Mohammad Anis · Naseem Ahmad
Editors

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ISBN 978-981-10-1916-6 ISBN 978-981-10-1917-3 (eBook)
DOI 10.1007/978-981-10-1917-3

Library of Congress Control Number: 2016954918

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Science+Business Media Singapore Pte Ltd.

Preface

The extinction of plant species is progressively taking place due to their being trapped in the vicious circle of ever-increasing industrialization, deforestation, global warming, climate change, and also unscrupulous human activities. The situation warrants acceleration of efforts to develop methods for their germplasm preservation. In this context, the importance of in vitro morphogenesis cannot be overemphasized, as the interplay of morphogenic factors, which can precisely be managed in vitro – grown plant system cannot be done ex vitro. Furthermore, its application for germplasm preservation becomes imperative, particularly in case of hybrids which must be propagated vegetatively, where seeds are not produced, the plant is systemically infected, or the plant material is very limited. The application of micropropagation techniques has witnessed major advances and numerous benefits over the last few decades and is the only aspect of biotechnology that has been convincingly documented with regard to its feasibility for mass-scale propagation commercially.

Molecular biology and biotechnology have now become an integral part of tissue culture research. The tremendous impact generated by genetic engineering and consequently the generation of transgenics has helped in the manipulation of plant genomes at will. There is indeed rapid development in this area with commendable success in India. It has, therefore, become increasingly difficult to author a book on the subject. Hence, this edited volume would hopefully prove informative to readers. The book provides a source material to researchers intending to initiate work in these areas.

The editors acknowledge the unstinted support received from contributors who spared valuable time in writing chapters for this volume and also sincerely thank the publishers for their cooperation in making this book a reality. We are indebted to Dr. H. C. Chaturvedi, former emeritus scientist (CSIR), and Dr. A. K. Sharma, former head, Tissue Culture Lab, CSIR-National Botanical Research Institute (NBRI), Lucknow, who introduced us to plant tissue culture and to the intricacies of the technique.

While preparing this book, we have received unflinching support from colleague and research students in the Plant Biotechnology group. Prof. Altaf Ahmad extended full cooperation and read a number of manuscripts. Postdocs of the Plant Biotechnology Laboratory, Dr. Ankita Varshney, Dr. Nigar Fatima, Dr. Ruphi Naz, and Dr. Saad Bin Javed, and doctoral students, Ms. Afsheen Shahid, Ms. Mehrun

Nisha Khanam, Mr. Sheikh Altaf Husain, Mr. Anees Ahmad, and Mr. Naushad Alam, extended full cooperation and solicited timely help.

Once again, thanks to all those who helped in various ways.

Aligarh, Uttar Pradesh, India

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Professor Mohammad Anis possesses about 31 years of teaching and research experience on Biotechnology and Cytogenetics. He has published over 185 research and review articles in Journals of International repute. He is recipient of National (1992) and Overseas (1994) Biotechnology Associateship of DBT, Govt. of India, INSA Visiting Scientist to Frankfurt University (1998) and Institute of Genetics and Plant Biotechnology, Nitra (2006), Slovak Republic, Eminent Scientist Award (2007) by National Environmental Science Academy, New Delhi, Vigyan Ratan Samman (2010) of Council of Science and Technology, U.P., Professor P. Maheshwari Medal (2013) and Mid-Career award of UGC (2015) for his significant contribution in Medicinal Plant Biotechnology. He has been Program Coordinator of several major projects like DST-FIST (I and II), UGC-DRS (I and II) and DBT-HRD.

Dr. Naseem Ahmad is Young Scientist SERB-DST, New Delhi in the Department of Botany, Aligarh Muslim University, Aligarh. He has more than 6 years of Postdoctoral experience in the area of Morphogenesis, Plant Tissue Culture and Molecular Biology. He has published about 37 research papers in Journals of International repute, 03 book chapter and 41 abstracts in various conferences. He is a member in the Editorial Board of various journals, holds Life Membership of many Learned/scientific societies and is a Fellow of Indian Botanical Society (FBS). He is recipient of "National Scholarship" of Slovak Republic (2013), Yuva Vaigiyanik Samman (2009), CST, UP; Young Scientist (2010) by NESA, New Delhi, Rashtriya Gaurav Award (2015) by India International Friendship Society, New Delhi and Young Scientist of the year (2015) by International Foundation for Environment and Ecology (IFEE), Kolkata. He has received Post Graduate Merit Scholarship in M.Sc. and Best oral presentation awarded at BBAU (Central University) Lucknow and has availed Senior Research Fellowship (SRF) of CSIR, New Delhi.

