In Vitro Propagation of Two Economically Important Plants: *Actinidia deliciosa* A. Chev. (Actinidiaceae) and *Saurauia punduana* Wallich (Actinidiaceae)

By

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Contents

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DECLARATION

I, Mr. P. Kadunlung Gangmei bearing Ph. D. Registration No. 475/2012 dated October 14, 2011 hereby declare that, the subject matter of my thesis entitled *'In vitro* propagation of two economically important plants: *Actinidia deliciosa* A. Chev. (Actinidiaceae) and *Saurauia punduana* Wallich (Actinidiaceae)' is the record of original work done by me, and that the contents of this thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. This thesis has not been submitted by me for any Research Degree in any other University/Institute.

This thesis is being submitted to the Nagaland University for the Degree of 'Doctor of Philosophy in Botany'.

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Chapter – 1

Introduction

Earth has a unique form, where life existed since more than two and half billion years ago. During this period life forms have changed from simple, unicellular organism, to the present day multi-cellular organisms, perhaps through long evolutionary trend by natural selection. Plants, animals and the microbes are the main domains of the earth found living as individuals, populations and community in the ecosystem of biosphere almost in all the sphere of planet earth. Thus the variability among living organisms from all habitats such as terrestrial, aerial, marine and other aquatic ecosystems and the ecological complexes which includes genetic diversity, species diversity and of ecosystems diversity. Of these, plant is one, found equally diverse in their geographical distribution and its organization, which makes the men depend directly or indirectly with significant complexity manifested in supporting of life as sources of food, cloths, houses, medicines, fuel, decorative articles, recreation and even the remarkable intellectual and

spiritual inspirations, which influence to lead mankind diverse in traditional and cultural practices.

The account of living organisms in the world is approximately 15 million, comprises of plants, microbes and animals. Of which about 1.9 millions of species of varied forms have been identified (Chivian and Bernstein, 2010). These plant and animal species, their products are of immense important where few hundred of species were agriculturally employed in food industry. However, about twelve plant species serves nearly 75% of total food supply and 90% of global domestic livestock production, from fifteen mammals and bird's species. These diverse forms of life are also in the course of extinction where it is estimated that 15 species have became extinct since the inception of men on planet Earth. The diverse forms so also the ways of uses serves benefits in growth and development contribute to men's society. Thus complex relationships of dependency form the services, where some society of mankind highly valued while others not. It involves the direct provision on material and non-material goods provide by directly with the presence of particular species of plants, animals or micro organisms, and also always been an integral part of long human experiences and moral conduct (Daiz *et. al*., 2006). Man's life become more comfortable and beauties than ever since with the use of plants species, remarkably by the rapid scientific and technological advancement exploitation, perhaps leads to decline their vast world at very fast rate to under various threats and restrictions.

Man brought about habitat destruction, environmental pollution, excessive exploitation of economically important species, introduction of invasive alien species; climate change etc increases with the ages for its own benefits rather response passively to other living forms which are actively involves in Earth's life-support systems. Biodiversity is sensitive to global changes which effect the interactions among every

ecosystem and in turn its services to men are losing at high. Study suggests that three main drivers (climate change, land-use pattern and nitrogen deposition) affects the organisms, particularly vascular plant species at larger scales, where their relative abundance may decrease by 12–16% by 2050 relative to species present in 1970 (Sala *et. al*., 2005). It was reported that between 1990 and 2010, global natural forest area estimated decline 3.4 percent area (1.4 million square kilometers) roughly the size of Mexico (Normander, 2012). It also reported that the global forest areas decrease roughly half over the past three centuries and 25 nations virtually lost their forest cover and 29 countries with more than nine-tenths of their forest cover. Drastically, tropical forests are disappearing at 130 000 km^2 per year which mostly through expansion for agricultural land annually by an average of 5 million ha/year between 2000 and 2010 (Fischer *et. al*., 2002). In the world's rich biodiversity of central Amazonia, inhabitant by Waimiri-Atroari Indians use 79% of tree species from single 1 ha of forest plot (Milliken, 1998). Similarly, 1748 of the ~8000 angiosperm species of Himalayan region were used as medicine and for other purposes (Samant *et. al*., 2011). In large areas of protected tropical forest land many illegal logging, hunting, poaching and agricultural extension etc. is still prevalent (Normander, 2012). Thus, for future the main drivers require an effective integrated effort of measures to improve ecosystem services at both global and regional levels by climatologists, ecologists, social scientists, and policy makers (Sala *et. al*., 2000).

IUCN enlisted 34 biodiversity hotspot regions in the world, of which 13 are in Asian-pacific. Of the 34 biodiversity hotspots, India is the home of four of them viz. the Himalaya, the Indo-Burma region, the Western Ghats and the Sunderland. The hotspots' boundaries have been determined by `biological commonalities'. Each of the areas features a separate biota or community of species that fits together as a bio-geographical

unit. According to IUCN, hotspots are geographically rich in species with high endemism, and are under constant risk (threat of extinction). The region must contain at least 1,500 species of vascular plants (> 0.5 percent of the world's total) as endemics to that region and has to have lost at least 70 percent of its original habitat. Anthropogenic threats include habitat loss, overexploitation for subsistence or commercial use and introduction of exotic species. However, this various threats expanded the understanding of the distribution of species diversity and the phenomena of speciation and extinction as from the study of island biogeography and population biology (Sodhi and Ehrlich, 2010). Thus IUCN Red List Data serves a significant role in determining the positive steps concerning on conservation where by many workers remarks its importance (Hayward, 2011).

With the increasing human population, exploration on plant community keeps men broader their knowledge in manipulating plants inherent potentiality, couples with increase in production demand that never end rather keep adding. As reported by the UNPD 2015 Report, the world had 2.5 billion populations in 1950 and almost tripled in 2005 with 6.5 billion people, while in 2015 increase to 7.3 billion which further projected rise to more than 9.7 billion by 2050. The world's human population exponential growth and spatial expansion brought about the changes of land use pattern, pollution, and overexploitation of natural resources which in turn endangered, loss of species and fluctuation of ecosystem functionality. Kleijn *et. al*. (2006) reported that the main drivers of land use is through agricultural practices and urbanization expansion which threats to terrestrial and aquatic biodiversity causes habitat fragmentation and destruction in protected and unprotected areas as well.

Another integral part of population explosion is migration, where there will be nearly 2 billion new urban residents by 2030, reflects further habitat destruction with

various means of pollution on environment. Thus world demographic change is likely to unfold in post-2015 population development pressurize on different types of disturbances, leads to climate change, global warming and so forth. Climate change and global warming drive extinction of many plant pollinators causes loss of genetic diversity, habitat fragmentation in local and global forest and even in the biodiversity hotspots (Ngezahayo and Liu, 2014; Evju *et. al*., 2015). However, the same degree of negative impact turns on humanity in terms of ecosystem services (Cardenale *et. al*., 2012). Discussions and case studies of several researchers focused on various challenges, including spatial and temporal scale, predictive climate, land use, soil type and topography, were ensemble of global climate and bioclimatic models results demographic structures which are realistic and species-specific but still that makes species vulnerable to climate change. The main cause of loss of biodiversity is through intensive agricultural extension which is a global problem attracted attention worldwide, with increase greater danger in species rich areas (Weibull *et. al*., 2003). Many species are remains in small size populations, at the most threatened which are the highest importance for conservational aspect. Therefore, idea of conservation management is urgent from habitat destruction, overexploitation, introduction of alien species and diseases, pollution and global climate change. IUCN purposely developed the most comprehensive dataset on conservation status 'Red List Data' to assess extinction risk of species (Rodrigues *et. al*., 2006; Hoffmann *et. al*., 2010). The data serves useful in conservation planning, policy and management, prioritizing sites, biodiversity evaluation and monitoring etc. Extensive pragmatic works of determining and evaluating of plant species under threaten were demonstrated by many workers (Borkowski and Podlaski, 2011; Fordham *et. al*., 2012). Today, no doubt the impact of human population on nature is big, leads to drastic loss of biodiversity (Sala *et. al*., 2000; Pringle *et. al*., 2015). The

biotic degradation brings concerns to authorities and environmentalists worldwide, subjected to increased human activities impact on natural ecosystems, disturbing the structural and functional natural system causing loss and changes to biodiversity components (Alho and Sabino, 2011). Thus importance of biodiversity to human well-being alerted the need for conservation and rational use of resources for sustainability which comes with unconditional.

India comes under the realms of 17 mega diversity of the world, geographically an area of 3,287,263 sq. km, where the mainland extended between 8°4'-37°6' N latitude and 68°7'-97°25' E longitude, home for many endemic species accounting to one-third of world flowering plants, confined in 2.4% of world's total land. The endemism of Indian flora is 1.5%, where Himalaya shared 3165 species, peninsular region with 2045 species. The diversity of the region manifested where large number of plants of other countries are found. Among plants, species endemism is estimated at 33% with ca. 140 endemic genera but no endemic families. Hooker (1875) described five elements of Indian floristic diversity-The Malaysian, The European-Oriental, The African, The Tibeto-Siberian and The Chino-Japanese elements. Due to rich floristic region Takhtajan (1969) (International Centre for Integrated Mountain Development, report) named '*The Cradle of Flowering Plants*'. India lies in subtropical region of northern hemisphere, where climate heterogeneity ranging from dense tropical rain forest of Andaman, Eastern Himalayas, arid hot tropical desert of Rajasthan, hot humid plain of South-Central Indian plateau of Andhra Pradesh, Maharashtra, to temperate and cold climate of northern mountainous region of Himalaya, Himachal, Jammu and Kashmir. North-Eastern India, under Indo-Burma region is one of the *hot-spot*, having diverse vegetations such as grassland, swamp, mixed deciduous forest, tropical forest, temperate forest and alpine vegetation. India harbor many economical important plant species medicinal,

horticultural, spices, ornamental, and timbers of immense valued. About endowed 17,000 species of angiosperms, of which 20% of 20,000 global medicinal plants are found in India (Laloo *et. al.*, 2006; Rai and Lalramnghinglova, 2011). The country also has long famous age old medicinal practices '*Ayurvedic System of Medicine*' still prevail today mostly among the tribal communities. Plant community dynamics and species diversity is an important aspect of forest ecology which entails contribution of various species in determining structure of specific habitats (Kumar and Bhatt, 2006). Studies on distribution and diversity pattern of plant communities reveals their structure but way to identify by their distribution, characterization, identification, classification and their utility is a challenging work Bhatt, 1993 (*Studies on the Flora of Western Kachchh*). Many horticultural important species like mango, banana, apple, jackfruit, gooseberry, oranges are found in abundance. The major horticultural crops producing state in India are Gujarat, Haryana, West Bengal, Andhra Pradesh, Kerala, and Maharashtra. Moreover, India is the largest mango growing and export country in the world.

North Eastern region of India harbor large number of plant species, mentioned in the manual of Indian Forestry (Bor, 1940). The region support luxuriant growth of many medicinal, ornamental, spices, timbers and horticultural important plant species in wild accounting to almost half of India's biodiversity (Mao *et. al.*, 2009). The region comes under eastern Himalaya and Indo-Burma, forms a distinct floristic region where it is also the meeting ground of Indo-Malayan, Afro-tropic, Indo-Chinese, the Himalayan and Peninsular Indian elements which formed when the peninsular plate struck against the Asian landmass, after it broke off from Gondwana land. Large number of angiosperm plants particularly horticultural plants like Gooseberry, Mango, Cherry, Kiwi, *Saurauia*, Guava, Passion fruit, Oranges, Pineapple, and Jackfruit etc are found growing in wild and domesticated as well.

Geographically, North Eastern region of India covered an area of 2, 62,000 sq. km which extended between 21°34´-29°50´N latitude and 87°32'-97°52´E longitude, where the region are predominated by the tribal community with varied cultural and traditional practices. Agriculture is the main occupation of the region, where tribal practices of agriculture like '*Slash and Burn'*/Jhum cultivation and terrace cultivation are found, with much dependency on forest products like wild edible leaves, fruits, medicines etc are part of the community activities. Other developmental activities like village establishment, industrial set up and many unplanned human activities leads to fragmentation in many parts of forest in many areas. Thus large areas of natural vegetation is under great threat or even extinct from its existence. Large areas of forest cover have many endemic species with much restriction, and often the most vulnerable to human disturbances particularly 'Jhum cultivation'. These species in the field margins are the most vulnerable and the first to be hit by extinction processes, and hence need rapid and effective conservation action. Similarly Brook *et. al*. (2008) suggested that presence of rare and threatened species makes habitat quality and serves as an indicator for conservation priorities therefore such sites with such range of threaten specie receive more attention than sites with common species dominant. Need arises to address the species under varied threats, identifying their crucial issues for further studies and follow-up action, among which, species from Actinidiaceae are one of them. In the region, the members of the family Actinidiaceae are found in the wild, which also been reported in the 'Flora of British India' by Hooker (1875). These species are horticultural important and are one of the endemic to the region, enlisted with threatened in the wild. Immediate intervention of advance techniques of germplasm employed in various scientific experimental and research projects could ensure to save the risk from endangered. Advance technique of plant propagation like *in vitro* technique would help

in conservation of economically important and threatened species as shown from the works of (Temjensangba and Deb, 2005; Yadav *et. al*., 2012; Deb and Pongener, 2013). Therefore, vegetative propagation of these threatened species through *in vitro* technique could substantiate its quantity in short period of time.

Since visionary researched of Haberlandt (1902) with the discovery of plant hormones (auxins in 1930s and cytokinins 1950s) and synthetic plant growth regulators and applicable nutrient media formulations in the 1960s, made revolutionaries in 'Plant Tissue Culture Technique'. Winkelmann *et. al*. (2015) listed scientific applications of the techniques, their importance in plant breeding, molecular biology, research tools in botany, pharmacology and secondary metabolite production, phyto-pathology, and vegetative propagation. Initially the technique aims to use for research purposes, but commercial utilization followed soon, particularly in floricultural and horticultural crops. *In vitro* culture techniques enable to produce large scale of planting materials on small area under controlled conditions and free from microorganisms, which are more vigorous than those propagated by conventional methods. Plant tissue culture areas ranges from micropropagation of ornamental and forest trees, production of pharmaceutically interesting compounds, and plant breeding for improvement of nutritional value of staple crop plants, including cryopreservation of valuable germplasm. All biotechnological approaches like genetic engineering, haploid induction, or somaclonal variation to improve traits strongly depend on an efficient *in vitro* plant regeneration system.

Quality control is an essential to assure high quality plant production, where selection of explants source, time of growth etc to produce diseases free material, authenticity of variety, elimination of somaclonal variants are some of the critical parameters. The rapid production of high quality, disease free and uniform planting stock is only possible through micropropagation. New opportunities has been created for

producers, farmers and nursery owners for high quality planting materials of fruits, ornamentals, forest tree species and vegetables. Since last two decades, efforts made in the use of plant cell cultures in bio-production, bioconversion or biotransformation and biosynthetic studies were intensive. The potential commercial production of fruits, pharmaceuticals products have grown large with much care on environmental impact in the cell culture industry. The technique is a noble approach obtaining substances in large scale, perhaps the most significant role to play in the future with transgenic plants. The ability to accelerate multiplication is of great benefit to mankind. Another scale on *in vitro* storage and cryopreservation are being progress to resolve the problems inherent in field gene banks by securing duplicate collection. These are the means the future generations relies, in place of simple conventional propagation programmes. They play significant role in agricultural development and productivity through rapid growth and propagation and also means in recovery and maintenance of vegetation restoration programmes.

The transgenic fruit plants and their commercialization show obstacles in many areas particularly in both regulatory and social hurdles (Rai and Shekhawat, 2014). Moreover, micropropagation technology is expensive as compared to conventional methods of propagation by means of seed, cuttings and grafting etc. Therefore it is essential to adopt measures to reduce cost of production, effective practices and optimal use of equipment. This can be achieved by improving the process efficiency and better utilization of resources such as substratum employed (Ogero *et. al.*, 2012). The bioreactor based plant propagation increase the speed of multiplication and growth of cultures with much reduce space, energy and labor requirements. However, needs special care and handling, which is comparatively sophisticated method. In fact, *in vitro* culture is high costs equipments with manual works, needs special treatments for

acclimatization, risks of somaclonal variation and physiological aberrations (e.g. hyperhydricity) etc which result high price to produce propagules compared to traditionally multiplied plants. Thus the techniques bring together breeders and growers of ornamental plants, tissue culturists, molecular biologists to discuss problems of past and current bottlenecks.

Commercial micropropagation expanded in the 1980s and 1990s. Orchids is the first to grows in commercial scale, and large number of lower wages orchids produced in South East Asian countries mainly Thailand (Gavinlertvatana and Prutpongse, 1991) and many other economical important plants like *Rosa* sps., *Prunus sp*, *Clematis*, Kalmia, but problems occurred on quality of plants, timing of production, and the mixing of cultivars. There are two broad culture methods, on solidified or liquid medium; in liquid medium either permanently immersed systems or temporary immersion systems employed (Etienne and Berthouly, 2002). The method offers the advantages of rapid growth and propagation, to scaling-up and automation.

Ornamental, timbers, spices and many other important plants species are micro propagated *in vitro* by using various explants such as shoot meristem (Kadhimi *et. al*., 2014; Kanwar and Kumar, 2009; Janarthanam and Seshadri, 2008; Medina *et. al*., 2009) etc. for commercial as well as conservation aims. However, axenic cultures and regenerative potential of pseudo bulbs as explants has been less explored as compared to the other explants (Kanjilal *et. al*., 1999; Sunitibala and Kishor, 2009; Kaur and Bhutani, 2010; Hong *et. al*., 2010; Kumar *et. al*., 2011; Naik and Naik, 2011; Niknejad *et. al*., 2011; Baskaran *et. al*., 2013), leaf explants (Ishii *et. al*., 1998; Anike *et. al*., 2012; Kumar *et. al*., 2015)

Gomes and Canhoto (2003) suggested that the position of micropropagation is best option when it is difficult to attain spread over several conventional approaches,

persistence rejuvenation difficulties and under required of growth rate. Janarthanam and Samathi (2010) propagated through apical shoot as in *Exacum travancoricum*. And among several methods of micropropagation, axillary shoot proliferation considered as the most favored technique as reported in Ericaceae clonal propagation (Jain and Häggman, 2007).

Some of the successful propagation and regeneration of plants through somatic embryogenesis are reported in Manchurian ash *Fraxinus mandshurica* Rupr (Kong *et. al*., 2012; Yang *et. al*., 2013a); in *Pinus pinaster* (Huma´nez *et. al*., 2012). Direct organogenesis using hypocotyl segments and seeds as explants has been reported in many other species (Basalma *et. al*., 2008; Ghnaya *et. al*., 2008; Rattana *et. al*., 2012). Explants used in direct organogenesis is important in efficient regeneration system (Babaoglu and Yorgancilar, 2000; Koroch *et. al*., 2002; Raghu *et. al*., 2006; Uranbey, 2010). Besides, many other plant species were propagated like *Acacia mangium* Willd., *Cedrus*, *Eucalyptus*, tea, *Curcuma longa* L., *Rosa rugosa* Thunb., *Curcuma amada* Roxb., *Alpinia galangal* Linn., *Cannabis sativa* L., hops, *Allium ampeloprasum* L., olive, *Pinus thunbergii* Parl., *Piper longum* L., barley, rice, *Dendrobium longicornu* Lindl., and *Mahonia leschenaultii* Nutt. So far, tissue culture studies in *Curcuma amada* are restricted to adventitious plantlet formation from rhizome and leaf sheath explants (Prakash *et. al*., 2004; Das *et. al*., 2010). Efficient regeneration through node has been done as in *Withania somnifera* (Kumar *et. al*., 2011).

For more than half century, in many parts of the world, focus was on horticultural crops through axillary bud culture adopted for the mass micropropagation from various plant parts. Successful micropropagation has been reported as in Guava, *Zizyphus mauritiana* (Kakon *et. al*., 2008; Abbas *et. al*., 2014). *In vitro* regeneration in *Passiflora foetida*, *Pyrus communis* (Rosa and Dornelas, 2012; Yousefiara *et. al*., 2014), in

blueberry (Guang-Jie *et. al*., 2008), shoot multiplication through subculture in rootstocks of cherry (Vujović *et. al*., 2012), from shoot tips in banana (Bhonsale *et. al*., 2011) from young and mature leaves of mangosteen (Qosim *et. al*., 2013), induction of somatic embryogenesis in date palm (Almusawi *et. al*., 2015; Al-Samir *et. al*., 2015).

Micropropagation starts with the selection of plant tissues/explants from a healthy, vigorous mother or donor plant, where any part of the plant (leaf, apical meristem, bud and root) can be used as explants to obtain plants with uniformity in their genetically resources and particular to vegetative propagated crops which prove ineffective propagation by conventional methods, and plants which do not produce fertile seeds. Genetic engineering proved effective on crops improvement in number of species, developed varieties with high yield potential and resistance against pests, drought and many other diseases as in apple (Braniste *et. al*., 2008; Blažek and Krelinová, 2011). Thus, the technology relies on plant tissue culture and molecular biology for the following aspects: production of disease-free plants (virus), production of secondary metabolites and production of varieties tolerant to salinity, drought and heat stresses. Many horticultural important plant species were preserving through cryopreservation technique as in cherry rootstock, strawberry etc.

