for 4–6 weeks at 25 ± 30 °C	2.0 μM				5.0 μM	Malabadi et al. (2009)
Hypocotyl, leaf, nodal, and root from in vitro germinated seeds	MS	Cultures were incubated at 25–28 °C under 16 h photoperiod	6.96 μM					Aslam et al. (2009)
Leaves were harvested from plants growing in gardens	NN	Different light conditions		8.0 μM		8.0 μM		Al-Khateeb et al. (2009)
Stem explant from mature plants	MS	Cultures were maintained at 25 ± 1 °C with a 16 h photoperiod	1.0 μM		1.0 μM			Kalidass et al. (2010)
Hypocotyl explants from 10–15-day-old seedlings grown in greenhouse	MS	Cultures were maintained at 26 °C with completely dark conditions		1.0		1.0		Singh et al. (2011)

Leaf explants	MS	Dark conditions		2.0		3.0		
Epicotyl explants	MS	Dark conditions	2.0			0.1		
Root explants	MS	Dark conditions		1.0		1.5		
Segment of shoots and roots from in vitro germinated seeds	B5		1.0		1.0			Fathalla et al. (2011)
	MS Subculture				1.0			
Leaves were collected from third node from the apex of garden-grown plants of *Vinca rosea*	MS	Cultures were incubated in a light regime of 18 h followed by 6 h dark period at 25 ± 2 °C	1.0		0.1			Negi (2011)
Nodal segment were collected from garden-grown plants of *Vinca rosea*	MS				1.0	1.0		
Young leaves, from mature plant	MS	Cultures were maintained in a culture room at 18 h photoperiod at 25 °C		1.25		0.2		Zulkepli and Samad (2011)
Young shoot tip from plants cultivated in a greenhouse	MS	Cultures were incubated at 25 ± 1 °C under complete darkness	1.5		0.5			Saifullah and Khan (2011)
		Cell suspension cultures on a gyratory platform shaker at 100 rpm and 25 ± 1 °C with a 16 h photoperiod						
Leaf explants from in vitro germinated seedlings	MS	½ MS; cultures were incubated under a 16 h photoperiod at 25 ± 20 °C	0.5			1.0		Verma et al. (2012)
Leaves that are still actively growing at 3–4 leaves from the apex shoots were used as the explants	MS	Cell aggregate cultures were incubated at room temperature and agitated at a speed of 120 rpm		2.0	0.2			Pandiangan et al. (2013)

(continued)