Part I

In Vitro Regeneration

Plant Tissue Culture: A Journey from Research to Commercialization

1

Mohammad Anis and Naseem Ahmad

Abstract

Tissue culture was a subject of academic interest for a long time. In recent years, it has become a useful tool for agriculture and medicine. It has therefore been a popular area of biological research. Considerable amount of literature has been generated, but it is not commensurate with the results obtained. The continuous and non-organized exploitation has resulted in many medicinal plants becoming rare, and a good number have even become extinct. Therefore, tissue culture has emerged as a science with a vast potential for human welfare ranging from large-scale plant production in horticulture and forestry, human health, plant protection as well as environmental protection. In vitro rejuvenation holds remarkable potentials for the production and superior plant-based medicine. There are mainly four approaches for in vitro germplasm preservation, which may lead to development of a tissue bank; cryopreservation, normally growing and multiplying shoot culture, slow-growth culture and regenerative long-term excised root culture. The main parameter for evaluating the worth of these approaches includes practicability, prolonged retention of regenerative potentiality and the least chances of genetic instability.

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M. Anis, N. Ahmad (eds.), *Plant Tissue Culture: Propagation, Conservation and Crop Improvement*, DOI 10.1007/978-981-10-1917-3_1

Abbreviations

BA	6-benzyl adenine
2iP	2-isopentenyl adenine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	6-furfurylaminopurine
PGRs	Plant growth regulators
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthalene acetic acid

1.1 Introduction

Biodiversity is nature's fabric of life. The economic prosperity of any country depends on this natural capital. Today we are in a global battle for the conservation of this natural wealth. Being driven primarily by climate disruption, habitat changes and over-exploitation, biodiversity loss is pushing earth towards the sixth mass extinction. Continuous and often indiscriminate collections of medicinal plants in bulk quantity from diverse ecosystem, coupled with destruction of natural habitats, are resulting in irreplaceable loss of valuable genetic diversity. Medicinal plants account for one-third of the species in the 'Red Data Book of India'. The country's rich biodiversity has been sadly and seriously affected with the increasing human population. The in vitro multiplications of protocols offer a potential technique of generating sufficient material for commercial planting. Its utilization in forestry, agriculture and horticulture is growing worldwide. Large numbers of plants have been recovered initiated from a sole entity in a comparatively short instance and space (Bhojwani and Razdan 1983). Micropropagation is fast, uses little quantity of shoots and succeeds when other methods fail (Fay 1992). The technique has been used globally for monitoring of secondary metabolite at various stages of growth and differentiation.

Tissue culture becomes a popular area of research with most laboratories jumping on the bandwagon, changing their names or opening a new section to include tissue culture. Conventionally these plants take a long time for multiplication and have a low rate of fruit/seed set and poor seed viability/germination, and often roots/rhizome of few years old plant contains the effective principle. Thus, in order to obtain active ingredients from storage organs, often whole plants are dug out which eliminate its chances of survival and perpetuation in nature.

Recent advancement in biotechnological methodology of culturing plant cell and tissues has provided new means of rapidly propagating and conserving the endangered and other vulnerable plant species.

As per directives of the University Grants Commission (UGC) in 2003, the curricula for both UG and PG were revised where greater emphasis was made on the courses related to Plant Biotechnology and Molecular Biology. Prof. M. Anis, the

group leader, established a laboratory and study programme in Plant Biotechnology in the Botany Department, AMU, Aligarh. On the basis of the research output made in this area during the last 15 years, he was instrumental in arranging huge grants from various government agencies. Based on the overall achievements and progress, the department was supported under the Special Assistance Programme DRS-I (2009–2014) and DRS-II (2016–2021), DST-FIST-I (2006–2010), DST-FIST-II (2011–2016) and DBT-HRD (2008–2013). This has paved the way for establishing a Plant Molecular Biology and Nanobiotechnology lab with an aim to expand academic operations by offering new courses and upgrading programmes to attract a wider spectrum of students and researchers.