Plant tissue culture technique has great impact on agriculture, industry and conservation strategy. It is an indispensable tool and practices at a rate without precedent in the production and propagation of genetically homogeneous, disease-free plant material in large scale as in blackberry through thermotherapy (Cheong *et. al*., 2014). *In vitro* propagation has been introduced several decade back to propagate sterile interspecific hybrids and molecular analysis as reported in *Cyclamen persicum*, *C. purpurascens* (‘Odorella’); disease-free strawberries and sweet cherry (Di Vaio *et. al*., 2015) and propagated found in seedling-derived leaf explants in *Embelia ribes*. Thus,

Intervention of biotechnological approaches for *in vitro* regeneration, mass micropropagation techniques and gene transfer studies has been successful in many horticultural plant species *Citrus reticulata* cotyledonary explants (Sarma *et. al*., 2011), shoot tips in Cherry, Craneberry, Lingonberry (Sedlák and Paprštein, 2008; Paprštein and Sedlák, 2015), shoot tip of Banana (Ngomuo *et. al*., 2014) and also in *Calamus* (Kumar *et. al*., 2012).

Embryo culture

Embryo culture is a type of plant tissue culture used to grow embryos from seeds and ovules in a nutrient medium through directly from embryo or indirectly through callus, and then subsequent formation of shoots and roots. The technique employs to break seed dormancy, test of seed vitality, production of rare species and haploid plants, in short breeding cycle of plants. Recently a successful protocol of *in vitro* propagation has been developed on *Khaya grandifoliola* from excising embryos of mature seeds and from immature seed embryos of palm (Pereira *et. al*., 2012).

The past decades tissue culture technique produces large number of secondary plant products which occupied an increasing demand in productive systems worldwide. The method also used in production of proteins and other medicinal substances; antibodies and vaccines on commercial scale which represent an economical alternative to fermentation-based production systems. Plant-made vaccines or antibodies are striking, as they are free of human diseases, thus reducing screening costs for which number of farmers incorporated transgenic plants into their production systems, increased approximately from 11 million in 2007 to 13.3 million in 2008. Thus many researchers use *in vitro* technique of propagating medicinally important plant species such as *Aloe barbadensis*, *Artimesia* (Hailu *et. al*., 2013; Sahoo and Rout, 2014).

Sometimes variability in genetic materials arises as soma clonal variation reported in *Solanum melongena* (Naseer and Mahmood, 2014).

Somatic embryogenesis

Somatic embryogenesis method of plant regeneration widely used projecting somatic cells or tissues develop into differentiated embryos. These somatic embryos develop into whole plants directly or indirectly by callus. Plant regeneration via somatic embryogenesis occurs by the induction of embryogenic cultures from zygotic seed, leaf or stem segment and further multiplication of embryos. The method applied successful on direct somatic embryogenesis in palm leaf and zygotic embryos cultured on MS medium enriched with 10 mg L^{-1} 2, 4-D, 2ip and picloram from female inflorescences (Ibrahim *et. al*., 2009; Silva *et. al*., 2012). Researchers believed that auxins such as 2, 4-D, NAA, Picloram, Dicamba, 2, 4, 5-T and endogenous hormone metabolism, genetic, physiological and environmental affects play a key role in somatic embryogenesis in different plant species (Fujimura, 2014; Roamei *et. al*., 2014).

Somatic embryogenesis has been employed in many horticultural plant species. In the process, different factors governed for regeneration like, type and orientation of culture explants, the medium and plant growth regulator combinations (Tang *et. al*., 2008; Usman *et. al*., 2012), gelling agents, light and dark phases (Leblay *et. al*., 1991), organic carbon sources and antibiotics (Predieri *et. al*., 1989) or sometimes with silver nitrate (Liu and Pijut, 2008). However, in grapevine which showed higher plant regeneration in liquid medium. Cytokinins activities are vital for inducing and developing somatic embryos. Similar reports are available on somatic embryo regeneration protocols of citrus undeveloped ovules (El-Sawy *et. al*., 2005), unfertilized ovules (Pasquali and Biricolti, 2004), anthers (Benelli *et. al*., 2010), from styles and stigmas (Meziane *et. al*., 2012), from leaves, epicotyls, cotyledons (Kiong *et. al*., 2008).

Somatic embryogenesis shows special characteristics features in plant regeneration aspect due to its morphological advantages of separate identical entities from the mother cultured tissue (He and Gang, 2014).

Organogenesis

Organogenesis refers to the production of plant organs i.e. roots, shoots and leaves that may arise directly from the meristem or indirectly from undifferentiated cell masses (callus). Plant regeneration via organogenesis involves callus production and differentiation of adventitious meristem into organs by altering plant growth hormones in nutrient medium as shows in micropropagation of *Psidium guajava* L. via direct organogenesis from nodal explants obtained from sterilized planting material (Rai *et. al*., 2009).

In vitro technique has successfully produced hybrid (GF677 Almond) from a suitable and compatible rootstock in almond and peach (Ehsanpour and Amini, 2003). Growth regulators such as BA, KN, Zeatin and 2ip have been exploited for shoot regeneration in *Prunus* spp. but, the number of shoots per explant reported is fairly low (Mant *et. al*., 1989). TDZ has been used for shoot regeneration from leaf segments of cherry (Hammatt and Grant, 1998). Moreover, BA was more efficient in shoot formation from leaves in cherry, sour cherry, peach and almond (Maciel, 2010; Azar *et. al*., 2011). In almond, BAP and TDZ have been successfully used to induce adventitious shoots (Namli *et. al*., 2011). Likewise, adventitious shoot formation is affected by the type and concentrations of the auxin used in regeneration media as in almond, in presence of IBA and NAA (Ainsley *et. al*., 2001). In cherry leaf segments, shoot regeneration was obtained from the media supplemented with NAA and TDZ (Bhagwat and Lane, 2004). Whereas axillary buds was better on MS containing BAP than combination of KN, 2iP and Zeatin in *Citrus sinensis*, while from foliar explants in *Brunfelsia calicina* (Liberman

et. al., 2010). Besides, other adjuncts like the antioxidant effect such as PVP and AC as in propagation of grape, palm (Alturki *et. al*., 2013). However, the broader spectrum on growth regulators applied in tissue culture is investigated in *Arabidopsis* and *Pinus* (Brunoni *et. al*., 2013).

Germplasm conservation

Several *in vitro* methods have been developed on genetic conservation of vegetative propagated crops. The methods involves slow growth using low temperature, darkness, low-light intensity, modification of minerals in the culture medium and use of osmotic agents and growth retardants etc where it carried out with wide range in duration of a subculture cycle which can be extended from a few weeks to 6–12 or more months. New cryopreservation technique of vitrification on different explants, as for cold-tolerant species, temperatures 0–5°C are employed, while for tropical species, 15–20° C are used. Protocols for preservation of vegetative tissues targets for conservation to avoid the loss from biotic or abiotic stress particularly plant species which do not produce seeds (sterile plants) or 'recalcitrant' seeds that cannot be stored for long period of time can successfully be preserved via *in vitro* techniques for the maintenance of gene banks. Widely cultivated, and highly consumes plum have a positive outcome by promoting germplasm exchange and rapid propagation (Annapurna and Rathore, 2010). Studies conducted in cycad seeds, which they appear to be recalcitrant, 'wet' and desiccation sensitive particularly during the protracted development of the embryo after seed shed (Chien *et. al*., 2012; Woodenberg *et. al*., 2014). The recalcitrant nature of cycad seeds shows that they are not suitable for *ex situ* long term storage and/or conservation and therefore methods other than seed propagation are urgently needed to conserve these plants.

Geographically, Nagaland lies in the North Eastern region of India of Indo-Burma biodiversity hot spot, extended between 25°06'-27°04'N Latitude and 93°20'-95°15'E Longitude, covering an area of 16,579 sq. Km with 8629 sq. Km of vegetation covered. In wild species of Bamboo, *Alnus sp.*, *Shorea sp.*, *Bombax sp.*, *Rhododendron sp.*, pears, Cherry, varied of orchids is found in abundance. The vegetations are experiencing the flavor of alpine forest, moist tropical rain forest, and sub-tropical rain forest where large numbers of economically important plants harbors. The rich biodiversity of the region shows an ideal environment factors for their growth and development of many species where many are extensively depleting by the anthropogenic activities. However, the region are predominant settle of 18 tribal communities, practice traditional methods of agricultural like '*Slash and Burn'* cultivation, hunting, fishing are common practices, besides these, village establishment, expansion of roads, mono-species farm and so on where large areas of vegetation covered were reduce at a fast rate, and brought the species at different levels of threats to their existence.

The state diverse vegetation are found to dwell large number of medicinal, ornamental, horticultural and timber plants are facing sensible impacts of depletion with the scaling-up population activities results in limited production of food and related industry. The limited food and cash crops are grown as farm/vineyard without standard agro-technological method of plantation, dissimilation, harvest and maintenance, and their small scale products available in the local markets with high demand of population. With meager technology and infrastructures, many horticultural important plant species like orange, pineapple, lemon, gooseberry, guava, passion fruit, mango are grow comparatively smaller in quantities compare with other parts of the country. Therefore, the approaches to avoid limited production of food, *in vitro* techniques of plant

propagation formed the basis of improvement in production both quantity and quality through micropropagation, breeding programs, conservations and related scientific research and biological investigations. Plant tissue culture technique is a powerful tool in mass production of economically important plants species and also conservation of threatened and endangered species worldwide. Micropropagation technique, direct organogenesis, somatic embryos are commonly practice methods, manifested in some of the successful works done by many workers (Misra *et. al*., 2011; Cruz *et. al*., 2013; Prakash *et. al*., 2014; Deb and Arenmongla, 2014).

Agriculture is the main occupation of the people of Nagaland. About 65% of the population depends on agriculture as per 2001 census. Shifting (Jhum) and terrace cultivation remain the dominant form of the land use pattern of the State. Till recently, for most farmers' horticulture has been meager activity, with busy throughout the year in cultivation of food crops. Besides, due to long gestation period in plantation, the cultivation of these crops has been confined to backyard gardens developed by almost every household. Only in the past decade, more focused has given to horticultural development in the State geared up impact on rural economy of the State. The geographical, diverse agro climatic conditions, varied soil types and abundant rainfall prevailing enables the cultivation of vegetables, spices, flowers, mushrooms, medicinal and many horticultural crops like banana, orange, peach, plum, kiwifruit, pineapple, passion fruit etc.

The total area of 36177 ha (2006-07) which represents 9.95% of gross cropped area 3.63 lakh per ha are under cultivation of horticultural crops. Both sub-tropical fruits such as pineapple, banana, citrus, guava, etc. and temperate fruits such as plum, peach, pear, passion fruit and various nuts have potential for exploitation. Among the fruit crops, pineapple, mandarin orange and passion fruit are already being produced on a

commercial scale. Amongst the different horticultural and economically crops, two species belongs to family Actinidiaceae (*Actinidia deliciosa* and *Saurauia punduana*) are very important one. Account of the two economical importance plant species:

Actinidia deliciosa A. Chev. (Actinidiaceae)

The species belongs to genus *Actinidia*, has about 60 species and 76 taxa. The species are wide spread in distribution and habitat throughout the temperate forests of the mountains and hills of south west China viz., Chang Jiang and Xi Jiang regions of Yangtse River valley in China while some found in Siberia and Indonesia. The genus *Actinidia* is a perennial vine, bearing berry fruits, cultivated all over the world. Of many species; *A. deliciosa* var. *deliciosa* and *A. chinensis* are the two species bearing edible fruits and widely eaten. Further the species *A. deliciosa* (A.Chev.) C.F. Liang *et* A.R. Ferguson, is commonly known as kiwifruit. The two edible species are *A. deliciosa* (hexaploid) and *A. chinensis* (diploid or tetraploid). Other species with economic potential are *A. arguta, A. eriantha, A. latifolia, A. chrysantha, A. melandra, A. kolomikta* and *A. polygama.* However, they are not commercial fruit crops as yet. Few decades ago, success in marketing of cultivars *A. deliciosa* has initiated breeding programmes with application of molecular technologies to improved new cultivars. However, dioecious nature and long juvenile period in kiwifruit are the other equally important constraints in breeding programmes. In 1920s, grafted plants of known sex were sold and selections for high quality fruit were made". As a result, the well-known cultivars of today, viz. Abbot', Allison', 'Bruno', Monty, Hayward' and Gracie', originated as seedling selections in New Zealand. Hayward' has now commercial importance, for its better shape, size, and long storability of fruits.

The first commercial cultivation of kiwifruit began in New Zealand and, thereafter mostly during 1970s. At Present, New Zealand has the best-developed

kiwifruit industry with 12,000 ha of land under its cultivation, out of total world cultivation of 22,900 ha. The land under kiwifruit plantations in New Zealand has further increased to 15,000 ha in 1992, producing almost 230,000 tons fresh fruit. The species is used in preparation of salad, pickle, chocolate in the food industry. Ever increase in demand than before, many countries started trade on kiwifruit, where its market is expanding in many European and American countries. Top kiwifruit producing countries are Italy 28%, China 23% New Zealand 20% Chile 10%, France 5% and Japan 3% as reported by Bano *et. al*. (2012), Kofoed (2008) in their research on export of kiwi in the world market. Thus, the prospects on production of large quantity depend on intervention of technological feasibility method which brings challenges where tissue culture technique stand to supplement on quality planting materials the future aspect of growing kiwi industry depend.

Actinidia deliciosa is a woody, climber shrub, puberulent, dioecious either Staminate or pistillate, grow in temperate cold mountainous region of high altitude **(Fig. 1 a, b**). Flowers during May and June, excellent source of potassium, folate and vitamin E and are high antioxidant content. The plant also well known for its medicinal importance from extracts of different parts of the plant, used for treating mange in dogs and has laxative effect, reported by Rush *et. al*. (2002) from ethno medicinal research. The presence of glutathione in the fruit juice also account for reduction in mutagenesis. *Actinidia deliciosa* is one of the species of the genus *Actinidia* having more than 50 different species. However, the species was named *Actinidia chinensis* till 1984. In India, kiwifruit was introduced in 1960s in parts of the Himalayan region. Where grown initially successfully, only seven cultivars of *A. deliciosa* var. *deliciosa* were imported from New Zealand and USA. However, the commercial potential and suitability in the mid and foothills of Himachal Pradesh further led to their spread to other parts of the country.

Figure – 1

Figure 1: a. *Actinidia deliciosa* (kiwifruit) growing in the farm showing its vegetative parts; **b.** Branches are bearing fruits**; c & d:** *Saurauia punduana* growing in the wild open secondary forest. **c.** Showing its vegetative parts; **d.** Branch bearing few reddish (mature) and (immature) brownish white color flowers.

Saurauia punduana Wallich (Actinidiaceae)

Saurauia punduana belongs to family Actinidiaceae. It comprises about 250 species distributed in the tropics and subtropics of Asia, and South and Central America. It is also the only extant genus within the Actinidiaceae whose natural distribution includes areas outside of Asia (tropical South and Central America). IUCN in 2004 reported that *Saurauia punduana* is identified in the Red List of threatened Species as critically endangered, assessment done by China Plant Specialist Group 2004.

Saurauia punduana Wallich (Actinidiaceae) are woody tree measure about ca. 6 m tall (**Fig. 1 c)**. Branches pubescent to glabrescent with minute scurfy hairs intermixed with unguicular scales. Petiole measures 3-5cm with pubescence as branches. Leaf blade narrowly elliptic to narrowly obovate, ca. 33 × 11 cm, thinly leathery, abaxially densely brown scurfy-puberulent, with sparse scales on mid vein and lateral veins, Inflorescences ca. 5 cm, axillary, 1-3-fascicled, 2- or 3-flowered, glabrous, scaly; pedicels slender; bracts broadly elliptic, ca. 4 mm (**Fig. 1 d)**. Flowers during June and July, mature flowers are pinkish white, large, 1.8-2 cm in diam. Sepals: outer 2 broadly elliptic, inner 3 narrowly elliptic to orbicular, enlarged when in fruit. The species is eaten as vegetable which helps in building immunity.

The species are perennial, grow mostly in the wild in open secondary and tertiary forest, and are identified as endangered in the natural habitat. The species experience high pressure to their existence similar with many other species in the region. The region bless with large number of economical important plants found growing in wild as well as domesticated where many are found depleting due to anthropogenic activities due to indiscriminate collection, local population practice of the so called ‘Slash and Burn’/Jhum cultivation, mono species farming, establishment of villages, roads, industries and many other anthropogenic activities destroying the vegetation in large area

of land cover. Many unplanned activities like logging of timber, rampant hunting and seasonal forest burn are the common activities which affects directly on vegetation of the region. Therefore need of efficient advanced techniques to produced quality planting materials required, for which present thesis emphasized to substantiate planting materials in large quantity through plant *in vitro* technique as demonstrated from the work of Vinoth and Ravindhran (2013).

Realizing the importance of these two species I have worked for my Ph. D. degree on in micropropagation and production of quality planting materials of these two species with the following objectives:

1. Study the habit and habitats to identify the best season for plant collection.
2. Develop protocols for *in vitro* mass multiplication from different explants sources.
3. Acclimatization of regenerates.
4. Introduction of regenerates in the poly house and wild.

Chapter - 2

In Vitro Propagation of *Actinidia deliciosa* A. Chev. (Actinidiaceae)

Totipotency of plant cells, tissues or organs is the gateway to *in vitro* techniques where the knowledge substantiates and applies particularly to the plants of economically important ones. However, in broader sense '*In Vitro Culture*' is widely used where it applied mostly vegetative parts of the plant, perhaps those plants their seeds are recalcitrant in nature and conventional methods of propagation shows less effective. Conventional propagation of plants through seed encounter varied difficulties such as seed viability, its dispersal efficiency and certain physiological factors on germination. The technique relies on mostly on plant materials, aseptic environment and medium. Planting materials may be a cell, small tissue or even an organ excised from plant parts/explants such as- root, stem, petiole, leaf or flower etc. However, meristematic cells give better result due to actively dividing. The initiation culture depends on

physiological state of the explant therefore, healthy parent plant is recommended. Choice of nutrient medium mostly depends upon the species; therefore solid or liquid medium with different salts or growth regulator concentrations prefer matters, even at different stages of growth and development. Among different types of media are available, of which White's medium is the first formulated plant tissue culture media but most suitable and widely use medium is Murashige and Skoog (MS) medium established in 1962. Other media include B_5 medium, Nitsch's medium, White medium etc. The cultured cell or tissue express dedifferentiation and has to undergo regeneration to form plant or its organs. It may be through either of two processes: Organogenesis or Embryogenesis.

Organogenesis involves formation of new organs like shoot buds etc. from callus. Organogenesis depends on many factors, differ for different plant species. However, mostly it is promoted by cytokinins. Skoog and Miller (1957) demonstrated that relative ratio of cytokinins and auxin is important in determining nature of organogenesis as in tobacco pant culture where high level of cytokinins favors shoot bud formation while auxin favors rooting. But there have been studies in other plant species which do not follow this concept of auxin/cytokinins ratio. However in many species shoot regeneration affects by abscisic acid but this variable response is due to their endogenous levels of growth regulators which differ in different plant species and also plant materials. Other factors govern organogenesis are size and source of the explant.

Actinidia deliciosa A. Chev. (Actinidiaceae) has wide spread in distribution and habitat throughout the temperate forests of the mountains and hills of south west China viz., Chang Jiang and Xi Jiang regions of Yangtse River valley in China. The genus *Actinidia* is a perennial vine, bearing berry fruits, cultivated all over the world. The first commercial cultivation of kiwifruit began in New Zealand and, thereafter mostly during

1970s. At present, New Zealand has the best-developed kiwifruit industry with 12,000 ha of land under its cultivation, out of total world cultivation of 22,900 ha". Fruits are used in preparation of salad, pickle, chocolate in the food industry. Ever increase in demand than before, many countries started trade on kiwifruit, where its market is expanding in many European and American countries.

In India, kiwifruit was introduced in 1960s in parts of the Himalayan region. Where grown initially successfully, only seven cultivars of *A. deliciosa* var. *deliciosa* were imported from New Zealand and USA. However, the commercial potential and suitability in the mid and foothills of Himachal Pradesh further led to their spread to other parts of the country. Present study aims at development of *in vitro* protocol for production of clonal planting materials of this economically important species.

Materials and Methods

Plant Materials and Processing

***Nodal explants*:** Choice of explant in tissue culture is a crucial step to establish successful means of plant propagation. Widely vegetative organs or tissue are mostly employed, particularly shoot tips or sometimes tendered nodal segment in many horticultural plant species. For the present investigation nodal segments/nodal explants from *in vivo* source was used as starting materials. The plant materials of *Actinidia deliciosa* were collected from the cultivated farm at Aosakhelimi, of Zunheboto District in Nagaland, a distance of about 15 km from the Botany Department of Nagaland University, Lumami (location: Altitude 1495 m, N: 26°08’42.2” and E: 094°31’35.6”). Plants materials were collected round the year at monthly interval. Nodal segments were harvested after 6-7 wk of flushing of new shoots. The explants were cut off from the branches of healthy parent plants, wrapped in fresh banana leaf segment. Further the banana leaf-wrapped explants were moistened by sprinkle water and packed in poly bags

and brought to the laboratory for further processing. The scales and darts were removed by washing under running tap water and stored at 4-5˚C in the refrigerator for 1-2 days before initiating culture.