A number of reproducible protocols originated from tissue culture studies using different morphogenic pathways on plants belonging to different categories have been established for the multiplication and conservation of phytodiversity. In addition, evaluation on the effect of different light intensities on photosynthesis and antioxidant enzymes during acclimatization of in vitro regenerated plants has been carried out. Since the possibility of occurrence of genetic variation (somaclonal variation) during in vitro process cannot be ruled out, we have been focusing on enhanced axillary shoot proliferation which is least prone to somaclonal variation.

The group of Plant Biotechnology in the University, Botany Department, has made significant contributions towards the development and progress of tissue culture technology in the country for mass propagation and morphogenic studies on large number of plants, including recalcitrant species that are difficult to be propagated from seeds. The research team has made pioneer and excellent contribution towards the mass propagation of several ornamental, medicinal, fruit and woody trees including endangered plants.

The present communication describes various approaches of in vitro manipulation for plant regeneration in selected plants, with medicinal/economic importance. The results are of great practical significance for their mass propagation with conservation and have been published in journals of international repute.

1.2 *Salix alba* L.

Salix alba (*Salicaceae*) is a large tree with olive-green, yellow or purple branches frequently cultivated in Western Himalaya up to an altitude of 2400 m. It is vegetatively propagated by cutting during February to March and used mostly in post and planks, house building, packing boxes, furniture, agricultural implements, etc. The regeneration potential of various explants was evaluated by manipulating various culture conditions. Among the various treatments of different cytokinins (BA, Kin, 2iP) singly on woody plant medium, BA was found superior in comparison with others in nodal explant. However, amalgamation of auxins (IAA, IBA or NAA) with optimum doses of BA was superior in the production of a maximum of 12.77 shoots with 1.83 cm average shoot length induced from nodal explants.

The presence of various additives, viz. silver nitrate, glutamine, ammonium nitrate or adenine sulphate, favours the production of good-quality shoots. Among

all, 2.0 mg/l of $AgNO_3$ was found to be the optimum for proper growth and development of shoots.

In vitro isolated shoots from the cluster were transferred to the media containing different auxins (IAA, IBA or NAA) at various doses for root induction. Among the various treatments tested, 0.5 μM IBA was found best for highest root induction. Plantlets with proper root and shoot systems were acclimatized using standard procedure. All the regenerants were lastly shifted to pots in the net house where they grew well lacking any noticeable morphological dissimilarity. No somaclonal variation among regenerants was observed as confirmed by PCR-based DNA markers (Khan 2014).

1.3 *Erythrina variegata* L.

Erythrina variegata L. (*Fabaceae*) is highly medicinal and is being used in traditional medicinal system in various parts of Asia, for liver disorder treatment to leprosy. Its extracts have been reported to show hypoglycaemic, antidiuretic, antihyperlipidaemic and sedative properties. It is a salt and drought tolerant and even grows in waterlogged conditions, giving it the ability to survive even near the seashores. It also possesses aesthetic value because of its beautiful inflorescence and is in high demand in the international market. Its vegetative propagation is, however, limited because of the requirement of large cutting which is difficult to transport. Therefore, it is a very good candidate for in vitro and physiological studies which are not possible using conventional methods. For developing an efficient regeneration system, nodal explants were incubated on MS culture medium fortified with different doses and amalgamations of plant growth regulators (PGRs). Combination of 5.0 μM 6-benzylaminopurine (BA) and 0.5 μM 1-naphthaleneacetic acid (NAA) was found to be most effective and induced maximum number of shoots (~13) per explant with average (4.8 cm) mean shoot length in 93.6 % cultures. Addition of cobalt (≤50 μM) to the medium significantly enhanced the growth parameters of the culture, increasing the number of shoots to more than 16 shoots/explant after 8 weeks of culture on the standardized medium. Rooting in in vitro obtained shoots was proficiently induced on full-strength MS medium fortified with 2.5 μM indole-3-butyric acid (IBA) which yielded more than three roots/shoots with mean root length of 3.2 cm. The cultures transferred from the media supplemented with optimized cobalt concentration showed better rhizogenic competence as compared to the one transferred from medium lacking cobalt. Cobalt exposure increased the percentage (83.5 %) of explants showing root induction on the similar medium as compared to unexposed cultures (74 %). Genetic characterization of the regenerants was also done using PCR-based DNA markers, to ensure that cobalt exposure or the use of plant growth regulators in the protocol has not compromised the genetic integrity of the progeny plantlets. Screening of 570 bands produced by DNA-based ten selected ISSR primers did not record any polymorphism among the regenerants, establishing their clonal nature. Thus, the developed regeneration protocol for *Erythrina variegata* can be used for its propagation and conservation and in other in vitro manipulations for plants' improvement (Javed and Anis 2015).

1.4 *Withania somnifera* L. (Dunal)

Withania somnifera (winter cherry) commonly called as ashwagandha belongs to family *Solanaceae*. It is also known as Indian ginseng and considered as highly representative of plant kingdom in the Indian system of medicine. It is an erect greyish shrub growing up to the height of 75 cm. It is composed of about 12 alkaloids, 40 withanolides and several sitoindosides. The major constituents are mainly present in the leaves. The roots are also composed of glucose, starch, dulcitol, reducing sugar and withanol, and currently an estimation of withaferin A and withanolides D was reported by HPLC analysis (Ganzera et al. 2003).

It is commonly used in the Ayurvedic system of medicine and chiefly claimed to possess potent aphrodisiac rejuvenative and life-prolonging properties. It is also used as a memory enhancer and in other gastric problems (Williamson 2002). It also helps various ailments like chronic fatigue, weakness, teeth loose, impotency, dehydration, weakness in the bones, premature ageing, muscle tension and emaciation. Leaves and fruits are also helpful in various problems like tumour, carbuncle, ulcers and tubercular glands.

Roots are also useful in rheumatism, general ability, nervous exhaustion, memory loss, spermatorrhoea and constipation. In Ayurveda, the roots are also prescribed for gynaec disorders, bronchitis, inflammation, skin diseases, fever rheumatism, etc. (Fatima 2013).

It propagates through seeds, but seeds possess very short viability with low germination rate which resists its propagation via seeds even after stratification. Due to indiscriminate use and ruthless collection from the wild, the species is now getting the category of endangered.

Therefore, an efficient propagation system is necessary for the propagation and commercial utilization of important medicinal plant. In order to reduce the pressure on natural population and also to provide an alternative method for the production of planting material, the present experiments were taken into consideration in order to develop an efficient in vitro method which can be utilized for mass propagation of selected medicinal plant.

Direct shoot bud emergence was noticed on the Murashige and Skoog (1962) culture medium with the supplementation of different doses of various cytokinins (BA, Kin, 2iP) either single or in combination with different auxins (IAA, IBA or IAA) in nodal and shoot tip explants. However, both explants failed to show any micropropagation response on control MS medium devoid of growth regulators.

Among various treatments tried, maximum shoot regeneration was achieved on MS medium supplemented with BA (2.5 μM) and NAA (0.5 μM) in nodal segments. One hundred percent rooting frequency was observed in cultured shootlets on rooting media composed of NAA (0.5 μM), on one-half MS medium. Histological examinations also confirm the induction of various shoot primordia in both nodal and shoot tip explants. The obtained complete plantlets with rooted shoots were acclimatized with green house and transferred to natural light with 95 % survival rate (Fatima and Anis 2012).

In order to improve the regeneration potential, the effect of phenyl urea derivative, thidiazuron (TDZ), was also studied using nodal explants. MS culture medium containing TDZ (0.0–10.0 μM) was successful in producing shoot and retaining high shoot development rate and subsequent elongation on hormone-free MS medium. TDZ at a dose of 0.5 μM was mainly effective in bud break which formed optimum multiplication frequency (98 %) and a number of shoots (23.8 ± 0.33) with shoot length (4.83 ± 0.66 cm), after 4 weeks of culture. These induced shoots when transferred onto the MS medium lacking TDZ showed the greatest shoot number (32.4 ± 0.24) with shoot height (7.66 ± 0.08 cm) latterly of the fourth subculture passage. Among the various doses of IBA (50–500 μM) experienced for ex vitro rooting, the maximum percentage of rooting was obtained in Soilrite™ when the lower end of the isolated shootlets was employed with 200 μM (IBA) for 15 min, which induced maximum roots (18.3 ± 0.16) with root length of (7.63 + 0.08 cm) per shoot. After proper hardening in the plant growth chamber, the regenerated plantlets were transported to the net house where they grow well, reach maturity and show normal flowering (Fatima and Anis 2011).