The nodal segments were trimmed off its leaves and cut off the internodes and collected only the nodal segment ca 2 cm. The trimmed nodal segments were surface sterilized by scrubbing by soft brush with 'Labolene' (a commercial laboratory detergent at 1:100, v/v) followed by washing under running tap water for 10-15 min. Subsequently explants were sterilized by treating with aqueous solution of $HgCl_2$ (0.3%, w/v) for 2-5 min followed by washing 4-5 times with sterilized pure water. The sterilized nodal segments were finally rinsed with 70% (v/v) ethanol for 30 sec followed by 2-3 wash with sterilized water and nodal segments were kept moistened with sterilized distilled water till culture on initiation medium/media.

In vitro foliate and nodal explants: Besides *in vivo* source nodal segments, cultures were also initiated from 5-8 wk old *in vitro* leaves and nodal segments harvested from the pre-existing axenic and used as planting materials for its subsequent regeneration cultures. The micro shoots were harvested and the leaves were carefully separated from the micro shoots. Further the micro shoots were cut into uninodal segment, while, the leaves were divided into three parts viz. normal full intact leaves, intact-injured leaves and segmented horizontally into 2-3 pieces and partially injured/scrubbed leaves. Different sets of normal intact leaves, injured or partially scrubbed intact leaves, segmented foliate and nodal segments were cultured on initiation medium fortified differently.

Tissue Culture Medium

For initiation of cultures from different explants Murashige and Skoog medium (MS) (Muraghige and Skoog, 1962) was used as basal nutrient medium with differential

fortification. The basal medium was supplemented with different concentrations of plant growth regulators (PGRs) like NAA, KN, BA (0-15 µM) either singly or in combination, three different organic carbon sources (viz., dextrose, glucose and sucrose) at different concentrations (0-4%, w/v). Besides these the basal medium was also fortified with three different antioxidants: polyvinyl pyrollidone (PVP), citric acid (CA) and activated charcoal (AC) at different concentrations (0-700 mgL^{-1}) were incorporated in the initiation medium. The medium was gelled using bacteriological grade agar (0.8%, w/v, make: Hi-media) and *p*H was adjusted to 5.8 with 0.1N NaOH and HCl before autoclaving at 1.05 kg cm^{-2} pressure and 121^{o}C for 20 min. In each borosilicate test tube (150x25 mm) ~15 ml medium was dispensed and plugged with non-absorbent cotton before autoclaving the medium. Similarly the *in vitro* leaves were cultured in full strength MS medium fortified with sucrose (3%), agar (0.8%). The medium was supplemented with different levels of cytokinins BA and KN (0-15 µM, v/v) singly.

Initiation of Cultures

Nodal explants: The sterilized nodal segments were taken out in the laminar flow cabinet and cultured on MS medium fortified with different organic carbon source (dextrose, glucose and sucrose, 0-4%), different antioxidants (CA, PVP and AC, 0-700 mgL^{-1}) and PGRs (BA, KN, NAA, 0-15 µM either singly or in combination). For each treatment 20 explants were cultured. In each test tube one segment was cultured and maintained at 25±2˚C under cool fluorescent light at an intensity 40 µmol $m^{-2}s^{-1}$ for 12/12 hr (light/dark) photo periods.

Leaf and nodal segments from *in vitro* source: Leaves was isolated from shoots grown in culture and kept moistened with sterilized distilled water till culture. Set of isolated leaves were section transverse into ~1.0 cm long and partially scrubbed/injured with blade, similarly sets with injured and without injured intact leaves. The foliar explants

(intact, injured and segmented) and nodal segments were cultured on different strengths of MS medium (0, 1/4th, ½, 3/4th and full strength) fortified with sucrose (0-4%), and PGRs such as KN, BA (0-15 µM) singly. For each treatment 20 explants was maintained for initiation of culture.

Callus Mediated Morphogenesis

The calli developed in the different initiation and regeneration medium from nodal segments, foliar explants were separated from the shoot buds, and maintained cultured separately on MS medium fortified with sucrose (3%), PGSs (NAA, BA and KN (0-15 µM) either singly or in combination for inducing morphogenesis. The cultures were sub-cultured at regular interval. The shoot buds developed in the callus mass were separated and transferred to regeneration medium for plant regeneration and culture proliferation.

Experimental Design and Statistical Analysis

Completely randomized experimental design was followed in all the experiments. In each experiment 20 explants were cultured for each treatment and all the experiments were repeated five times. The cultures were maintained at 25±2^{o}C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} intensities and 12/12 hr each (light/dark) photo period unless mentioned otherwise. All the cultures were sub-cultured at 5-6 wk interval. The cultures were monitored regularly and data collected at weekly interval. *In vitro* response was evaluated based on the percentage of explants responded and number of propagules formed in the culture after specific period of time (as stated in the table) and data was expressed as the mean of replicates ± standard error. Data was further analyzed by one way ANOVA using the General Linear Model procedure in SAS Statistical Package (SAS Ins.) and standard deviation from mean was worked out and compared using Least Significant Difference (LSD) test at $p \leq 0.05$.

Plant Regeneration and Culture Proliferation

The micro shoots developed directly from the cultured explants (nodal explants from *in vivo* and *in vitro* source, foliar explants) and from the cultured callus were maintained for another two passages under optimum initiation medium for further development. The shoot buds /micro shoots were separated and transferred to fresh MS medium containing different carbon sources like glucose, dextrose and sucrose (0-4%), PGRs such as BA, KN (0-15µM) and NAA (0-6 µM) either singly or in combination for plant regeneration and culture proliferation.

Rooting of the Regenerates

Though there was some roots formation in some regenerated shoots on regeneration medium, but roots were not fully developed and shorter in length. About 4-5 cm long plantlets/ micro shoots with well expanded leaves from the regeneration medium were selected for inducing rooting following different technique as stated below:

1. One set of micro shoots was pulse treated with IBA (0-50 µM) and NAA (0-20 µM) for 0-24 hours (0, ½ h, 1h, 24h and 24h) followed by culturing on MS plain medium containing sucrose (3%). The selected auxins concentrations were sterilized in the autoclave at 121°C under the pressure of 1.05 kg cm^{-2} for 20 min. before pulse treating the micro shoots. The cut ends of the micro shoots was dipped in PGRs solution for the stipulated durations and transferred to fresh plain nutrient medium. While in other set the PGRs (NAA and IBA) (0-15 µM) was directly incorporated in the rooting medium for inducing of roots in the culture micro shoots.

2. In another set of experiment, effort was made to study the effect of foliation/defoliation on *in vitro* rooting of micro shoots. For the purpose, the leaves of

the micro shoots were removed using scale blade from one set of micro shoots and maintained in the rooting medium and maintained in the normal laboratory condition.

Hardening of the Regenerates

The well rooted plantlets were collected from the rooting medium and subjected to hardening. For the purpose the regenerates were transferred to $1/4^{th}$ strength of MS medium adjunct with sucrose (1%) and maintained in the normal laboratory condition for 7-8 wk. In the last 2 weeks, a part of the culture vials were exposed to normal day light for 4 h a day followed by transfer to normal laboratory condition. Besides agar gelled medium regenerates were also maintained on other agar substitutes like chopped coconut coir, saw dust, forest litter with the objective to look for agar alternative and reduce the production cost.

***Substrata for hardening medium*:** Dry coconut husk, forest leaf litter were chopped into small pieces (~0.1-0.5 cm size). These were soaked in laboratory detergent for 1-2 hr and rinsed with water several times and sun-dried for 4-5 hr. Besides coconut coir and forest litter, saw dust was also employed as alternative substratum. Saw dust was collected from the local carpentry workshop. The substratum was sun-dried and soaked in laboratory detergent for 2-3 hr and rinsed thoroughly under running water followed by sun dried. About 10-12 grams of chopped coconut husk, forest litter and saw dust were transferred test tubes separately and autoclaved at 121° C temperature for 30 min at the pressure of 1.05 kg cm^{-2}.

About 12-15 ml $1/4^{th}$ strength MS liquid medium enriched with sucrose (1%) was dispensed in all the test tubes and autoclaved under 121° C temperature at 1.05 kg cm^{-2} pressures for 15-20 min. These medium filled test tubes with different substrata were used for hardening of the rooted regenerates.

Potting Mix and Transplantation of the Regenerates

With the help of spate, soil was dug out from the thick mud on the ground, hand-picked the litter contained in the mud and crushed into small pieces (size: 0.1-0.3) with flat surface wooden block on clean cemented floor. Crushed soil was well mixed with fine sand, collected from the building construction site. Dried rotten wood powder from local forest was collected, thoroughly mixed with the mixer of sand and soil in the ratio 2:1:2. The collected potting mix materials were transferred in poly bags (size: 15x12) and plastic cups, stored it in shade for 1 wk and used for plantation of hardened plantlets.

The partially hardened plantlets from the cultured vials was taken out and rinsed with running tap water and transferred onto poly bags containing soil, sand, decayed wood powder mix with forest litter at 2:1:2 ratios. The planted poly bags were covered with holed transparent bigger poly bags and watered alternate days with $1/5^{th}$ MS salt solution once in a week for 1 month. The covered poly bags were removed from the pots and maintained in partial shade ca.50% for 2-3 wk and finally exposed to normal day light. The acclimatized transplants was transferred to the experimental bed and monitored regularly for three months.

Results

Initiation of Culture

Nodal explants: The newly flushed shoots of *A. deliciosa* were collected from the mature plants throughout the year. The *in vitro* morphogenetic response was greatly influenced by various factors like post harvest treatments, seasonal effect of explants collection, strengths of basal medium, quality and quantity of organic carbon sources, PGRs, incorporation of antioxidants in the initiation medium.

***Seasonal effect of explants collection*:** Optimization of explants collection, from the field grown plants is a necessary step for *in vitro* establishment of any plant species since

plant growth are significantly influenced by season, either growing or dormant seasons. The nodal segments of the species *Actinidia deliciosa* of 7 wk old branch were collected for culture initiation. The nodal explant collection was periodically carried out in every month for consecutive three years. Variations were observed on the influence of different factors on morphogenetic responses of the *in vitro* culture establishment was found. With the seasonal variation in a year, during July-August, maximum rainfall and high temperature were observed which favored the production of phenolic compounds and other exudates in the explants, while in winter particularly, December leaching activities found to be decrease considerably, thus showed the optimum morphogenetic response of the species. Of all the seasons of collections, the midsummer during the months of July found to be least responsive with only 8.3% explants responded positively and most tissues were necrotic and degenerated on culture medium. Compare to summer, winter season was found to be suitable

Table 1: Seasonal effect of *in vivo* Kiwi nodal explant collection on *in vitro* morphogenetic response

Month of collection	Time of initial response (d)*	% response (±SE)#	Morphogenetic pathway$	Type of response@
January	A	50.0 (1.2)^{c}	Sb	Dark green shoots, broad leaves with moderate growth rate.
February	A	41.7 (1.2)^{d}	Sb+Ca	Shoots light green, long internodes and smaller curly leaves.
March	B	37.5 (1.1)^{e}	Ca+Sb	Light green shoots, unhealthy growth rate with curly leaves.
April	C	20.8 (1.1)^{g}	Ca+Sb	As above, with stunted in growth rate.
May	C	25.0 (0.8)^{f}	Sb+Ca	Callused, curly smaller leaves, shoots with long internodes and necrotic.
June	C	16.7 (0.9)^{h}	Ca+Sb	As above.
July	C	08.3 (0.6)^{j}	Ca+Sb	Shoots light green, slow growth rate, light to brownish smaller leaves.
August	C	12.5 (0.8)^{i}	Ca+Sb	Shoots with long internodes, curly light green leaves.
September	B	25.0 (1.3)^{f}	Sb+Ca	Light green smaller leaves, unhealthy growth rate.
October	B	50.0 (1.3)^{c}	Sb+Ca	Dark green shoots, light green leaves, slower growth rate.
November	**A**	**75.0 (1.5)^{a}**	**Sb**	**Dark green shoot buds, broad dark green leaves, healthy growth**.
December	A	62.5 (1.2)^{b}	Sb	Dark green shoots and leaves but moderate growth rate.

Newly flushed shoot of ~7 wk old

* A: 0-5 d, B: 5-10 d, C: 10-15 d

#±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

$: Ca-Callus, Sb- Shoot bud, Ca+Sb-Callus and Shoot bud,

@ On MS medium supplement with Sucrose (3%, w/v), agar (0.8%, w/v), BA (6 µM, v/v) and PVP (300 mg l^{-1}).

Data represented the mean of five replicates.

season for nodal explants collection (**Table 1)**. First sign of morphogenesis was recorded within 5 days of culture initiation as swelling of cultured nodal segments. Of the different seasons studied, explants collected during February to October yielded poor *in vitro* response, with much lesser callusing and fewer shoot buds formation, however the explants collected during winter (November–January) supported healthy shoot buds formation with broad, dark green leafy. Under the given culture conditions nodal explants collected during November exhibited optimal *in vitro* morphogenic response where ~75% nodal segments responded positively and formed shoot buds on MS medium fortified with BA.

***Effect of antioxidants*:** The sterilized nodal explants were cultured on MS medium fortified differently with different adjunct including different antioxidant sources (AC, CA and PVP) at different strengths to prevent the phenolic exudation of explants. The nodal segments cultured on medium freed from any antioxidant released phenolics in the medium and tissues turned necrotic and degenerated. Incorporation of antioxidants in the medium improved the condition. Of the different quality and quantity of antioxidants used in the present study, PVP at a concentration of 300 mg L^{-1} found to be most suitable supported ~75% of cultured nodal explants affected with healthy growth rate and controlled browning of medium (**Table 2**). Compared to PVP, other two antioxidants (AC and CA) found to be less effective as far as controlling of browning is concerned across the concentrations tested. Though both AC and CA could reduce the phenolic oxidation but did not support healthy initiation of culture. At lower concentrations of all the three antioxidants failed to reduce the browning of medium while at higher concentrations (>300 mg L^{-1}) though the browning could be stopped but affected adversely the morphogenetic response of the nodal segments.

Table 2: Role of quality and quantity of antioxidants on *in vitro* initiation culture of Kiwi nodal explant from *in vivo* source

Antioxidant type & Conc. (mg l^{-1})*			**% response (±SE)#**	**Morphogenetic response@**
AC	CA	PVP		
0	0	0	-	Release of phenolic and browning of medium.
100	-	-	33.3 (0.7)^{f}	Release of phenolic exudates and explants degenerated gradually.
300	-	-	50.0 (1.5)^{c}	Exudation of phenolic and lighter browning of medium.
500	-	-	41.7 (1.1)^{d}	Dark green shoot bud with broad leaves, no browning, healthy growth.
700	-	-	25.0 (1.2)^{g}	Controlled browning, light green shoots, long and curly leaves.
-	100	-	41.7 (1.1)^{d}	Shoot bud slight brownish with small curly light green leaves, stunted growth, moderate browning.
-	300	-	50.0 (1.2)^{c}	Swollen ends, light green tissue formed at base. Dark green shoots with smaller, longer leaves.
-	500	-	41.7 (1.4)^{d}	Controlled browning, healthy shoots with curly leaflets.
-	700	-	33.3 (0.9)^{f}	Slight brownish shoot buds with curly leaves, controlled browning.
-	-	100	41.7 (1.2)^{d}	Moderate browning, leaves dark green & slight curly and shoots with long internodes.
-	**-**	**300**	**75.0 (1.5)^{a}**	**Healthy shoots with broad dark green leaves. Controlled browning medium**.
-	-	500	66.7 (1.8)^{b}	Moderate browning, shoots dark green with stunted growth.
-	-	700	25.0 (0.6)^{g}	Poor growth, leaves dark green smaller with controlled browning.

* AC-Activated charcoal, CA-Citric acid, PVP-Polyvinyl pyrolidone.

±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

@ On MS medium supplement with agar (0.8%, w/v), sucrose (3%, w/v) and BA (6µM, v/v).

Data represented the mean of five replicates.

Table 3: Effect of quality and quantity of organic carbon sources on *in vitro* morphogenetic response of nodal explant of Kiwi from *in vivo* source

Organic carbon source & Conc. (%)*			Time for initial response	% response (±SE)#	Morphogenetic response@
D	G	S			
0	0	0	0	00	No response
1	-	-	10	08.3 (0.4)^{h}	Light green shoot buds, but degenerated.
2	-	-	9	25.0 (0.6)^{f}	Shoots light green smaller, stunted growth rate.
3	-	-	8	33.3 (0.8)^{e}	Moderate shoots growth with light green small leaflets.
4	-	-	10	16.7 (0.7)^{g}	Shoots smaller, support low growth rate.
-	1	-	7	16.6 (0.9)^{g}	Low growth rate with stunted brownish shoots.
-	2	-	9	33.3 (1.0)^{e}	Shoots light greenish and support moderate growth rate.
-	3	-	11	41.6 (0.8)^{d}	Shoots curly leaves with moderate growth rate.
-	4	-	10	16.6 (0.6)	As above.
-	-	1	9	41.6 (0.7)^{d}	Moderate growth rate, shoots small, long light green leaves.
-	-	2	6	65.6 (0.9)^{b}	Dark green shoots, support slower growth rate.
-	-	**3**	**5**	**75.0 (1.5)^{a}**	**High growth rate with shoots dark green, broad and dark green leaves**.
-	-	4	12	50.0 (1.0)^{c}	Dark green shoot with curly and long dark green leaves and moderate growth rate.

* D-Dextrose, G-Glucose, S-Sucrose,

±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

@ On MS medium supplement with agar (0.8%, w/v), BA (6µM, v/v) and PVP (300 mg l^{-1}).

Data represented the mean of five replicates.

Table 4: Effect of quality and quantity of cytokinins on direct organogenesis of nodal explants of *Actinidia deliciosa* from *in vivo* source

Cytokinins (µM) BA	KN	Time for initial response (d)	% response (±SE)*	No. of meristematic loci per node	Morphological response of shoots@
0	0	-	-	-	Explants degenerated
3	-	6	50.0 (1.3)^{c}	3	Few dark green broad leaves, short internodes shoot buds.
6	-	**5**	**75.0 (1.5)^{a}**	**4**	**Swollen cut ends, no calli with dark green shoots, few dark, broad leaves**.
8	-	6	58.3 (2.3)^{b}	2	Broad dark green leaves, shoot buds with longer internodes, few advantages shoots.
10	-	7	50.0 (1.3)^{c}	3	As above.
12	-	8	41.7 (1.4)^{d}	3	Swollen and crack, shoots are light green with long narrow leaves.
14	-	8	33.3 (0.9)^{e}	1	Shoots light green with slight curly light greenish leaves.
16	-	9	25.0 (1.2)^{f}	1	Crack open cut ends, swollen cut ends. Shoot with few leaves, light green long and curly.
-	2	7	33.3 (0.9)^{e}	1	Swollen basal ends and turn slight dark brownish. Shoots with few dark green, broad leaves.
-	4	8	25.0 (1.5)^{f}	2	Protruded dark green shoot, few broad light green leaflets.
-	6	6	41.7 (2.1)^{d}	1	As above.
-	8	7	50.0 (2.1)^{c}	1	Crack nodal explants, light greenish shoot with few longer leaflets.
-	10	8	41.7 (2.3)^{d}	3	Basal ends swelled, shoots with light green curly leaflets and unhealthy.
-	12	11	33.3 (1.5)^{e}	1	Light green shoot with few green leaves, long internodes, vitrified looks.
-	14	10	33.3 (1.7)^{e}	1	Dark green shoots with long, curly leaves. Poor plant growth.
-	16	13	25.00 (2.0)^{f}	1	Swollen basal cut ends and developed light green curly leaves.

* **±SE:** Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

@ On MS medium supplement with sucrose (3%, w/v), agar (0.8%, w/v) and PVP (mg l^{-1}).

Data represented the mean of five replicates.