The morphogenetic response of copper sulphate and zinc sulphate on nodal segment was also studied. Inclusion of micronutrients $CuSO_4$ (25–200 μM) and $ZnSO_4$ (50–500 μM) in an already standardized MS medium showed better response in shoot bud formation and lengthening. $ZnSO_4$ gives a better response in comparison with $CuSO_4$; about 61 and 66 shoots per explant were obtained with 100 μM $CuSO_4$ and 300 μM $ZnSO_4$, respectively. Rooting in micropropagated shoots was achieved on one-half MS + NAA (0.5 μM). Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content in the micropropagated plants increase with the increasing copper and zinc concentration up to optimum dose of 100 and 300 μM of $CuSO_4$ and $ZnSO_4$, respectively, in the medium. Micropropagated plantlets were hardened by the following standard procedure with 95 % survival rate. All the regenerated plants were morphologically similar (Fatima et al. 2011).

Non-embryogenic, synthetic seeds were produced by encapsulating nodal segments (containing axillary buds) of *Withania somnifera* L. in calcium alginate hydrogel comprising MS culture medium. A 3% sodium alginate with 100 μM calcium chloride $CaCl_2$ was found to be the optimum concentration for the creation of consistent syn seeds. The effect of different treatments, i.e. MS medium containing different doses of cytokinins (0.5, 1.0, 2.5, 5.0 and 10.0 μM) along with optimum dose of auxins NAA (0.5) on in vitro regeneration response of synthetic seeds, was assessed. The optimum percentage (86.2 %) of the transformation of calcium alginate-coated nodal segments into plantlets was obtained on MS medium composed of BA (2.5 μM) and NAA (0.5 μM) after 4 weeks of incubation. Rooted plantlets were achieved on one-half MS supplemented with 0.5 μM NAA. Plantlets obtained from stored synthetic seeds were hardened accordingly. Significant enhancement in the pigment contents (chlorophyll, carotenoids) and net photosynthetic rates with an increase in acclimatization days may be due to the proper working of photosynthetic machinery. Activities of antioxidant enzymes, i.e. superoxide dismutase, catalase and peroxidase, were significantly increased which suggest their preventive role in membrane oxidation and damage to biological molecules.

Also, an enhanced level of lipid peroxidation, as indicated by MDA content, is a sensitive diagnostic index of oxidative injury, clearly indicating its positive determining role in combating oxidative stress. The generated RAPD and ISSR patterns from regenerated plantlets with the mother plant were similar which confirms the genetic stability among the clones. The synthetic seed technology could possibly pave the way for the conservation, short-term storage and germplasm exchange with potential storability and limited quarantine restrictions (Fatima et al. 2013).

1.5 *Cuphea procumbens* Orteg.

The stimulatory effect of different doses of three cytokinins, BA, Kin and 2iP, on in vitro shoot bud induction, proliferation and multiplication of a potential medicinal herb *Cuphea procumbens* was investigated. Young nodal explant excised from 15 days old using cotyledonary node explants excised from 15-day-old sterilized seedlings and multiple shoots were induced on MS medium augmented with different doses of cytokinins. Maximum shoot regeneration frequency (70 %), mean number (9.33) of shoots per explant and the highest shoot length (4.16 cm) were obtained on MS medium enriched with 2.5 μM BA along with 0.5 μM NAA after 4 weeks of incubation. The addition of 200 mg/l casein hydrolysate to the standardized medium increases regenerants' growth. Microshoots of 4 cm length were successfully rooted on one-half MS medium supplemented with different concentrations of IBA. The in vitro raised healthy plantlets with properly developed roots and shoots were acclimatized and maintained in the net house with 80 % survival. Random amplified polymorphic DNA (RAPD) marker analysis of ten randomly selected in vitro raised plantlets confirms their genetic fidelity with the mother plant. The results suggested that the culture environments used for explant multiplying are suitable for clonal propagation of the selected remedial plant as these do not seem to hinder with genetic integrity of regenerants. High multiplication rate associated with observed genetic stability clearly indicates the efficacy of the present in vitro clonal propagation protocol of their valuable plants of high commercial value (Fatima et al. 2012).

1.6 *Syzygium cumini* L.

Syzygium cumini (*Myrtaceae*) is a large evergreen tree, native to the Indian subcontinent and adjoining regions of Southeast Asia. It possesses antioxidant, antimicrobial, anti-inflammatory and antiamnesic activities and is used in various neurological disorders. The fruit has various promising therapeutic values (antidiabetic properties) with various phytoconstituents, such as tannins, alkaloids, steroids, flavonoids, terpenoids, fatty acid and vitamins. The plant is propagated with seeds and vegetative methods. Vegetative methods are less effective and seed propagation may result in genetic variation. The seeds have short dormancy period and lose viability after maturation. A study was conducted to evaluate the effect of metatopolin (an aromatic cytokinin) at different concentrations with IAA, IBA or NAA on MS medium.

Among the various concentrations (0.5–10.0 μM) tested, 5.0 μM was found to be the optimum single treatment. However, the mean number of shoots per explant increased considerably when the combination of optimum metatopolin with different auxins was tried. Among all the tested concentrations, metatopolin (5.0 μM) + NAA (2.0 μM) proved to be the best treatment for induction of maximum shoots (25.37) with shoot length (6.54 cm) per explant (Naaz et al. 2014).

For rooting, isolated shoots (4 cm) from clumps were excised and transferred to the rooting medium containing various concentrations of IBA and NAA on full- or half-strength MS medium. Of the different treatments evaluated, the best rooting response (85%) with average root number (6.33) and root length (7.13 cm) was observed on half-strength MS medium containing NAA (5.0 μM). Properly rooted plantlets with four to five fully expanded leaves were successfully hardened off in growth room and finally to the normal environmental conditions. No detectable variation among the potted plants in respect to morphological and growth characteristics was observed. The genetic integrity among regenerants was also confirmed by using PCR-based DNA markers (RAPD/ISSR) (Naaz 2015).

1.7 *Albizia lebbeck* L. (Benth.)

Albizia lebbeck (*Fabaceae*) is indigenous to tropical Southern Asia and found mainly in India, Australia, Bangladesh, etc. It is a deciduous, hermaphrodite woody tree, attaining a height of 30 m. It is used to treat boils, cough, eye flu, gingivitis, lung problem and abdominal tumours. In addition, antiprotozoal, hypoglycaemic, anticancer and analgesic properties have also been reported.

Conventionally, it is propagated through seeds or microcuttings. Propagation through seeds is not useful due to the long seed dormancy. Moreover, the progeny from seeds is not homogeneous. Therefore, tissue culture technique is applied for propagation which provides an alternative for mass production of plants with uniform characteristics.

The manipulations of various culture conductions were carried out for in vitro production of plantlets from different explants, viz. seedling-derived cotyledonary node, node, cotyledon, hypocotyls, root and mature nodal explants. Among the various experiments carried out, hypocotyl explants excised from 15-day-old aseptic seedling produced an optimal shoot regeneration frequency (81%) and number (22) of shoots on MS medium supplied with 7.5 μM BA after 4 weeks of incubation.

Further, excellent response in shoot multiplication was recorded when shoot clusters were subcultured to a medium augmented with 7.5 μM BA and 0.5 μM NAA, producing highest number of shoots (34) per hypocotyl explant with mean shoot length of 6.3 cm after 8 weeks of culture.

Adventitious root induction in in vitro isolated shoots was readily achieved with various auxins (IAA, IBA or NAA) at different concentrations. The maximum root regeneration frequency was achieved on MS medium supplemented with IBA (2.0 μM) which produced an average of 5.2 roots with mean root length (4.4 cm) per shootlet. The micropropagated plantlets were acclimatized in soil with 80%

survival rate. Various physiological parameters during hardening were also evaluated. The estimation of photosynthetic pigments and antioxidant enzyme analysis has been an important parameter in determining the ability of the plants to survive oxidative stress and played an important role for better adaptation of regenerated plantlets transferred from in vitro to ex vitro environment (Perveen 2013).

1.8 *Acacia gerrardii* (Benth.)