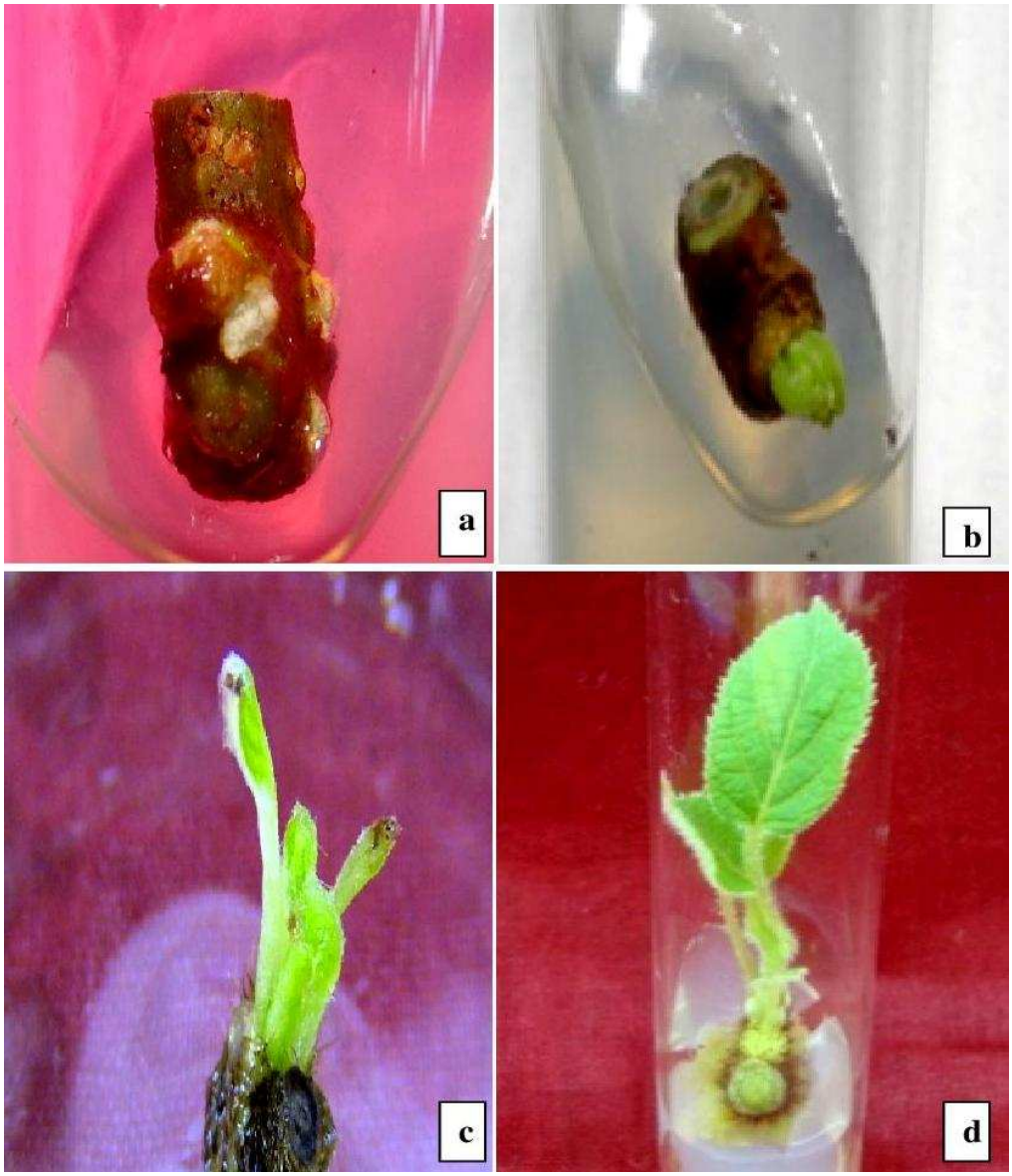

Figure – 2

Figure 2: Direct organogenesis of *in vivo* nodal explant of *A. deliciosa* cultured on initiation medium showing various developmental stages. **a.** Swelling of nodal explant on initiation medium; **b**. Meristematic loci/shoot buds developed from nodal segments with undifferentiated leaflets; **c.** Young shoot buds releasing leaves; **d.** Differentiated shoot bud with fully opend leaves.

***Effects of organic carbon sources*:** Different concentrations of various organic carbon sources viz., dextrose, glucose and sucrose (0-4%, w/v) were also incorporated in the initiation media. Incorporation of one of the organic carbon in the initiation medium was prerequisite for successful for induction of morphogenetic response. There was no morphogenetic response on organic carbon controlled media. The morphogenetic response was very poor on medium fortified with dextrose and glucose as organic carbon source across the concentrations. Of the different concentrations of different organic carbons tested in the present study, 3% concentration is found to be most effective in all, while at this concentration; dextrose was found affected ~33% of the explants responded direct bud development with moderate growth rate and smaller leaves. Whereas glucose fortified medium supported ~41% response. But in sucrose (3%) fortified medium ~75% of nodal explants responded positively with healthy shoot buds development (**Table 3)**. At lower concentration of sucrose, fewer shoot buds formed while at higher concentration, the response was comparatively poorer.

***Effects of PGRs*:** In order to initiate *in vitro* culture, cytokinins such as BA and KN were incorporated singly at different concentrations (0-16 µM) to induce the formation of micro shoot buds. On the two cytokinins tested at different concentrations, KN was found to be inferior across the concentrations compared to BA where in most of the cases only one shoot bud invoked per node and fewer explants registered *in vitro* response (**Table 4)**. On BA rich medium explants exhibited swelling and cracking as first sign of response after 5 days of culture, which differentiated into axillary and advantages shoot buds formation within 3 weeks of cultured was noted (**Fig. 2a, b**). Under the given conditions optimum response was recorded on medium fortified with BA (6 µM) where as many as 4 shoot buds per node was resulted from ~75% of explants cultured (**Table 4**) on MS medium fortified with sucrose (3%), PVP (300 mg L^{-1}). The

shoots buds elongated on the same initiation medium in subsequent sub-culture (**Fig. 2c)**. The shoot buds converted into micro shoots within 2 passages of 5-6 intervals on the optimum initiation medium with well expanded leaves (**Fig. 2d**).

***Synergetic effect of BA-NAA and KN-NAA*:** Besides singly treatment of two cytokinins as described above, combined treatments of BA-NAA and KN-NAA were also studied for *in vitro* initiation of culture as shown in table 5. Of the two groups of combination, BA-NAA combination was found superior over KN-NAA combination though both the combinations were far inferior compared with singly treatments of both BA and KN. The synergistic effect of BA-NAA and KN-NAA exhibited varied morphological responses, such as calli formation, development of somatic embryos and shoot buds development. Whereas, in absence of cytokinins tissue/explants gradually degenerated without any positive morphogenetic responses. However, at higher than optimum concentration, explant developed smaller shoot buds with long curly leaves. Under the experimental condition, effect of BA-NAA shows better result in development of micro shoot via loci formation which at concentration of 6 µM BA with 3 µM NAA gives 3 loci per explant, stood at the figure of ~37.5% explants were responded after 5 wk from cultured (**Table 5**). Dark green with broad leaves of micro shoots were developed which was allowed to culture in single BA treatment further proliferation in the proliferation medium.

Table 5: Synergistic effect of BA-NAA and KN-NAA on *in vitro* morphogenetic response of Kiwi nodal segments from *in vivo* source

PGRs Conc. (µM) B/N	K/N*	% response (±SE)[#]	Morphogenetic pathway[$]	No. of loci/ explants	Type of response[@]
0	0	-	Ca	-	Callused, subsequently degenerated.
3/3	-	25.0 (0.5)	Ca+Sb	2	Light green calli & shoot buds with long internodes.
6/3	**-**	**37.5 (0.7)**	**Ca+Sb**	**3**	**Light green calli, shoot buds with broad light green leaves**.
9/3	-	43.8 (1.1)	Ca+Sb	1	Moderate dark calli, dark green shoot bud with few leaflets.
12/3	-	31.3 (0.9)	Ca+Sb	2	Swollen base, light green calli, shoot buds light greenish.
15/3	-	25.0 (0.7)	Ca+Sb	2	High dark green calli and spotted white granular calli, shoots with long internodes.
-	3/3	18.8 (0.5)	Ca	0	Swollen ends, moderate light green calli, few white granular patches.
-	6/3	25.0 (0.8)	Ca+Sb	2	High dark green calli, shoots with longer and curly leaflets.
-	9/3	43.8 (1.4)	Ca+Sb	2	Shoots and leaves dark green, light green calli formed.
-	12/3	50.0 (0.9)	Ca	0	Swollen base, light green calli.
-	15/3	31.3 (0.8)	Ca+Sb	1	Light green and low brown soft calli, shoot buds greenish, longer leaflets.

*: K- Kinetin, B- Benzyl Adenine, N- α-Naphthalene acetic acid.

±SE: Standard error from mean.

$ Ca: Callus, S: Shoot buds.

@ On MS medium supplement with sucrose (3%, w/v), agar (0.8%, w/v).

Data represented the mean of five replicates.

Table 6: Effect of quality and quantity of cytokinins, leaf segmentation and leaf injuries on *in vitro* morphogenetic response of *Actinidia deliciosa*

Cytokinin type & Conc. (µM)		No. of buds formed per intact leaf	% of response (±SE)*	No. of buds formed of per leaf segment	% of response (±SE)*	No. of buds formed of per segmented scrubbed leaf	% of response (±SE)*
BA	**KN**						
0	0	-	-	-	-	-	-
3	-	3	41.7 (0.6)[b]	3	25.0 (0.5)[d]	3	33.3 (0.9)[b]
6	-	4	25.0 (0.9)[d]	4	37.5 (0.5)[b]	5	35.0 (0.8)[b]
9	-	**5**	**50.5 (0.7)[a]**	**7**	**41.7 (1.1)[a]**	**7**	**41.6 (1.0)[a]**
12	-	3	33.3 (0.3)[c]	5	37.5 (0.7)[b]	6	33.3 (0.7)[b]
15	-	2	33.3 (0.6)[c]	4	12.5 (0.6)[e]	4	25.0 (0.7)[c]
-	3	2	25.0 (0.9)[d]	2	08.3 (0.6)[f]	2	16.6 (0.6)[d]
-	6	3	41.6 (1.1)[b]	2	25.0 (0.7)[d]	3	33.3 (0.5)[b]
-	9	2	16.7 (0.7)[e]	3	25.0 (0.5)[d]	2	41.6 (0.8)[a]
-	12	4	33.3 (0.7)[c]	4	33.3 (0.4)[c]	4	33.3 (0.7)[b]
-	15	3	25.0 (0.6)[d]	2	16.6 (0.9)[e]	3	25.0 (0.6)[c]

* Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

On MS medium, supplement with sucrose (3%, w/v), agar (0.8%, w/v) under normal laboratory condition.

Data represented the mean of five replicates.

Table 7: Effects of MS salt solution strength on *in vitro* morphogenetic response of *in vitro* raised Kiwi nodal and foliar explants

Explants type	MS strength (%)	% resp. (±SE)*	No. of loci formed/ explants	Type of response @
Nodal explants	Water agar	-	-	No response.
	1/4th	18.8 (0.8)^{d}	1	Slow growth rate with longer internodes.
	1/2	25.0 (1.1)^{c}	2	As above.
	3/4th	31.3 (1.2)^{b}	3	Dark shoots with broad leaves, support partial stunted growth.
	Full	**58.3 (1.2)^{a}**	**6**	**Shoots dark green with few branches, broad dark green leaves and support healthy growth rate.**
Foliar explants	1/4th	12.5 (0.5)^{d}	1	Leaves smaller and curly, light green, unhealthy growth.
	1/2	18.8 (0.5)^{c}	2	Slight greenish shoots with long internodes and stunted growth.
	3/4th	25.0 (0.9)^{b}	3	Curly long leaves, light green shoots but stunted growth.
	Full	**50.5 (0.7)^{a}**	**5**	**Shoots with dark broad leaves, support healthy growth rate.**

***±SE:** Standard error from mean; Data with the same *letters* in the column for each explants are not significantly different at 5% level.

@ On MS medium supplement with BA (3 µM, v/v) for nodal explants and BA (9 µM, v/v) for foliar explants. Data represented the mean of five replicates.

Figure – 3

Figure 3: Different steps of *in vitro* organogenesis of foliar explants of *A. deliciosa*. **a.** Intact cultured leaf initiating callus development at leaf base and its terminal end; **b.** Responding leaf folded up with numerous bulbous structures formation; **c.** Young micro shoots developed from cultured leaf; **d.** Partially injured lead folded up; **e.** Numerous light green bulbous structures developed from the injured cultured leaf; **f.** Few young micro shoots formed from the cultured leaf.

Initiation of Culture from Foliar Explants and *In Vitro* Grown Nodal Segments

***Effect of cytokinins and priming*:** The micro shoots with fully expanded leaf blades were harvested from the *in vitro* grown cultures. The leaves were collected from the *in vitro* grown micro shoots and nodal explants segmented uninodal. The leaves were grouped in three parts viz., intact leaf, leaf segments, and injured/scrubbed leaves. The leaf/leaf segments and nodal segments were cultured on different strengths of MS medium (0, 1/4th, 1/2., 3/4th and full strength) fortified with BA and KN singly (0-15 µM) as presented in Table 6 and 7. The cultured leaves/segments showed distinct swelling, more prominent along the leaf base and mid ribs after 6 days of cultured (**Fig. 3a, b, c**). Varied responses were manifested with development of calli formation, somatic embryos and shoot buds development was observed within 4 weeks (**Table 6**). Of all the designed cultured, maximum number of shoot buds development was recorded with BA at concentration of 9 µM in uninjured intact leaves with 5 shoot buds accounted to ~50.5% response. Though the morphogenetic response in terms of invocation of meristematic loci were higher in leaf segments and injured leaves where as many as 7 shoots buds developed in both the cases (**Fig. 3 d, e, Table 6)**, but percent morphogenetic response was comparatively higher in intact leaves. The morphogenetic response was initiated with curling of the leaf followed by callusing of whole leaf and shoot bud formation (**Fig. 3 f**). But in case of leaf segments and injured leaves, response initiated from the cut ends followed by callusing and shoot buds formation. In all the three leaf explants types, optimum response was registered on full strength MS medium fortified with BA (9 µM), sucrose (3%) (**Table 6, 7**). Under this condition 50.5, 41.7 and 41.6% intact leaves, leaf segments, and injured leaves respectively responded positively on full strength MS medium and developed shoot buds. At lower strength of nutrient medium fewer shoot buds formed per explants and per cent response was significantly

poor compared to full strength of MS medium. The shoot buds further elongated and formed micro shoots in subsequent sub-cultures on the optimal initiation medium.

Similarly healthy morphogeneic response from *in vitro* sourced nodal segments was achieved on full strength MS medium (**Table 7)** where ~58% cultured nodal segments responded positively and developed shoot buds and callus on medium fortified with BA (3 µM) (**Fig. 4 a, b**). Under optimal condition as many as 6 shoot buds accompanied by callusing at the basal part (**Fig. 4 b)**.

Callus Mediated Regeneration

The sterilized nodal segments (**Fig. 5 a**) were also cultured on MS medium fortified with sucrose (3%) and BA-NAA and KN-NAA in combination. The cultured maintained on different initiation medium explants responded differently like callusing (**Fig. 4 b, 5 b, c)** and shoot bud formation (**Table 8**). The resultant callus from the nodal segments, foliar explants and nodal segments from *in vitro* source were maintained on the optimal initiation medium for callusing were maintained for 2-3 passages under normal laboratory condition. On this medium shoot buds developed (**Fig. 5 d, e**). On the different combinations of PGRs tested in the present study a combined treatment of BA-NAA (3 µM) resulted healthy shoot bud formation (**Table 8**). The shoot buds were singled from the initiation medium and transferred on culture proliferation and regeneration medium (**Fig. 5 f**). On the medium multiple shoots developed along with few roots in some micro shoots.

Figure – 4

Figure 4: Regeneration of plantlets from *in vitro* source nodal explants of *A. deliciosa* on MS medium containing cytokinins BA. **a.** Cultured nodal explant; **b.** Multiple shoot buds developed from cultured nodal segment. **c.** Multiple micro shoots with fully open leaves on regeneration medium; **d.** Healthy micro shoots ready for rooting.

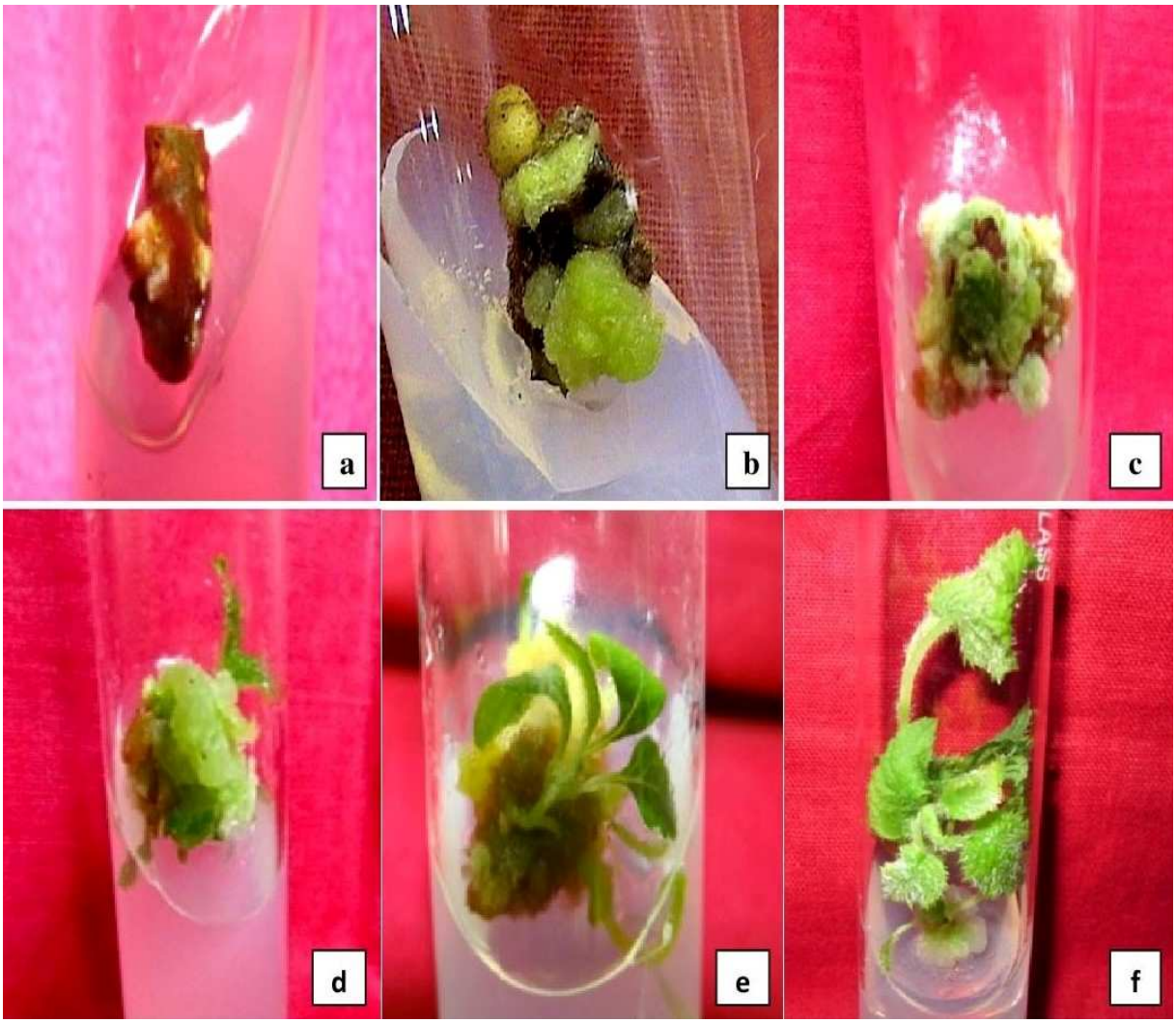

Figure – 5

Figure 5: Callus mediated plant regeneration from *in vivo* nodal segments. **a.** Swelling of node as first sign of callusing; **b-c.** Light and dark green calli developed from the nodal segments; **d-e.** Shoot buds formed from callus; **f.** Isolated micro shoot from calli mass on regeneration medium.

Table 8: Effect of PGRs on callus mediated regeneration of *Actinidia deliciosa* from *in vitro* sources

PGRs Conc. (µM) NAA	BA	KN	% response (±SE)*	Morphogenetic pathway #	Type of response @
0	0	0	-	-	-
3	**3**	**-**	**58.3 (1.1)[a]**	**Ca+Sb**	**Shoots dark green with shorter internodes, broad dark green leaves and healthy growth**.
3	6	-	50.0 (0.9)[b]	Ca+Sb	As above but slower growth rate.
3	9	-	41.6 (1.2)[d]	Ca+SE	Dark broad few leaves, few light green somatic embryos, shoots with longer internodes.
3	12	-	41.7 (1.1)[d]	Ca+Sb	Light green leaves, longer internodes and shoots light brownish to green.
3	15	-	33.3 (1.2)[f]	Ca+Sb	Shoots light greenish, long internodes, broad leaves, but unhealthy growth.
3	-	3	25.0 (0.6)[h]	Ca+Sb	Dark green shoots with broad leaves, slower growth rate.
3	-	6	41.6 (0.8)[d]	Ca+Sb	Light greenish shoots, few broad leaves and longer internodes but unhealthy growth.
3	-	9	33.3 (0.6)[f]	Ca+Sb	Longer internodes, light greenish leaves, slow growth rate.
3	-	12	25.0 (1.2)[h]	Ca+Sb	Smaller, slight brownish leaves and long internodes, shoots unhealthy growth rate.
3	-	15	16.7 (1.5)[i]	Ca+Sb	As above.
6	3	-	16.7 (1.2)[i]	Ca	Low light green and high brown calli without shoot bud developed.
6	6	-	33.3 (1.5)[f]	Ca+SE	Few light green somatic embryos, shoots light greenish with longer internodes, slow growth and unhealthy.
6	9	-	31.3 (2.1)[g]	Ca+Sb+SE	Shoots, few dark green broad leaves, moderate dark green calli and few somatic embryos.
6	12	-	43.8 (2.1)[c]	Ca+Sb	Dark greenish shoots with few broad light green leaves, long internodes and slow growth rate.
6	15	-	37.6 (1.1)[e]	Ca+Sb	As above.
6	-	3	50.0 (0.8)[b]	Ca+R	White granular patches developed on dark green calli. No shoot developed but rooted.
6	-	6	25.0 (0.7)[h]	Ca+Sb	Few shoot buds, light green broad leaves but long internodes with unhealthy growth.
6	-	9	31.3 (0.6)[g]	Ca	High light green calli with brown calli moderate without shoot formation.
6	-	12	33.3 (0.7)[f]	Ca+Sb	Light and brown calli formed, shoots with long internodes and light green leaves.
6	-	15	41.7 (0.8)[d]	Ca+Sb	Shoots dark green with broad leaves, high light green calli formed.

***±SE**: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

Ca-Callus, Sb- Shoot bud, SE- somatic embryos, R- root.

@ On MS medium supplement with sucrose (3%, w/v) and agar (0.8%, w/v).

Data represented the mean of five replicates.

Table 9: Effects of cytokinins on shoot proliferation of Kiwi from *in vitro* raised nodal explants

Cytokinin Conc. (µM) BA	KN	% response (±SE)*	Pathway of response #	No. of shoot buds/ explants	Type of response @
0	-	00.00	Ca	0	Moderate light green calli formed.
3	**-**	**58.3 (1.2)[a]**	**Ca+Sb**	**6**	**Swollen ends, shoots few dark green leaves and dark green calli. Healthy growth rate**.
6	-	50.7 (1.1)[b]	Ca+Sb+SE	4	Dark green shoot buds with few leaflets longer internodes. Light green calli, few somatic embryos.
9	-	33.3 (1.2)[d]	Ca+Sb	2	Swollen base, light green calli, few shoot buds with dark green leaves.
12	-	33.3 (1.1)[d]	Ca+Sb	3	As above.
15	-	41.7 (2.1)[c]	Ca	0	White granular and brown gelatinous calli, no micro shoots.
-	3	50.6 (2.1)[b]	Ca	0	Light and dark green calli formed.
-	6	33.3 (1.2)[d]	Ca+Sb	1	Soft brown calli, dark green shoots bud.
-	9	50.0 (1.4)[b]	Ca+Sb	2	White granular brown calli, shoot buds with dark green leaflets.
-	12	41.4 (1.4)[c]	Ca+Sb	3	Light green calli, micro shoots with dark green leaves.
-	15	33.3 (1.1)[d]	Ca+Sb	1	Light green calli, shoot buds green, long internodes.

±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

Ca-Callus, Sb-Shoot bud, SE-Somatic embryos.

@ On MS medium supplement with cytokinins (BA-Benzyl adenine, KN-Kinetin), sucrose (3%, w/v) and agar (0.8%, w/v).

Data represented the mean of five replicates.

Table 10: Effects of quality and quantity of organic carbon sources on morphogenetic response of *in vitro* raised nodal explants of *A. deliciosa*

Organic Carbons (%)	% response (±SE)*	No. of loci evoked /explants	Micro shoot lenght (cm)	Type of response @
Control	-	-	-	No response
Sucrose				
1	25.0 (0.5)^{d}	2	2.3	Micro shoots developed but stunted growth
2	41.7 (0.9)^{b}	4	3.7	Smaller shoot buds and slight stunted in growth.
3	**58.3 (1.2)^{a}**	**6**	**4.5**	**Dark green leaves, multiple shoot buds, healthy growth.**
4	33.7 (1.5)^{c}	3	3.0	Shoot buds smaller and curly with stunted growth rate.
Glucose				
1	16.6 (1.1)^{e}	2	1.2	Light green shoot buds with long, curly leaflets, etiolated.
2	33.3 (1.2)^{c}	1	1.7	Swollen base, micro shoots light greenish and unhealthy growth.
3	41.3 (2.1)^{b}	3	2.5	Shoot buds light green with high brown calli formed. Poor growth.
4	25.0 (1.6)^{d}	2	2.1	Shoots with brownish long internodes with light green leaves.
Dextrose				
1	16.7 (1.4)^{e}	2	1.0	Light green shoots with curly leaflets, long internodes.
2	25.0 (1.6)^{d}	1	1.8	Leaves smaller, long and curly, support unhealthy growth.
3	41.6 (1.3)^{b}	2	2.0	Shoots and leaves are small and light green.
4	33.3 (0.9)^{c}	3	1.6	Dark green shoots with small, curly leaves and stunted growth.

*±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

@ On MS medium, supplement with cytokinins (BA 3 µM, v/v) and agar (0.8%, w/v).

Data represented the mean of five replicates.

Culture Proliferation and Plant Regeneration

The shoot buds developed from cultured nodal segments (*in vivo* and *in vitro* sourced), leaf explants were transferred on MS medium fortified with different quality and quantity of organic carbon sources viz. dextrose, glucose and sucrose (0-4%) and PGRs like BA and KN (0-15 µM) and cultured under normal laboratory condition for culture proliferation and plant regeneration for 2-3 passages.

***Effects of PGRs*:** The cultures maintained on PGR free medium exhibited retarded growth of the shoots/micro shoots, but in the presence of PGRs recorded varied morphogenetic responses. Treatment with cytokinins KN and BA with varied concentrations (0-15 µM) of single treatment were used. Of the two, BA performed superior over KN, as many as 6 shoot buds developed per explant across the concentrations of 3 µM within 5 wks of cultured which account to ~58.3 percent of explants responded positively (**Table 9**) with healthy growth rate. The micro shoots thus produces were further sub cultured for another 2 passages for culture proliferation and multiple shoot formation (**Fig. 4c, d**) on MS medium fortified with BA (3µM) while on the same medium fortified with KN ~50% shoot buds proliferated to micro shoots bud failed to produce multiple shoots.

***Effect of quality and quantity of organic carbon on culture proliferation and plant regeneration*:** *In vitro* nodal explants and shoot buds were cultured on MS medium conjunct with different organic carbon sources viz. dextrose, glucose and sucrose at different concentrations 0-4% (**Table 10**). Of the three carbon sources tested, sucrose outperformed the other two in loci formation noted as much as 6 loci per explant and the responded explant percentage which were recorded to ~58.3%, also corresponding to the micro shoots growth length of 4.5 cm after 4 wk of cultured in 3% sucrose concentration. With higher concentration ≤3% sucrose, generated dark green micro shoots via loci

formation but leaves are smaller and curly looks. The carbon source dextrose performed the least outcome of the test recorded.

Rooting of Micro Shoots

The shoots buds developed from different cultured explants were treated for root induction under different auxins at varied levels. It was also observed that even in the regeneration medium some micro shoots developed few roots (**Fig. 4d**) but not well developed and shorter in length. About 4-5 cm long plantlets/ micro shoots with well expanded leaves from the regeneration and proliferation medium were selected for inducing rooting under following conditions:

1. Set of micro shoots was pulse treated with IBA (0-50 µM) and NAA (0-20 µM) for 0-24 hours (0, ½ h, 1h, 24h and 24h) followed by cultured in MS plain medium containing sucrose (3%).

2. While in other set the PGRs (NAA and IBA) (0-15 µM) was directly incorporated in the rooting medium for inducing of roots in the micro shoots.

3. In another set of experiment, in similar conditions, effort was made to study the effect of foliation/defoliation on *in vitro* rooting of micro shoots. For the purpose, the leaves of the micro shoots were removed using scale blade from one set of micro shoots and maintained in the rooting medium and maintained in the normal laboratory condition.

Table 11: Effect of defoliation of micro shoots and auxins on *in vitro* rooting of *Actinidia deliciosa*

Auxins conc. (µM)		Micro shoots with normal leaves			Defoliated micro shoots		
NAA	**IBA**	**% response (±SE*)**	**No. of roots/ shoot**	**Average root length (cm)**	**% response (±SE[a])**	**No. of roots/ shoot**	**Average root length (cm)**
0	0	-	-	-	-	-	-
3	-	18.75 (1.2)^{f}	2	1.2	12.50 (1.1)^{h}	1	1.4
6	-	25.00 (1.3)^{e}	3	1.5	25.00 (1.2)^{f}	3	1.5
9	-	37.50 (1.1)^{c}	3	1.9	31.25 (1.6)^{d}	4	2.8
12	-	43.75 (1.7)^{b}	4	2.0	50.00 (1.8)^{b}	5	2.1
15	-	31.25 (1.4)^{d}	3	1.7	43.75 (1.6)^{c}	3	1.6
-	3	16.66 (1.2)^{f}	2	1.3	18.75 (1.6)^{g}	2	2.2
-	6	37.50 (2.1)^{c}	4	1.9	31.25 (1.6)^{d}	4	2.0
-	**9**	**50.00 (2.1)^{a}**	**5**	**2.1**	**56.25 (1.9)^{a}**	**7**	**2.5**
-	12	43.75 (1.3)^{b}	4	2.0	50.00 (1.7)^{b}	5	1.9
-	15	31.25 (1.7)^{d}	3	1.6	43.75 (2.3)^{c}	4	2.0

Investigation has been done on MS medium supplement with sucrose (3%, v/v), agar (0.8%, w/v).

PGRs have been directly incorporated in the nutrient medium.

Data presented after 6 wk of culture

*±**SE**: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level;

Data represented the mean of five replicates.

Table 12: Effect of pulse treatment with IBA and NAA and treatment duration on *in vitro* rooting of micro shoots of *Actinidia deliciosa*

Auxins conc. (µM) IBA	NAA	Pulse treatment duration (h)	% response (±SE)*	Average of roots/ explants	Type of Response**
0	0	-	-	-	-
10	-	1/2	06.2 (0.2)	2	Low roots formation, moderate brown soft calli developed.
		1	12.5 (0.2)	4	Moderate light green and brown calli formation, low root formed.
		24	25.0 (0.3)	3	Light green calli formation, few roots.
20	-	1/2	12.5 (0.3)	2	Moderate roots formation and its length, light green calli formed moderately.
		1	31.2 (0.5)	3	Low light green calli formed. High roots formation with moderate length.
		24	37.5 (0.7)	4	Moderate roots and light green calli formation.
30	**-**	1/2	25.0 (0.3)	2	Moderate light green calli with rhizogenesis.
		1	**56.2 (0.6)**	**5**	**High rooting, moderate shoot length and low callusing.**
		24	43.7 (0.6)	4	Low callused with moderate rooting.
40	-	1/2	25.0 (0.8)	2	Low roots formation, moderate light green and brown calli formation.
		1	50.0 (0.5)	4	Rhizogenesis and high brown calli formation.
		24	37.5 (0.7)	3	Few roots and mostly callused at the base.
50	-	1/2	18.7 (0.7)	2	Low calli and root formation.
		1	43.75 (0.5)	4	Moderate roots and light green calli formation.
		24	37.5 (0.8)	3	Moderate rooting, but high callusing.
-	10	1/2	18.7 (0.6)	1	Few roots formation and moderate calli formation.
		1	18.7 (0.6)	1	Moderate callused with fewer roots.
		24	31.2 (0.6)	2	High light green calli formation, but roots formation low.
-	20	1/2	18.7 (0.3)	1	Moderate roots formation and its length. Light green callus.
		1	25.0 (0.4)	3	Moderate light green calli and roots formation, but shorter roots.
		24	31.2 (0.8)	2	Moderate rooting and green callusing.
-	30	1/2	25.0 (0.6)	2	

		1	43.7 (0.6)	3	Fewer long roots formed.
		24	31.2 (0.4)	4	Roots formation moderate, but higher light green callusing.
-	40	1/2	37.5 (0.7)	2	Moderate roots length and moderate brown calli.
		1	50.0 (0.5)	4	Low light green calli and root formation, with moderate brown callused.
		24	43.7 (0.7)	3	Moderate roots and light green calli formation.
-	50	1/2	25.0 (0.6)	2	High light green callused with moderate, few long roots developed.
		1	37.5 (0.3)	3	Light green callusing with fewer roots.
		24	31.2 (1.1)	2	Low roots and brown calli development.
					Roots development low with high light green callus.

*±**SE**: Standard error from mean., ** On MS medium supplement with agar (0.8%, w/v) and sucrose (3%, w/v).

Data compiled after 7 wk of culture

Data represented the mean of five replicates.

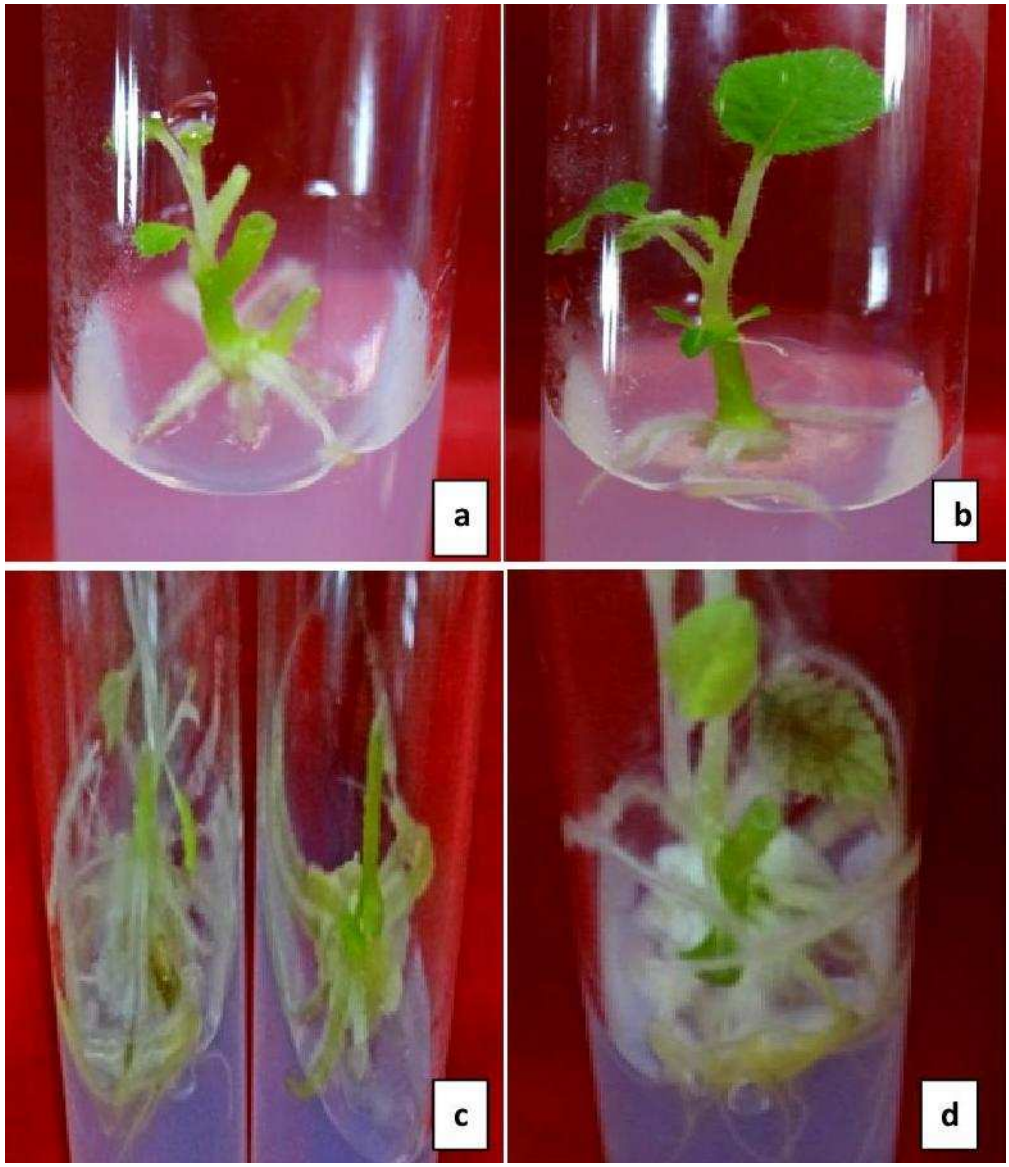

Figure – 6

Figure 6: Rooting of micro shoots of *Actinidia deliciosa.* **a.** Defoliated micro shoot on rooting medium after 3 wk of culture showing initiation of rooting; **b.** Micro shoot with normal leaves in rooting medium; **c.** Healthy rooting of defoliated micro shoots, **d.** Callus formation in the basal part of the micro shoot at higher concentration of PGRs.

Figure – 7

Figure 7: Hardening of plantlets in partially chopped coconut coir, **a.** Well rooted plantlets of *Actinidia deliciosa* transferred for hardening; **b.** New shoots and roots developed during the hardening stage; **c.** Plantlets showing formation of secondary roots from stem; **d.** Hardened plantlets after 7 wk of hardening ready for transfer to potting mix.

During the present study, it was found that defoliation of micro shoots before transferring to rooting medium was effective as defoliated micro shoots resulted more roots and better root growth (**Table 11**, **Fig. 6a, b**). In the defoliated micro shoots there was no or very little callusing and healthy root morphology while, normal micro shoots (foliated shoots) resulted fewer roots and callused at the base (**Fig. 6 c, d**). Of the different quality and quantity of PGRs (NAA and IBA) used for rooting of micro shoots, IBA was found suitable over NAA for induction of rooting and root growth. While, NAA fortified medium supported fewer root formation and poor root growth. Each micro shoots of *A. deliciosa* resulted as much as 7 roots and average length of each root was measured as 2.5 cm from defoliated micro shoots on medium enriched with 9 µM IBA. While medium with 9 µM NAA supported 5 roots per micro shoots and root length was 2.1 cm (**Table 11**).

Besides incorporation of PGRs directly in the rooting medium, micro shoots were also pulse treated with IBA and NAA (0-50 µM) for 0-24 h and maintained on MS plain medium. Of the two PGR tested at different levels for different periods, pulse treatment with IBA (30 µM) for one h supported better rooting where as many as 5 roots per micro shoot developed in ~56% of cultured micro shoots (**Table 12)**. Prior to inoculations, micro shoots were pulse treated with different auxins (IBA and NAA) at varied concentrations (1-50 µM) for different durations (0-24 hr). In the treatment with IBA, at a concentration 30 µM of 1 hr pulse treated resulted 5 rootlets per micro shoot where ~56% micro shoots responded positively (**Table 12**) while, pulse treatment with NAA, optimum response was registered at 40 µM concentration for one h gives considerable affects, where ~50% micro shoots resulted rooting and 4 roots per micro shoot developed within 7 wk of culture. The well rooted micro shoots were subjected to *in vitro* hardening. The well rooted micro shoots plantlets were taken out from the rooting medium (**Fig. 7 a)** and transferred to hardening condition.

Table 13: Effects of different substrata on Kiwi plantlets in hardening

Substrata*	% survival (±SE**)	No. of adventitious root(s) formed per plant	Increase on plant height (cm)	Type of response
Agar gelled medium	30.43 (1.2)	1	0.9	New root development absent, and stem and branches looks light greenish and much soft.
Chopped coconut coir	**82.60 (2.4)**	**3**	**1.0**	**Greenish and granular white spots calli. New branches developed with no new root formation**.
Saw dust	56.52 (2.1)	1	0.7	No new root formation and few branches with light green, soft calli.
Forest litter	69.56 (2.4)	1	0.4	New branches developed and No new root formation.

* Added 1/4th MS salt solution fortified with sucrose (1%).
** ±SE: Standard error from mean.
Culture condition: Normal laboratory condition.
Data collected after 7 wk of culture.
Data represented the mean of five replicates.