Acacia gerrardii (*Fabaceae*) is a small-size tree legume commonly available in arid river valleys. Its unusual papery bark, ample shade and spring flowers make an excellent tree to mix with traditional and landscape species. Generally, it is propagated through seeds but seeds are recalcitrant with short seed viability. Vegetative propagation method through cutting is rather slow, and a good number of cuttings died during transportation and plantation. Various treatments containing cytokinins (BA/Kin) at different concentrations either single or in augmentation with auxins (IAA, IBA or NAA) have been evaluated on MS medium in order to establish an in vitro method for its propagation from CN explants excised from aseptically raised seedlings. The explants failed to show any response on control MS medium devoid of plant growth regulators. However, the addition of cytokinin helped in shoot bud induction in CN explants. Among all the concentrations tested, 5.0 μM BA exhibited 5.5 shoot per explant in 90 % cultures. Kin (5.0 μM) was found to be least effective. A combination of auxin and cytokinin showed synergism in shoot bud induction and proliferation. The combined effect of BA (5.0 μM) and NAA (0.5 μM) resulted in an increase in shoot number (Varshney et al. 2013).

The regenerated shootlets were rooted well in vitro on a medium containing full-strength MS salts and IBA (2.0 μM). The in vitro regenerated plantlets were successfully acclimatized and established in normal garden soil under full sun with 70 % survival rate.

1.9 Current Status of Plant Tissue Culture Commercialization in India

The response of various explants from different genotypes to different plant growth regulators clearly shows where tissue culture is today and where it is heading as an equal partner with molecular biology, as a tool in basic plant biology and in various other areas of application. Knowledge of tissue culture has contributed greatly to our understanding of the factors responsible for growth, metabolism, differentiation and morphogenesis of plant cells. The techniques of plant tissue culture have been employed as an important aid to conventional methods of plant improvement. These have been used as a tool for the propagation of genetically manipulated superior clones and for ex situ conservation of valuable germplasm.

In recent years, there has been an explosion in the number of commercial plant tissue culture units in India. Till date, 95 commercial tissue culture production units

have been recognized by the Department of Biotechnology, Government of India under the National Certification System for tissue culture-raised plants (NCS-TCP 2016). The potential for the domestic market is enormous, and by conservative estimates, it is around Rs. 200 crores with an annual growth rate of 20 %. The production capacity of commercial tissue culture units ranges between 0.5 million and 10 million plants per annum with an aggregate production capacity of about 200 million plantlets per year.

Micropropagation industry in India is providing major support to the Indian agriculture in four crop groups: fruits, ornamentals, spices and forestry/plantation crop. Banana is the largest selling tissue culture food crop. TC papaya plants are now marketed for extraction and processing of papain. TC anthuriums, orchids and gerberas have attained commercial importance. TC rose plants are used as pot plants. Nearly 500 ha are under tissue culture cardamom cultivation in Southern India recording 20–30 % increase in yield. Vanilla cultivation is expected to increase from the existing 50 ha to more than 400 ha in the coming years using TC plants. Sugar companies have in-house units of micropropagation of sugarcane. 'Jain Tissue Culture', working since 1995 for propagation and supply of 'Tissue Culture Planting Material' in the country, is the biggest laboratory for banana, pomegranate and strawberry in the world. There is a growing demand for bamboo and eucalyptus for selective reforestation. Thus, from few research laboratories several years ago, tissue culture is rapidly becoming a commercial industry in the country. Today, micropropagation and in vitro conservation have been standardized for various plant species. It no longer remains an empirical science and is now being employed in studying intricate pathways of plant metabolites and molecular genomics of plants.

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2 Selection of Elites and In Vitro Propagation of Selected High-Value Himalayan Medicinal Herbs for Sustainable Utilization and Conservation

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Abstract

Reduction in the forest cover from the Indian Himalayan region (IHR), due to overexploitation, has resulted in decreased availability of non-timber forest products, including medicinal plants of high economic value. With the ever-increasing human population and growing demand for plants and plant-derived products,

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M. Anis, N. Ahmad (eds.), *Plant Tissue Culture: Propagation, Conservation and Crop Improvement*, DOI 10.1007/978-981-10-1917-3_2

there has been tremendous anthropogenic pressure on these primary producers. Many plant species are a source of high-value drugs; due to increasing global demand for the 'naturals', they are being subjected to reckless, often illegal harvesting, well beyond the natural regeneration capacity. This has led to many species being listed in the Red Data Book or in various IUCN threat categories. Improper harvesting (season and/or age of the plant/plant parts) not only results in uneconomical yields due to low content of active ingredients but also adversely affects the process of natural regeneration. There is, therefore, an urgent need for commercially important species to be subjected to improved management practices and regulated harvesting to generate better economic benefits on one hand and to encourage cultivation for sustained utilization as well as economic development of the region on the other. This twin strategy would also help to improve the conservation status of such species.