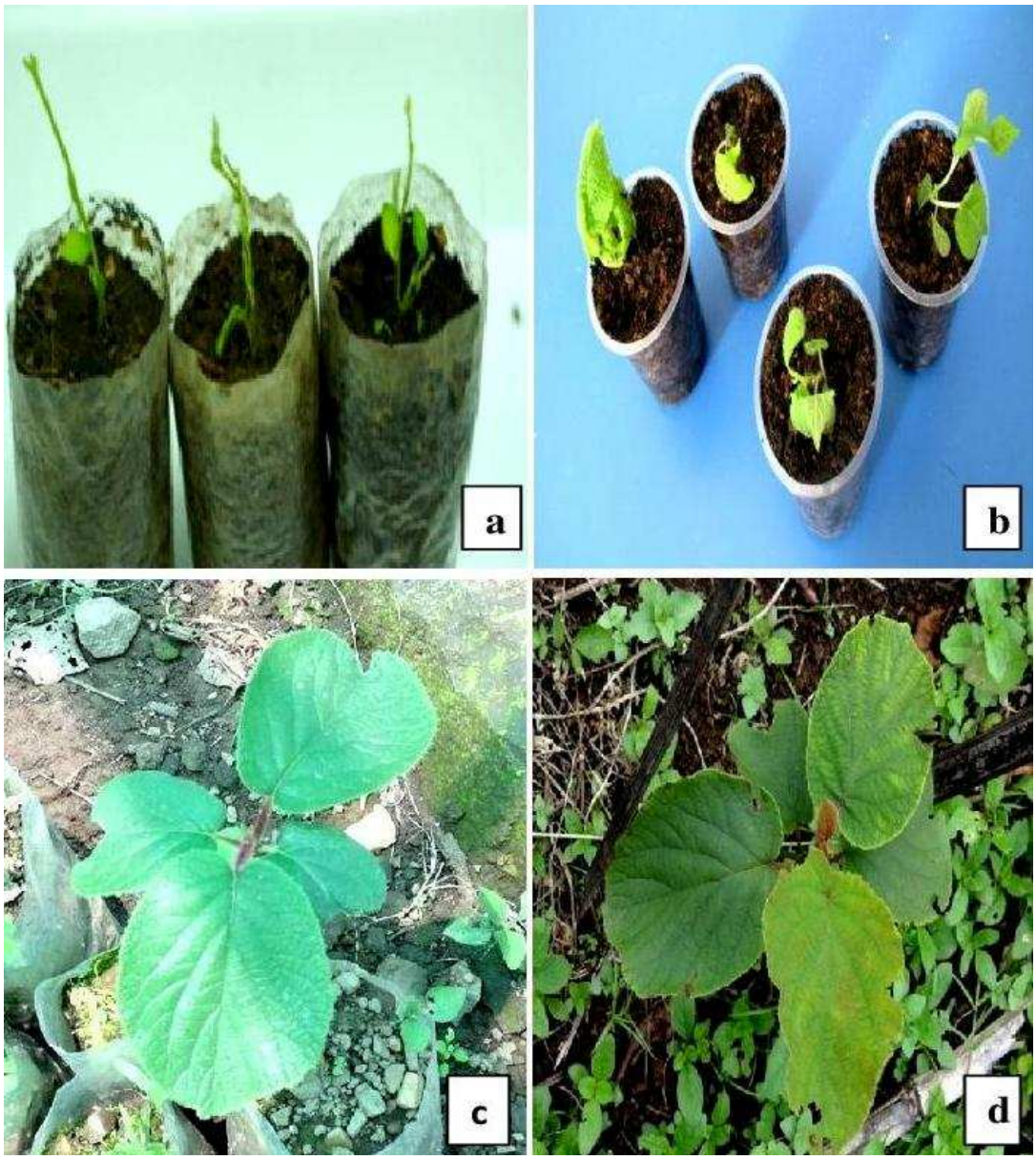

Figure – 8

Figure 8: Potting and transplantation of regenerates of *Actidinia deliciosa*. **a.** Defoliated hardened plantlets transferred to potting mix; **b.** Transplants started producing new leaves in the pots; **c.** Kiwi plantlets developed dark green expanded leaves shoot in potting mix; **d.** Transplant established in the field.

Hardening of Regenerates and Transplantation to Potting Mix

The well rooted plantlets were taken out from cultured vials, washed with luke warm water to remove any intact trace of agar remains in the roots, and transferred it into liquid 1/4th strength MS medium containing 1% sucrose without any PGRs (**Fig. 7 a**). Sets of processed dry coconut coir, forest litter and saw-dust were used as substrata, and maintained for 5-6 wk in the normal laboratory conditions followed by exposing the cultures to the normal day light for 4 h a day. Within 6-7 wk of transfer in the hardening condition the hardened plantlets started producing new shoots, roots and secondary roots from the stem which is the characteristics of kiwi plant in natural condition (**Fig. 7 b).** The cultures exposed to normal day light was hardier compare to the plants maintained only to the normal culture condition. It was observed that the cultures exposed to normal day light produced secondary roots from the stem, glands and scale in the stem (**Fig. 7 c, d)** which was achieved in about 82% regenerates within 7-8 wk (**Table 13**). Of the different substrata tested in the present study with *A. deliciosa* chopped coconut coir was found to be most suitable for hardening where ~82% regenerates survived under hardening condition. Plantlets turned slightly light green in color and smaller young new leaflets with considerable increased in its height (1.3 cm). Compared to coconut coir, agar gelled medium, saw dust and forest letter were found to inferior for the purpose (**Table 13**).

The suitably hardened plants were taken out from the culture vials and transferred to potting mix contained mixtures of soil, sand and decaying wood power in the ratio 1:1:1 ratio in poly bags (15x12 cm) (**Fig. 8 a**). The potted plants were maintained for 5-6 wk under partially controlled conditions of light Ca. 70% sunlight. Regular interval of water was feed, once a wk for 5- ½ months, followed by exposed for 2 hr interval a day under natural day light for 1 wk, where finally left under natural day light. Within 2-3

wk of transfer in the potting mix the new leaves started emerging (**Fig. 8 b).** Established transplants were maintained for 2-3 months in the poly house where leaves turned dark green (**Fig. 8 c)** before transferring to the cultivated plot **(Fig. 8 d)**. About 200 plants were transferred to the field of which ~62% transplants survived after two months of transfer.

Discussion

Initiation Culture

***Initiation of culture from nodal explants*:** Several woody plant species have been mass propagated by tissue culture technique especially fruits and ornamental trees. For initiation of aseptic culture with explants of *in vivo* source season of explants collection is the foremost and necessary step. Selection of explant becomes a primary task for successful propagation as different vegetative organs or tissues become more morphogenetic in culture particularly during the actively growing seasons in nature. Thus identification of right collection season is very crucial for *in vitro* establishment of explants and inducing morphogenesis. Plant growth is controlled by different physiochemical processes and interaction with PGRs. This may further influenced by environmental factors such as sunlight, humidity, temperature etc.

Actinidia deliciosa is cultivated commercially for fruits in the orchards. However, the big obstruction in the expansion of growing area is the scarcity of the quality planting materials. The species is propagated by cuttings/grafting or budding. Grafting is a general commercial practice as *Actinidia* cuttings roots readily from hardwood or softwood cuttings but takes long times for rooting. As a result, limited number of plants is produced, which is relatively small in relation to a huge demand. Therefore, the study was carried out to produce quality planting materials through micropropagation method. The study speculated on varied response encounter with

significant differences under the influence of various factors such as effects of PGRs, basal medium strength, light intensity, inorganic nutrients both macro as well as micro nutrients, organic carbon sources, antioxidants and substratum etc.

In the present investigation, culture was established from nodal explants from *in vivo* source. The *in vitro* morphogenetic response differed significantly from the explants collected during different seasons. Many plant species are found varied responses with the seasons, length of day light, temperature, humidity, rainfall and so on which directly linked with its active physiochemical factors triggered in the active growth phase (Deb and Arenmongla, 2012, 2014; Deb *et. al.,* 2014) . In the present study ~75% nodal segments responded positively cultured during November followed by December but the least response was obtained during the months of July and August. There was a gradual reduction in explant response following the month of January, February till June and least during July. Similar investigation was shown on other woody plant from the work of observed the best response in shoot regeneration and multiplication when the culture was initiated during the winter season in *Stevia* (Roy *et. al.,* 2004). Likewise seasonal variation of *in vitro* shoot induction was also reported in many other plant materials (Deb and Arenmongla, 2012; Deb *et. al.,* 2014). Several workers like Sutan *et. al*. (2010) investigated the influence of seasonal effects on micropropagation in *Fragaria* and *Potentilla* and reported the significant variation in response to seasons between both the varieties.

During summer with high relative humidity, rainfall, temperature and more light intensity shows less affective on *in vitro* morphogenetic response of nodal explants due to more leaching of phenolics in the medium but during winter browning of media was comparatively less and favored morphogenesis. The first notable sign of explants response was swelling of explants followed by cracking from the cut ends and

remarkably by protrude out axillary bud from its position, and after 3 wk of sprouting buds few dark green leaflets with oblong, healthy dark green shoot buds developed. On the contrary, explants collected during summer, particularly in the months of July and August affects only 8% of cultured explants responded after 17 days of cultured and mostly of explants degenerated.

***Effects of antioxidant on initiation of culture*:** A common problem on establishment of *in vitro* culture is browning of medium due to release of phenolic compounds from the explants, where morphogenetic response declines significantly due to tissue necrosis and browning. The release of exudates is partially or completely controlled by use of antioxidants. In the present study phenolic exudation could substantially control by use AC, CA and PVP. Active response with complete cease of browning medium was noted on medium enriched with PVP (300 mg L^{-1}) but at concentrations above 300 mg L^{-1} did not support healthy growth in all three antioxidants used. Study also revealed that AC and CA were inferior over PVP in controlling browning of culture. However, antioxidants like PVP and CA in combination at 200 mg L^{-1} and 100 mg L^{-1} respectively could successfully control browning in *Pinus kesiya* (Deb and Tandon, 2004). Use of antioxidants like AC acts on plant morphogenesis due to its irreversibly large area of adsorption capacity (large surface area) of inhibitory compounds in the medium affecting several physiological changes, such as stimulatory and inhibitory activities (Baker *et. al*., 1992). Browning of medium or darkening of culture media due to release of naturally present substances release, were adsorption along with vitamins, metal ions and plant growth regulators, gaseous ethylene etc (Thomas, 2008). It also affects the medium p^H, alter the ratio of its components and subsequently influence plant regeneration (Druart and Wulf, 1993), but it is ineffective toward glucose, sucrose, dextrose, meso-inositol etc in the medium (Yam *et. al*., 1990).

***Effects of organic carbon sources*:** In the present investigation, organic carbon such as sucrose, dextrose and glucose were used. The use sucrose as carbon source as food was studied by several investigators (Omar *et. al*., 1992; Deb and Arenmongla, 2012; Deb *et. al.,* 2014). While some investigators used glucose and fructose in the cultivation of date palm tissue (Abdel-Rahim *et. al*., 1998), but they did not demonstrate effective in promoting development.

Organic carbon, a vital role in plant as an energy sources for various metabolic activities and signify a key element for development *in vitro* (Caldas *et. al*., 1998). Quality and quantity of carbon affects different morphogenetic responses were observed in the present investigation. In the study, test was conducted to optimize the effects of different level of sucrose, dextrose and glucose influence on growth and development of plantlets regeneration. Sucrose has been used as a major carbohydrate source in the induction medium. It is the main source of energy for *in vitro* plant tissue cultures as this has insufficient autotrophic ability. Sucrose not only acts as an external energy source but also help to maintain osmotic potential of the culture (Nowak *et. al*.,, 2004; Siwach *et. al.,* 2011) which would permit the absorption of mineral nutrient present in medium, essential for optimal proliferation. Studies on *A. deliciosa* in the present investigation highest organogenesis frequency (75%) was obtained in medium having 3% (w/v) sucrose where within 5 days of culture morphogenetic response initiated on MS medium. The frequency declined considerably at lower concentrations of organic carbon sources with consistent findings of earlier workers (Pandey and Bhojwani, 1999; Deb and Arenmongla, 2012; Deb *et. al.,* 2014). Of the three carbon sources used, glucose supported least response in respect to its initial response. However, higher concentration (above 3%, w/v) of sucrose did not favor the initiation culture. Reinert *et. al.* (1977) suggested that sucrose level of 2-5% favor for rice anther culture. Naik and Nayak

(2005) used various concentration of sucrose 30, 60 and 90 g L^{-1} for *in vitro* bulblet production in *Ornithogalum virens* and found 60 g L^{-1} of sucrose was most effective. A significant effect of carbon source concentration has been reported by Garcia *et. al.* (2002) in *Olive*. Earlier workers have found that plant morphogenesis vary at different developmental stages on different sucrose concentrations (Temjensangba and Deb, 2005; Pongener and Deb, 2009; Deb and Arenmongla, 2012; Deb *et. al.*, 2014).

***Effect of PGRs on organogenesis*:** The method of induction of plant organs such as roots, shoots or leaves arise directly from the meristem or indirectly from callus. Plant regeneration involves the callus production and differentiation of adventitious meristem into organs by altering the concentration of plant growth hormones in nutrient medium. Skoog and Miller (1957) first who demonstrated that high ratio of cytokinins to auxin stimulate the formation of shoots in tobacco callus while high auxin to cytokinins ratio induced root regeneration.

In *A. deliciosa* direct shoot bud was successfully induced from the nodal explants cultured on initiation medium containing 6 µM BA under controlled conditions of organic carbon and antioxidant in the MS basal salts solution. Under this condition ~75% of cultured explants responded with vigorous growth and developed dark greenish leaves. From the cultured nodal explants, as much 4 micro shoot buds developed after 5 wk of culture.

Beside singly treatments of BA and KN, the synergistic effect of BA-NAA and KN-NAA were tested for induction of morphogenesis. The combination of BA with NAA and KN with NAA revealed the variation in their effect on explant response with shoot bud development as well as calli formation. The BA combined with NAA at concentrations of 6 µM and 3 µM respectively responded optimally where as many as 3 shoot buds developed after 5 wk of culture. Whereas, combined treatment of KN and

NAA treatment at 9 µM and 3 µM respectively shows significant result with 2 micro shoots but inferior to combination of BA and NAA in shoots number couple with more calli development. But the combined treatments of BA-NAA and KN-NAA were far inferior compared to singly treatment of BA and KN. In the past the positive synergetic effect of BA and KN with auxins such as NAA, IBA, and IAA in regeneration and proliferation programme where earlier workers reported in many horticultural plant species like pear on shoot tip and stem segment of peach (Hassanen and Gabr, 2012).

Leaf culture

In the study with foliar explants concentrations of different cytokinins and the priming on leaf explants had significant influence on morphogenesis. Instead of size and part of the leaf explants in the present study focus was on effect on partially scrubbed or injured leaf explants with segmentation. It was found that, BA was a better option over KN for organogenesis in foliar explants on MS medium fortified with sucrose (3%). In the past similar response was also reported in *Datura stramonium* (Amiri and Kazemitabar, 2011) and in *Plectranthus barbatus* (Thangavel *et. al*., 2011) where BA was reported to be superior over TDZ for shoot induction. The present study outcome on organogenesis from intact, non-injured leaf explant which produced 4-5 shoot buds affecting ~50.5% of cultured leaf explants while on injured explants, much higher shoot buds formation 6-7 shoot buds formed but responded only ~41.6% of explants. This shows that injured explants have higher cytokinin stimulatory effects on its tissue due to direct contact with the medium containing cytokinin (BA at 9 µM) and plant regeneration from leaf tissue.

In the past many workers reported the effect of leaf treatment, leaf segmentationon *in vitro* morphogenesis. Hamidoghli *et. al*. (2011) reported the effect of disc orientation as factor in determining the rate of shoot formation in *Primula*

heterochroma. However, Meng *et. al*. (2004) found that the abaxial leaf surface was more competent for shoot regeneration in 'Marion' blackberry. Lo *et. al*. (1997) suggested that the lower shoot regeneration on the leaf surface touching the medium is possibly the result of poor gas exchange and in such condition while Thorpe (1988) reported that lack of O_2 may result in inadequate supply of free energy for shoot induction and differentiation from the covered tissue.

***Effect of PGRs on callus mediated regeneration*:** The callus that developed from subsequent cultures of leaf and nodal explants cultures were maintained and induced shoot bud induction using PGRs particularly BA, KN and NAA in combination, where it was recorded ~58.3% of callus regenerated into shoots with healthy growth rate in the medium containing BA 3µM with 3µM NAA. However, KN with NAA at a concentrations of 6 µM and 3µM respectively yielded considerable result affecting ~50% of cultured explants which is significantly lower than combined treatment of BA and NAA.

Culture Proliferation and Plant Regeneration

***Effects of cytokinins*:** Plant growth regulators play an essential role in determining the development pathway of plant cells and tissues in culture medium. Besides natural compounds, synthetic chemicals with similar physiological activities developed correspond to the natural ones (Pierik, 1997). Quality and quantity of hormones determined the type of culture established or regenerated. Higher concentration of auxins favored root formation, whereas with cytokinins it promoted shoot regeneration and under balance leads to development of undifferentiated cells, callus. It also well demonstrated by many workers (Schuller *et. al*., 2000; Dobrev *et. al*., 2002).

The shoot buds obtained from different pathways such as direct organogenesis from nodal explants culture, from leaf explants and callus of *A. deliciosa* were isolated

and cultured in proliferation medium and maintained for 2-3 passages. In the present study using 3 µM BA singly yielded a maximum 6 micro shoots. Cytokinins in planting materials (nodal explants) were initially treated at higher dose at 6 µM of BA concentrations during the initiation culture which may have higher inducing affects in shoot proliferation of proliferated nodal explants. Meenakshi *et. al*. (2011) showed the efficient pathways for regeneration in banana using BA and other cytokinin.

***Effects of medium strength*: T**here is no standard basal medium so far for all the taxa *in vitro* technique. The MS basal salts solution is the most widely used media for shoot regeneration and callus formation. In the present study, it was found remarkable effects on different strengths of MS basal medium. Response was shown better result with full strength on initiation culture as it contains higher nitrates contains than other media. The other strength/concentrations performed inferior in culture proliferation. In the past many workers demonstrated the effect of nutrient media on culture proliferation and plant regeneration in different species viz., in date palm (Taha *et. al*., 2007), *Larix decidua* (Bonga, 2004), *Prunus insititia* L. (Andreu and Marin, 2005), *Elaeis guineensis* (Muniran *et. al*., 2008).

***Effect of organic carbon*:** Carbon is essential for all the plants as an energy give source, in various forms used in *in vitro* culture. Sucrose was reported to act as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots. Three different carbon sources at varied concentrations were used for study the morphogenetic response. Sucrose was found to be superior over other carbon sources at 3% (w/v) concentration, where explant could able to produced 6 micro shoots. However, the other two sugars supported 3 shoot buds formation coupled with slow in growth rate. Several workers demonstrated the role of sucrose on *in vitro* culture in many plant species viz., in *Cinnamomum tamala* (Deb *et. al*., 2014); in banana culture (Placide *et. al*., 2012).

Similar study was also done on *Musa* sp. in micropropagation (Morfeine, 2014). Thus all the studies performed with different prospective has concluded that sucrose serve the most efficient for plantlets regeneration and somatic embryos induction.

Rooting of Micro Shoots

The effects of plant regulators on rooting have been extensively studied in various plant species. For *in vitro* rooting of micro shoots of *A. deliciosa*, different concentrations of IBA and NAA were used. The first root formation was recorded after three wk of culture on medium fortified with 1.0 mg L^{-1} NAA while other concentrations could invoke rooting of micro shoots only after ~6 wk of culture and better roots response was observed at 9 µM IBA concentration in ~56.25% defoliated micro shoots compare to only ~50% response from foliated micro shoots after 5 week of culture. Under optimum condition as many as 7 roots per micro shoots resulted from the defoliated shoots against only 5 roots from the foliated shoots. Besides number of roots per micro shoot accompanying healthy plant growth. Further it was found that the micro shoots cultured with leaves promoted callusing at the base with fewer roots, but in the micro shoots without leaves/defoliated micro shoots there was no or very little callus formation in the base. There was a reverse correlation between callus formation and rhizogenesis and indicates that root development is direct organogenesis. This is possibly due to fact that the undifferentiated tissue/callus may interfere in development of normal vascular connection in the roots. It is believed that in the defoliated shoots, the endogenous auxin level was lesser compared to foliated shoots due to removal of leaves. Under this condition callus formation was negated and the available auxin was used for invocation of root meristem. This statement is based on the fact that at higher auxin content medium there was callusing from the base even from the defoliated micro shoots. In the past the stimulatory effect of IBA on rooting was reported in *E. agallocha*

(Rao *et. al*., 1998), *Melia azedarach* (Thakur *et. al*., 1998), *Bambusa vulgaris* (Aliou *et. al*., 2006). The findings of present study with *A. deliciosa* is in agreement with the past reports (Vengadesan *et. al*., 2002; Nanda *et. al*., 2004)) on superiority of IBA for rooting over other auxins.

Besides directly incorporating PGRs in the nutrient medium, the micro shoots were also pulse treated with different concentrations of IBA and NAA (0-50 µM) for 0-24 h followed by transferring onto MS hormone-free medium containing sucrose (3%) and maintained under normal laboratory condition. Of the different concentrations of growth regulators and treatment periods tested for the purpose, the micro shoots pulse treated for one h at 30 µM IBA supported as many as 5 roots per micro shoot where ~56% shoots registered response; while, ~43% shoots supported 3 roots per shoots with 30 µM NAA pulse treated for one h. This finding suggests that for *in vitro* rooting of regenerated shoots of *Actinidia deliciosa* IBA is an ideal hormone. Naija *et. al*. (2008) found that period of 5 days in rooting medium enriched with IBA was sufficient to induce 97% rooting in micro-cuttings of *Malus* × *domestica* Borkh. Root stock MM 106, but exposure to periods longer than five days results undesirable effects on shoots, such as callus formation and leaf necrosis. Similarly Metivier *et. al*. (2007) reported that the percentage of rooted shoots of *Cotinus coggygria* Mill increased with increasing time of exposure to the IBA containing medium, from 40% after 1 day of exposure, to 100% after 5 days.