In order to meet such challenges, in vitro propagation (tissue culture) techniques have provided a well-recognized potential for rapid multiplication of elite clones for the supply of much needed good-quality planting material for cultivation and also to achieve conservation objectives. Keeping these goals in mind, studies were taken up to assess the active ingredient content of plants/plant parts collected from natural populations growing in different locations/altitudes in the wild and to develop in vitro propagation methods for selected high-value alpine medicinal herbs (*Aconitum balfourii*, *A. heterophyllum*, *Picrorhiza kurrooa* and *Podophyllum hexandrum*). Using elite plant material, attempts have been made to establish tissue culture protocols that involved the induction of multiple shoots, improved rooting and subsequent development of suitable methods for hardening and field transfer. In a few cases, the survival and growth of tissue culture-raised (TCR) plants was also monitored to evaluate their field performance.

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
IHR	Indian Himalayan region
GA_3	Gibberellic acid
Kn	Kinetin
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
PGS	Plant growth substance
SR	Seed raised
TCR	Tissue culture raised
TDZ	(Thidiazuron): 1-phenyl-3 (1,2,3-thiadiazol-5-yl) urea

2.1 Introduction

The forest cover from the Indian Himalayan region (IHR) has been substantially reduced over the years, and it varies from 10.14% (Jammu and Kashmir) to 90.38% (Mizoram) across IHR states (Anonymous 2013). The recommended cover of 67% and above is not present in many of the Himalayan states. This has adversely affected the availability of non-timber forest products, including medicinal plants of high commercial and therapeutic value. The increasing human population and the growing demand for plants and plant-based products have collectively placed very high anthropogenic pressure on these primary producers. Many plant species are known sources of high-value drugs, and due to the increasing global demand for the 'naturals', they are being subjected to reckless, often illegal harvesting, well beyond their natural regenerative capacity. This has led to many species being listed in the Red Data Book and/or in various threat categories (Nandi et al. 2002; Anonymous 2003; Ved et al. 2003).

The life and economy of the hill people, to a large extent, depend on the plants, and thus any reduction in the forest cover does have a great negative effect on natural resources including their living conditions. Moreover, improper harvesting (season and/or age of the plant/plant parts) results in uneconomical yields due to the suboptimal content of active ingredients and also adversely affects the process of natural regeneration. There is, therefore, an urgent need for all such commercially important species to be subjected to improved management practices and regulated harvesting to generate improved long-term economic benefits on one hand and to encourage their cultivation for sustained utilization as well as economic development of the region on the other. Keeping these goals in mind, studies were taken up to assess the active ingredient content of plants/plant parts collected from natural populations of selected medicinal plants growing in different locations/altitudes in the wild and to develop in vitro propagation (tissue culture based) methods for these high-value alpine medicinal herbs (*Aconitum balfourii*, *A. heterophyllum*, *Picrorhiza kurrooa* and *Podophyllum hexandrum*). Using elite (in terms of high active principle content) plant material, attempts have been made to establish their in vitro (tissue) cultures, induce multiple shoots, improve rooting of shoots and subsequently develop suitable methods for hardening before field transfer. In a few cases, the survival and growth of in vitro-raised (IVR) plants was also monitored to evaluate field performance.

A brief description of all four species selected (Fig. 2.1) for in vitro propagation has been provided below.

Aconitum balfourii Stapf. [=*A. atrox* (Brhul) Muk.; family, Ranunculaceae; English name, aconite; local names, 'Meetha' and 'Bish'] is a highly valued medicinal herb endemic to the alpine and subalpine belts of the IHR and grows above 3200 m altitude (Samant et al. 1998). Its tuberous roots are used by various ethnic communities for curing different ailments (rheumatism, fever, etc.) and are important source of ingredients used in the preparation of Indian Ayurvedic medicines (Chopra et al. 1984; Anonymous 1988). The medicinal properties have been attributed to several diterpenoid alkaloids, mainly aconitine, balfourine, bikhaconitine and pseudaconitine, the latter being highly toxic and biologically 2.5 times more active than aconitine (Chopra et al. 1984; Khetwal et al. 1992).