Hardening/Acclimatization and Field Transfer of Regenerates

In vitro raised plantlets must experience the flavor of natural environment to get adjusted with its system through trial and error. For *in vitro* grown regenerates/plantlets it is important that the plants get adapted to new conditions from controlled conditions which are tagged with limited gas exchanges, high relative humidity, low light intensity

and up-taking of carbon sources (sugars) in the culture medium (Pospisilova *et. al*., 1999; Deb and Imchen, 2010; Deb and Pongener, 2013). Abnormal characteristics of *in vitro* plantlets in leaves of unusual stomatal structure, malfunction, less development of cuticle or receptacle wax on the surface which influences the factors contributing to excessive water loss resulting in the high mortality of plantlets or regenerates (Hazarika, 2006). Under control, energy was readily available by external carbon sources fortified in medium along with abundant moisture for physio-chemical activity. Many workers found that a reduction in relative humidity *in vitro* culture increased plant survival rate after transferring to soil (Deb and Imchen, 2010; Deb and Pongener, 2013). Similarly, Lamhamedi *et. al*. (2003) shows that decrease in relative humidity induced the epicuticular wax formation of plantlets. However, there are many factors affect the plantlets under greenhouse like planting bed or substrate, plantlets age and shading level (Rodrigues *et. al*., 2005; Hassanpanah and Khodadadi, 2009). Survival rate of *Prunus domestica* plantlets was found affected by the shoot height rather than the number and length of roots (Padilla *et. al*., 2003). While, Thomas (2008) reported that 3 weeks old *Vitis vinifera* plantlets was more advantageous with enhanced vigor than the 4 and 5 week-old plantlets.

In the present investigation well rooted plantlets were transferred from rooting medium to hardening condition onto $1/4^{th}$ MS basal salt solution with 1% sucrose (w/v) but without any plant growth regulators. In the nutrient medium partially chopped coconut coir, saw dust and forest litter were used as substrata and maintained in the normal condition for 7-8 wk. In the last two weeks the culture vials were exposed to the normal day light for 4 h followed by normal laboratory condition. It was found that the cultures exposed to normal day light during the hardening process were dark green, harder and produced secondary glands and secondary roots in the shoots which are

characteristics of Kiwi. These plants adapted better in the potting mix after transfer. Of the different substrata used for hardening purpose, regenerates from partially chopped coconut coir registered better survival rate of ~82.60%, however under control condition of agar gelled medium, it supports 30.43% survival rate which indicate that agar as substratum makes the rooted plantlets much softer, indicated from the shoots turning light greenish.

Well-hardened rooted plantlets were transferred to plastic bags containing a mixture of vineyard soil, sand, decayed wood powder (2:1:2 ratio) with few holes punctured. Under the condition, the first two week of transplantation, watered once a wk the plantlets were kept covered by a polyethylene tent to provide high humidity but allow sufficient light. Later the polyethylene cover was removed progressively whenever leaves appeared to be wet. The polyethylene covers were withdrawn completely after 3 weeks of transferred. After 3 weeks, the plants were transferred to partially shade for 1 wk and finally the acclimatized plants were shifted to field conditions, where 62% of them having survived with sprouting few broad hairy dark green leaves developed after 6 wk of field transferred. Thus the growth characteristics of plants raised *in vitro* did not show any significant morphological variations from those of the natural habitat.

Conclusions

Actinidia deliciosa popularly known as ‘Kiwi fruit’ is an economically important horticultural species grown in different parts of the world including Nagaland for its fruits (Kiwi fruits) which has high commercial value. Conventionally kiwi is propagated through stem cutting but rooting of stem cuttings takes long times and due to this the commercial cultivation is at times hampered due to non availability of the clonal planting materials. During the present study *in vitro* micropropagation protocols of *A. deliciosa* are developed from *in vivo* and *in vitro* grown nodal segments and leaf explants. During

the study the different physic-chemical properties controls the micropropagation of the species from different explants has been optimized starting from explants collection, culture initiation to transfer of regenerates to the potting mix. The protocols developed will be a boon for the local horticulturists for production of clonal planting materials for commercial cultivation.

Chapter - 3

Micropropagation of *Saurauia punduana* Wallich (Actinidiaceae)

Angiosperm plants are most efficient group of plant in reproduction due to their specialized morphological advantages over others. Reproductive organ plays an important role in its existence largely by seeds potentiality for perpetuation through favorable environmental condition in natural system. However, unfavorable stress strike many a times where natural means of reproduction significantly drop as natural systems are partially govern by many factors where its component do not remains static. In this condition, reproductions through natural means are not the path for its successful establishment. Thus artificial means such as plant biotechnology programme like plant tissue culture technique play an alternative means for its survival.

Past studies have demonstrated that protocols necessary for shoot growth and development vary considerably among genotypes and requirement of nutrient media and

plant growth regulator differ from species to species. For initiation of culture and plant regeneration, various explants types like shoot tips, nodal explants; foliar explants, axillary tips, roots etc. could be successfully used in large number of plant species. Plantlets from axillary buds or shoots have proved to be the most reliable method of true-to-type *in vitro* propagation. The two methods used either single or multiple nodes culture for which dominance of shoot apical meristem stimulated by growth regulator, particularly cytokinins.

Nutrient media like 'Woody Plant Medium' (WPM) (Lloyd *et. al*., 1988) and MS medium (Murashige and Skoog, 1962) were used for many species. In most micropropagation studies, Benzyl adenine (BA) has been effective cytokinins for shoot growth either singly or in combination with low concentrations of kinetin (KN) and auxin like indole-3- acetic acid (IAA), NAA, IBA etc. Thomas (2008) reported that, incorporation of antioxidants like activated charcoal found useful in controlling organic compounds exuded by explants in culture, although at times undesirable binding of growth regulators, particularly BA with antioxidants in culture.

Saurauia punduana Wallich belongs to family Actinidiaceae is an economically important plant. IUCN in 2004 reported that *Saurauia punduana* is identified in the Red List of threatened Species, assessment done by China Plant Specialist Group 2004. The species are perennial, grow mostly in the wild in open secondary and tertiary forest, and are identified as endangered in the natural habitat. The species experience high pressure to their existence similar with many other species in the Noth Eastern region of India due to various anthropogenic activities. The threat warrants developing alternate propagation technique and production of clonal planting materials.

Materials and Methods

Plant Materials Collection and Processing

Nodal explants: Newly flushed young shots of ~6-9 wk old were collected from the mature plants for consecutive 1-2 years at alternate months. The leaves, scales etc. were carefully cleared off from the young shoots. The materials were washed with running tap water, and wrap in fresh banana leaf section with sprinkle water to keep moistened and stored at 4-5°C in the refrigerator for 1-2 days. Materials were collected from the wild, open secondary forest of Lumami village, Zunheboto district of Nagaland near the Department of Botany, Nagaland University, Lumami.

***Seed explants*:** Fruits of different age range from 3-8 wk old were collected from the matured plants growing in wild. The fruits were plucked from the branches, kept in poly bags moistened with sprinkled water to avoid desiccation. Fruits were washed with running tap water and stored at 4-5°C in refrigerator for 1-2 days. Seeds were extracted from the sterilized fruits and cultured on germination medium.

***Leaf explants*:** Young leaves were excised from the *in vitro* raised micro shoots after 5-8 wk of sprouting in the laminar flow cabinet. The leaves were soaked in water till used.

Sterilization of Explants

Processed young shoots for nodal explants and fruits were washed with running tap water and soaked in diluted ‘labolene’ (a commercial laboratory detergent, 1:100 ratio, v/v) for 4-5 min. and scrubbed with soft brush except fruits. Shoots and fruits were washed under running tap water for 3-5 min. subsequently surface sterilized with aqueous solution of mercuric chloride (0.3%, w/v for nodal explants and 0.1% for fruits) for 2-3 min. and rinsed 4-5 times with sterilized distilled water. Following this they were dipped into 70% (v/v) ethanol for ~30 sec followed by rinsed with sterilized distilled water 2-3 times. Surface sterilized explants were kept moistened with sterilized distilled

water in the laminar flow cabinet until cultured in the medium. From the sterilized fruits in the laminar flow cabinet, seeds were extracted and gathered, moistened with water till used for cultured in the initiation medium.

Tissue Culture Medium

For culture initiation, full strength MS medium fortified with agar (0.8%, w/v) as gelling agent was used. The nutrient medium was fortified with three different organic carbon sources viz. dextrose, glucose and sucrose at different concentrations (0-4%, w/v), plant growth regulators (PGRs) benzyl adenine (BA), kinetin (KN) and α-naphthalene acetic acid (NAA) at different concentrations (0-15 µM) either singly or in combination. The nutrient medium was also conjunct with different antioxidants viz. activated charcoal (AC), citric acid (CA) and polyvinyl pyrollidone (PVP) at 0-700 mg L^{-1}.

The p^H of the medium was adjusted 5.6 using 0.1 N NaOH and 0.1 N HCl. About 12-13 ml medium was dispensed in each borosilicate test tubes (size: 25x150 mm) and pluged with nonabsorbent cotton before autoclaving at 121°C for 15-20 min at a pressure of 1.05 kg cm^{-2}. The autoclaved medium was solidified and used for initiation of culture only after 3 days of preparation.

Initiation of Culture

Nodal explants: Uninodal segments were excised from the sterilized young shoots and cultured on MS medium fortified differently with organic carbon sources, PGRs, antioxidants. For initiation of culture different concentrations of cytokinins like KN, BA at different concentrations (0-15 µM, v/v) either singly or in combination was used. For each treatment 20 nodal segments were cultured. The cultures were maintained at 25±2°C temperature under cool white fluorescent light at 40 µmol m^{-2} s^{-1} intensity with alternate 12/12 hr light and dark phases.

***Seeds explants*:** Seeds extracted from the sterilized fruits were cultured on MS medium fortified with sucrose at different concentrations (0-5%, w/v). Different PGRs such as NAA, 2, 4-D, KN and BA (0-4 µM, v/v) are used either singly or in combination Cultures were maintained at 25±2˚C under cool fluorescent light at 40 µmol $m^{-}2$ s^{-1} intensity for 12/12 hr (light/dark) photo periods for seed germination.

Leaf explants: Young *in vitro* leaves were isolated from the *in vitro* raised shoots and cultured on MS medium fortified with sucrose (3%, w/v), agar (0.8%, w/v) without further sterilization. The intact leaves were cultured in the medium supplemented with different levels of cytokinins BA and KN (0-15 µM, v/v) singly. For each treatment 20 leaves were cultured and the cultured were maintained at 25±2˚C under cool fluorescent light at 40 µmol $m^{-}2$ s^{-1} intensity for 12/12 hr (light/dark) photo periods.

Callus Mediated Morphogenesis

The calli developed in the different initiation medium with *in vitro* sourced nodal segments and foliar explants were separated from the shoot buds, micro shoots and maintained cultured separately on MS medium fortified with sucrose (3%), PGSs (BA and KN (0-15 µM) either singly or in combination for inducing morphogenesis. The cultures were sub-cultured at regular interval. The shoot buds developed in the callus mass were separated and transferred to regeneration medium for plant regeneration and culture proliferation.

Experimental Design and Statistical Analysis

Completely randomized experimental design was followed in all the experiments. In each experiment 20 explants were cultured for each treatment and all the experiments were repeated five times. The cultures were maintained at 25±2^{o}C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} intensities and 12/12 hr each (light/dark) photo period unless mentioned otherwise. All the cultures were sub-cultured at 5-6 wk interval. The

cultures were monitored regularly and data collected at weekly interval. *In vitro* response was evaluated based on the percentage of explants responded and number of propagules formed in the culture after specific period of time (as stated in the table) and data was expressed as the mean of replicates ± standard error. Data was further analyzed by one way ANOVA using the General Linear Model procedure in SAS Statistical Package (SAS Ins.) and standard deviation from mean was worked out and compared using Least Significant Difference (LSD) test at $p \leq 0.05$.

Regeneration of Plantlets and Culture Proliferation

The shoot buds/micro shoots and callus produced from the cultured explants were maintained for another two passages under optimum initiation medium for further development. The micro shoots/regenerates were separated and transferred to fresh MS medium containing sucrose (3%), BA and KN at different concentrations (0-15µM either singly or in combination) for culture proliferation and plant regeneration.

Rooting of Regenerates

The regenerated shoots/micro shoots of ~3-4 cm with well expanded leaves were harvested from the regeneration medium and subjected to inducing rooting. For the purpose MS medium was supplemented with sucrose (3%, w/v), different auxins such as IBA and NAA at varied concentrations (0-15µM) gelled with agar.

Hardening of the Regenerated Plantlets

***Substrata for hardening medium*:** Dry coconut husk, forest leaf litter were chopped into small pieces (~0.1-0.5 cm size). These were soaked in laboratory detergent for 1-2 hr and rinsed with water several times and sun-dried for 4-5 hr. Besides coconut coir and forest litter, saw dust was also employed as alternative substratum. Saw dust was collected from the local carpentry workshop. The substratum was sun-dried and soaked in laboratory detergent for 2-3 hr and rinsed thoroughly under running water followed by

sun dried. About 10-12 grams of chopped coconut husk, forest litter and saw dust were transferred test tubes separately and autoclaved at 121° C temperature for 30 min at the pressure of 1.05 kg cm^{-2}.

About 12-15 ml 1/4th strength MS liquid medium enriched with sucrose (1%) was dispensed in all the test tubes and autoclaved under 121° C temperature at 1.05 kg cm^{-2} pressures for 15-20 min. These medium filled test tubes with different substrata were used for hardening of the rooted regenerates.

***Hardening of the regenerated plantlets*:** Well rooted micro shoots were selected for *in vitro* hardening. Well rooted plantlets from rooting medium were transferred to the test tubes containing different substrata and 1/4th MS medium and were maintaining in the normal laboratory conditions for 6-7 wk before transferring to community potting mix.

Transplantation of Regenerates to Community Potting Mix

Preparation of potting mix: With the help of spate, soil was dug out from the thick mud on the ground, hand-picked the litter contained in the mud and crushed into small pieces (size: 0.1-0.3 cm) with flat surface wooden block on clean cemented floor. Crushed soil was well mixed with fine sand, collected from the building construction site. Dried rotten wood powder from local forest was collected, thoroughly mixed with the mixer of sand and soil in the ratio 2:1:2. The collected potting mix materials were transferred in poly bags (size: 15x12), stored it in shade for 1 wk and used for plantation of hardened plantlets.

Transplantation of regenerates: The partially hardened plantlets from the cultured vials were finally taken out and transferred onto perforated small community pots containing potting mix containing soil, sand, decayed wood powder mix with forest litter. Transplanted pots were covered with holed transparent poly bags and watered alternate days with 1/5th MS salt solution once in wk for two wk. The covered poly bags were

removed from the plantlets and maintained in partial shade ca.50% for 2-3 wk and finally exposed to normal day light followed by transferring to the wild.

Results

Initiation of Culture

Nodal Segments

***Seasonal effects of explants collection*:** Optimization of season of explants collection from the field grown plants is the prerequisite step for *in vitro* establishment of any plant species. Likewise, the nodal segments of the species (*Saurauia punduana*) were periodically collected all the months throughout the year. Findings of the present investigation reveal that seasonal variations of explants collection greatly influence the *in vitro* morphogenetic responses of nodal explants (**Table 14**). In the period of study, during the months of July-August, mid-summer found to be least responding where only 6.2% of explants after 12 d of culture responded. In most of the cases tissues turned necrotic and degenerated subsequently. Better morphogenetic response was registered during the early winter where within 5-6 days cultured nodal explants started responding by way of producing multiple shoot buds/meristematic loci. While, during December optimum responses were noted, with much earlier response of after 4-5 d of inoculation (**Table 14**) which affects as much as 56.25% of nodal explants with well developed axillary buds with little calli formation.

It was found that during July-August plant growth in nature was healthy but phenolic exudation was more compared to the physiologically unfavorable season like just before onset of winter. Nodal explants collected during winter exudates lesser phenolic compounds in the medium and resulted better morphogenetic response. Of the different seasons tested plant materials collected during November-December registered optimum morphogenetic response by way of producing shoot buds.

Effects of antioxidants: Field grown nodal segments released phenolics in the nutrient medium which resulted necrosis of explants and subsequent degeneration. It was necessary to incorporate antioxidant in the initiation medium for invocation of morphogenetic response. For initiation of culture, MS medium was conjunct with different antioxidants such as PVP, citric acid and activated charcoal range from 0-700 mg L^{-1} to reduce the browning of medium (**Table 15**). Incorporation of antioxidants in the medium improved the condition. Of the different quality and quantity of antioxidants used in the present study, PVP at a concentration of 300 mg L^{-1} found to be most suitable (**Table 15**). Compared to PVP, other two antioxidants (AC and CA) under performed as far as controlling of browning is concerned across the concentrations tested. Though both AC and CA could reduce the phenolic oxidation, did not support healthy initiation of culture. At lower concentrations of all the three antioxidants failed to reduce the browning of medium while at higher concentrations (>300 mg L^{-1}) though the browning could be stopped but affected adversely the morphogenetic response of the nodal segments.

Table 14: Seasonal effect of *Saurauia punduana* nodal explants collection from *in vivo* sources (9 wk after sprouting) on *in vitro* morphogenetic response

Month of collection	Time of initial response (d)*	% responses (±SE)#	Type of response@
January	A	43.7 (0.8)^{c}	Swollen at the cut ends, shoot buds dark green with broad leaves and no browning of medium.
February	B	37.5 (0.7)^{d}	No browning of medium, dark green shoot with slight curly leaves.
March	B	31.2 (2.1)^{e}	Cut ends swelled and cracked, shoots light green accompanied by poor growth, low browning of medium.
April	C	25.0 (0.9)^{f}	Low browning of medium, shoots light green and curly, poor growth.
May	C	18.7 (0.7)^{g}	Same as above
June	C	12.2 (0.9)^{h}	High browning and most of the explants degenerated subsequently.
July	C	12.2 (0.6)^{h}	As above
August	C	06.2 (0.3)^{i}	Browning of medium and tissue necrosis, light green shoot buds but poor growth.
September	B	18.7 (1.4)^{g}	Long internodes, light green leaves and partially support growth with low browning.
October	A	37.4 (2.1)^{d}	As above
November	A	50.0 (1.6)^{b}	Less browning of medium, healthy growth of shoots and dark green broad leaves.
December	**A**	**66.7 (2.1)^{a}**	**Healthy shoots developed without browning of medium but slower growth rate**.

Flushed shoot of ~7 wk old; * A: 0-6 d, B: 6-12 d, C: 12-18 d; On MS medium supplemented with sucrose (3%), BA (9 µM, v/v) and PVP (300 mg l^{-1}); # Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level; Data represented the mean of five replicates.

Table 15: Effect of antioxidants on *in vitro* culture initiation for nodal explant of *Saurauia punduana* from *in vivo* source

Antioxidants & Conc. (mgl^{-1})			% response (±SE)[#]	Morphogenetic response[@]
AC	CA	PVP *		
0	0	0	-	Release phenolic compounds.
100	-	-	25.4 (1.4)[d]	Moderate exudation of phenolics but explants degenerated.
300	-	-	41.6 (1.7)[b]	Controlled browning, shoots buds dark green leaves but smaller.
500	-	-	33.3 (1.4)[c]	Dark green shoots, curly dark green leaves, controlled browning.
700	-	-	16.6 (1.7)[e]	Shoots light green, curly leaves with long internodes, controlled browning.
-	100	-	33.3 (1.5)[c]	No medium browning, light green curly leaves and smaller, stunted growth.
-	300	-	41.6 (0.6)[b]	As above.
-	500	-	25.2 (0.9)[d]	Shoots dark green, smaller with slight curly leaves, controlled medium browning but stunted growth.
-	700	-	25.0 (1.0)[d]	Control medium browning but brownish shoots with curly leaves and poor growth.
-	-	100	41.6 (2.1)[b]	Shoots dark green, leaves smaller, long internodes, controlled browning.
-	**-**	**300**	**66.7 (2.1)[a]**	**Healthy growth rate with dark green shoots, broad green leaves and no browning of medium.**
-	-	500	33.3 (0.9)[c]	Shoots dark green, slight curly leaves, slow growth.
-	-	700	25.6 (1.5)[d]	Few leaves, light green smaller, brownish long internodes, no browning of medium.

* AC- activated charcoal, CA- citric acid, PVP- polyvinyl pyrollidone.

#±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

@ On MS medium supplement with agar (0.8%, w/v), sucrose (3%, w/v) and BA (9 µM, v/v).

Data represented the mean of five replicates.

Table 16: Effect of organic carbon source on *in vitro* morphogenetic response of *saurauia punduana* nodal explant from *in vivo* source

Carbon source* (%)			Time of initial response (d)	% response (±SE)#	Morphogenetic response@
D	G	S			
0	0	0	-	-	-
1	-	-	13	12.5 (1.1)^{g}	Long internodes with light green leaves, poor growth.
2	-	-	10	18.7 (1.7)^{f}	Small light green leaves, stunted growth.
3	-	-	9	25.0 (1.3)^{e}	Shoots with dark green slight curly leaves, slightly stunted growth.
4	-	-	10	31.2 (1.1)^{d}	Curly smaller leaves, long internodes, poor growth.
-	1	-	10	18.9 (1.4)^{f}	Brownish to light green smaller leaves and curly, support unhealthy growth.
-	2	-	8	25.6 (1.7)^{e}	Light green shoots with long internodes.
-	3	-	9	31.5 (1.9)^{d}	Dark green smaller leaves, slow growth rate.
-	4	-	10	31.5 (0.9)^{d}	Leaves dark green and broad, and stunted growth with long brownish internodes.
-	-	1	11	31.2 (1.7)^{d}	Slightly stunted growth with smaller light green leaves.
-	-	2	8	43.7 (1.6)^{c}	Dark green broad leaves with brownish long internodes but slower growth rate.
-	**-**	**3**	**6**	**66.7 (2.1)^{a}**	**Support healthy growth rate with dark green shoots and broad leaves.**
-	-	4	9	50.0 (2.1)^{b}	Dark green broad leaves, slower growth with brownish long internodes.

* D- dextrose, G- glucose, S- sucrose.

#±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

@ On MS supplemented with agar (0.8%, w/v) and BA (9 µM, v/v).

Data represented the mean of five replicates.

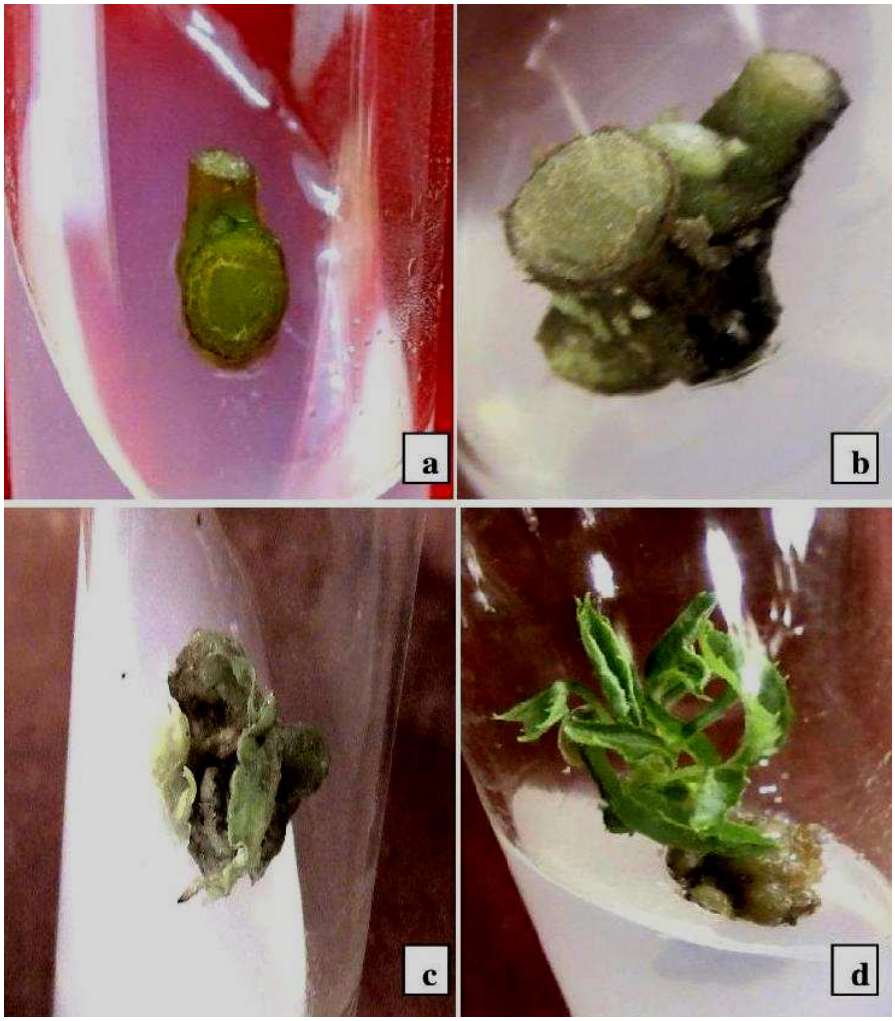

Figure – 9

Figure 9: Direct organogenesis from *in vivo* nodal explant of *S. punduana*. **a.** Swelling of nodal explant on initiation medium; **b.** Formation of meristematic loci from the swelled nodal zone; **c.** Few shoot buds invocked from the loci; **d.** Shoot buds with well developed leaves.

***Effects of organic carbon sources*:** Organic carbon plays important role on *in vitro* establishment of cultures. For initiation of culture from nodal segments nutrient medium was fortified with different organic carbon sources (dextrose, glucose and sucrose) at 0-4%. There was no morphogenetic response on organic carbon controlled media and cultures degenerated **(Table 16**). Of the three organic carbon sources tested at various concentrations, dextrose and glucose were found to be least effective compared to sucrose for *in vitro* morphogenesis. The morphogenetic response was very poor on medium fortified with dextrose and glucose as organic carbon source across the concentrations. Of the different concentrations of different organic carbons tested in the present study, 3% concentration is found to be most effective for glucose and sucrose while 4% dextrose fortified medium supported optimal morphogenic response. Under the given culture conditions, highest morphogenetic response was about 31% on media enriched with dextrose (4%) and glucose (3%) (**Table 16**). But in sucrose (3%) fortified medium ~66% of cultured nodal explants responded positively within 6 days of culture initiation and resulted dark green shoot buds with broad leaves. At lower concentrations of sucrose fewer explants responded and produced stunted shoot buds while at higher concentration (4%) slightly etiolated shoots developed.

***Effects of growth regulators*:** It was perquisite to incorporate at least one of the growth regulators to invoke morphogenesis from the cultured nodal segments. On PGRs controlled medium, all the explants degenerated without any response. The nodal segments were cultured on MS medium enriched with sucrose at (3%), PVP (300 mg L^{-1}). The nutrient medium was also further supplemented with two different cytokinins (BA and KN) singly at concentrations 0-15 µM. Within 6 days of culture initiation the first sign of *in vitro* morphogenic response was recorded as swelling of nodal segments and the invocation of meristematic loci from the nodal zone (**Fig. 9 a, b**). It was found that morphogenic response on BA and KN fortified media was significantly different

under identical condition (**Table 17**). Of the two PGRs tested, BA was found to be superior over KN for initiation of *in vitro* culture from nodal segments. Further it was found that KN fortified medium supported fewer shoot bud formation per node and callusing of the explants. Highest morphogenic response (50%) was recorded on medium containing 6 µM KN where only 3 shoots buds from each node. Under the similar culture condition on medium containing BA supported 4 shoot buds formation. In the present study under the given conditions, optimum response was registered on medium fortified with BA at 9 µM where ~66% of explants responded positively and as many as 5 shoot buds formed from each node without any accompanying callusing of the explants. Within 3-4 wk of culture these meristematic loci converted into shoot buds with distinct leaves (**Fig. 9 c**).

The resultant shoot buds were maintained for another two passages on optimum initiation medium (BA at 9 µM) for further culture differentiation and proliferation where the healthy shoot buds resulted with fully expanded leaves (**Fig. 9 d**).

Table 17: Effect of quality and quantity of cytokinins on *in vitro* organogenesis of nodal explants of *Saurauia punduana* from *in vivo* source

PGRs Conc. (µM) BA	KN	Time for initial response (d)	% response (±SE)*	Pathway of response#	No. of loci /node	Morphogenetic response@
0	0	-	-	-	-	-
3	-	8	25.0 (1.1)^{e}	Sb+Ca	2	Shoots dark green with broad leaves, slight light green callus.
6	-	6	33.1 (2.1)^{d}	Sb	4	Dark green shoots and broad leaves.
9	-	**6**	**66.7 (2.1)^{a}**	**Sb**	**5**	**Shoot buds with healthy growth.**
12	-	7	41.6 (1.4)^{c}	Sb+Ca	3	Dark green shoots, slow growth, light green callus.
15	-	10	16.6 (1.5)^{f}	Ca+Sb	2	Light green calli; shoot buds with broad leaves, long internodes.
-	3	9	33.3 (1.7)^{d}	Sb+Ca	2	Smaller leaves with long internodes, light green calli formed.
-	6	6	50.0 (1.8)^{b}	Ca+Sb	3	Shoots with long internodes, dark green calli, and growth moderate.
-	9	7	41.2 (1.1)^{c}	Sb	4	Smaller leaves and crowded, support moderate growth.
-	12	7	33.6 (0.9)^{d}	Sb+Ca	2	Light green leaves, long internodes, and slightly stunted growth.
-	15	12	25.0 (1.3)^{e}	Sb+Ca	1	As above.

*Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level.

Ca- callus, Sb- shoot bud.

@ On MS medium supplemented with sucrose (3%, w/v), agar (0.8%, w/v) and PVP (300 mg l^{-1})

Data represented the mean of five replicates.

Table 18: Effect of cytokinins on morphogenetic response of foliar explants of *S. punduana* from *in vitro* source

PGRs Conc. (µM) BA	KN	% response ±SE[#]	Response pathway[$]	No. of shoot bud (s) formed /explant	Type of response @
0	0	-	-	-	No response
3	-	31.25 (1.2)[c]	Ca+Sb	2	Smaller dark green leaves and shoots with stunted growth rate and moderate brown callused.
6	**-**	**56.25 (1.5)[a]**	**Ca+Sb+SE**	**4**	**Shoots healthy growth with dark green broad leaves shoot buds with few somatic embryos formed. Low dark green and brown calli formed.**
9	-	43.75 (1.5)[b]	Sb +Ca	3	Dark broad leaves with slower growth rate shoots, less branched and moderate light green calli formed.
12	-	31.25 (2.1)[c]	Ca+Sb	1	High light green calli with long internodes shoot buds, smaller leaflets and not support healthy growth.
15	-	25.00 (2.1)[d]	Ca+Sb	1	Curly smaller leaflets, high brown and moderate light green calli, stunted growth
-	3	18.75 (1.1)[e]	Ca	1	High brown calli formed with dark green shoots, broad leaves and long internodes.
-	6	31.25 (1.7)[c]	Ca+Sb	2	Shoots dark green leaves with long internodes, moderate light green and brown calli developed
-	9	25.00 (1.6)[d]	Ca+Sb	2	Dark green leaflets with slight curly and smaller shoots. Moderate light green and brown calli developed.
-	12	18.75 (0.9)[e]	Ca+Sb	1	Slower growth rate with smaller light green leaves with high brownish calli formation.
-	15	12.50 (0.8)[f]	Ca+Sb	1	Stunted growth with smaller curly leaflets and high brown calli.

#±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level

$ Ca-callus, Sb- shoot bud, SE- somatic embryo.

@ On MS medium supplemented with sucrose 3% (w/v), agar 0.8% (w/v).

Data represented the mean of five replicates**.**

Table 19: Effects of PGRs on morphogenetic response of *in vitro* raised foliar explants of *Suarauia punduana*

PGRs Conc. (µM) NAA	BA	KN	% response (±SE)*	Response pathway#	No. of shoot bud (s) formed/explant	Type of response @
0	0	0	-	-	-	No response
3	3	-	25.0 (1.1)[f]	Ca	0	Light greenish brown calli.
3	6	-	37.5 (2.1)[d]	Ca+ Sb	2	Shoots dark green with broad leaves, light green calli developed.
3	**9**	**-**	**54.2 (1.5)[a]**	**Ca+ Sb**	**4**	**Moderate dark green callusing accompanied by shoot buds with dark green leaves.**
3	12	-	43.7 (1.6)[c]	Ca+ SE	3	Light green calli with somatic embryos.
3	15	-	31.2 (2.1)[e]	Ca+ Sb	2	Shoots with curly light green leaves, with white granular calli.
3	-	3	50.0 (2.2)[b]	Ca	0	Brown and light green calli.
3	-	6	43.7 (1.6)[c]	Ca+ Sb	1	Moderate light green calli, shoots dark green with broad leaflets.
3	-	9	50.5 (2.4)[b]	Ca+ Sb	2	Broad dark green leaflets with long internodes shoots, slight callusing.
3	-	12	50.0 (2.6)[b]	Ca+ Sb	2	Mostly light green calli, shoot buds with long internodes.
3	-	15	37.5 (1.8)[d]	Ca+ Sb	1	Shoots with green curly leaflets, light green calli.
6	3	-	31.2 (1.7)[e]	Ca	0	Moderate brown to light green calli.
6	6	-	43.7 (1.4)[c]	Ca+ Sb	1	Shoots with curly leaflets with long internodes, poor growth.
6	9	-	50.2 (2.1)[b]	Ca+ Sb	3	Both light and brown callused high with etiolated shoot buds with smaller leaflets.
6	12	-	43.7 (1.5)[c]	Ca+ Sb+R	2	Light greenish white granular calli, brown rootlets, light green shoots.
6	15	-	31.4 (0.8)[e]	Ca+ Sb	1	Shoot buds with dark green leaflets, light green calli.
6	-	3	25.0 (1.2)[f]	Ca	0	Light green calli.
6	-	6	37.5 (1.1)[d]	Ca	0	As above.
6	-	9	50.0 (1.7)[b]	Ca+ Sb	2	Light green leaflets, stunted growth.
6	-	12	31.2 (1.7)[e]	Ca+ Sb	1	Shoot buds with light green curly leaflets, long internodes, green calli.
6	-	15	18.7 (1.9)[g]	Ca+ Sb	1	Light green calli with light greenish shoot buds, unhealthy growth rate.

* Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level; # Ca-callus, Sb- shoot bud, SE- somatic embryo; @ On MS medium supplemented with sucrose (3%, w/v), agar (0.8%, w/v); Data represented the mean of five replicates.

Figure – 10

Figure 10: *In vitro* culture of *S. punduana* leaf and regeneration of plantlets. **a.** Segmented foliar explant cultured on initiation medium showing curling; **b.** Cultured leaf explant swollen up and developed numerous dark green bulbous structures; **c.** Shoot buds and somatic embryos developed from the bulbous structures; **d.** Micro shoots developed with dark green leaflets after 7 wk of culture; **e.** Few healthy micro shoots developed with distinct dark green leaves ready form rooting.

Foliar explants

Foliar explants were harvested from the *in vitro* raised shoots formed from the cultured nodal segments. Foliar explants were divided into different grouped viz. 1. Partially-injured/scrubbed intact leaves, 2. Injured/scrubbed segmented leaves, 3. Intact leaves without injury, 4. Segmented leaves without injury. The foliar explants were cultured on nutrient medium fortified with sucrose (3%), BA and KN (0-15 µM) singly for *in vitro* morphogenesis (**Table 18**). The foliar explants were also treated with combined treatment of NAA (3 and 6 µM) and either BA (3-15 µM) or KN (0-15 µM) (**Table 19**). Within 4-5 days of culture, the cultured leaves/segments started curling (**Fig. 10 a)** as a first sign of response and within 2 wk time developed bulbous/globular structures from the curled explants (**Fig. 10 b**). These structures subsequently converted into shoot buds, or callus (**Fig. 10 c**). Morphogenetic response was very distinct in all the cases along the leaf base and mid ribs. However, injured intact leaves responded comparatively faster (after 5 days of cultured) than others. Varied responses were manifested with development of calli formation, somatic embryos and shoot buds development.

Effects of cytokinins: In the absence of cytokinins, foliar explants failed to invoke organogenesis. Of all the culture types, maximum number of shoot buds development was recorded with BA at concentration of 6 µM in the medium with 3% sucrose, where as much as 56% of explants responded positively where as many as 4 shoot buds per leaf accompanied by callusing and somatic embryo like structures (**Table 18**). While, KN fortified medium registered poorer response.

When compared the performance of both the PGRs, it was found that KN supported more light brownish callus formation with lesser shoots development at lower concentrations (<6 µM), while at higher concentrations (>6 µM) dark green calli

developed. Within 4-5 wk of culture these shoot buds differentiated on the same initiation or on fresh medium and converted into elongated shoots buds with distinct leaves (**Fig. 10 d**). These shoot buds differentiated further on regeneration medium and were used for rooting (**Fig. 10 e**).

***Effect of combined treatments of PGRs on in vitro foliar explants*:** Foliar explants show varied response under the influence of PGRs (BA-NAA and KN-NAA) at different concentrations. In general the combined treatment of cytokinins (BA and or KN) and auxin (NAA) found to be inferior for organogenesis from foliar explants over single treatment of cytokinin. Explants cultured on NAA rich medium along with BA and Kn across the concentration supported more callus formation (**Table 19**). Optimal morphogenic response (54%) was recorded on MS medium fortified with NAA (3 µM) and BA (9 µM).

Callus mediated regeneration from nodal segments

The shoot buds developed on the initiation medium were transferred to fresh optimum medium for culture proliferation and plant regeneration. Further the callus developed from the cultured explants was maintained separately on fresh medium for callus proliferation (**Fig. 11 a, b)**. On the subsequent subculture shoot buds developed on the same medium (**Fig. 11 c**). The resulted shoot buds thereafter transferred on fresh medium for micro shoot development (**Fig. 11 d**).

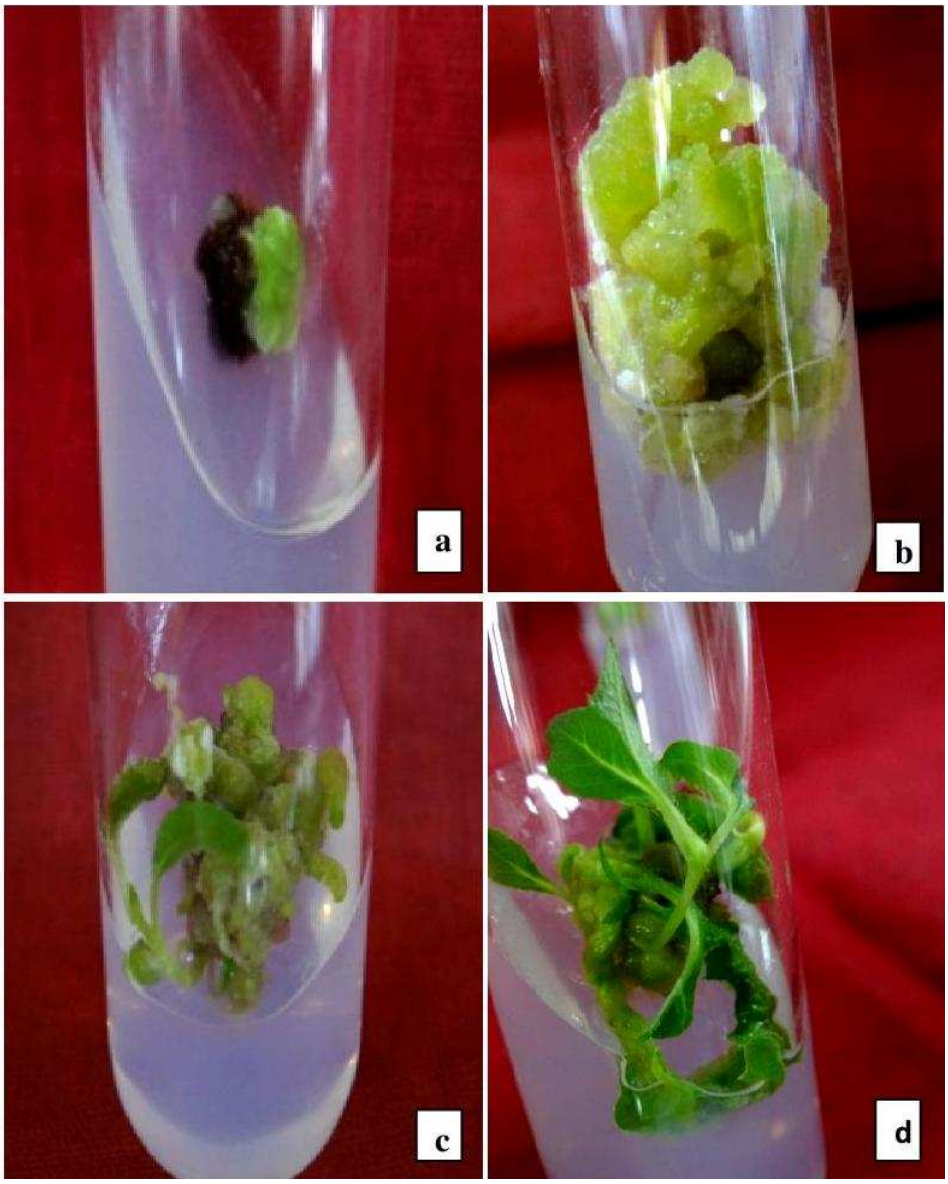

Figure – 11

Figure 11: Regeneration of *Saurauia punduana* from *in vivo* nodal explant of through callus culture. **a.** Cultured nodal explant swollen and developed dark green mass of callus; **b.** Dark green callus turned light green calli mass after 6 wk of culture; **c.** Few young shoot buds formed with few dark green leaflets; **d.** Healthy micro shoots with dark green leaves transferred in fresh regeneration medium.

Table 20: Effects of quality and quantity of auxins on rooting of micro shoots of *S. punduana*

Auxins Conc. (µM) IBA	NAA	% response (±SE#)	No. of root (s) formed/ micro shoot	Average root length (cm)	Type of response @
0	0	-	-	-	No response
3	-	12.5 (1.1)[e]	2	1.1	Moderate brown and low dark green calli, long internodes. Scanty root hairs.
6	-	25.0 (1.2)[c]	2	1.3	Brownish internodes, broad light green leaves and high light green calli formed.
9	-	31.2 (1.6)[b]	3	1.7	Low brown and dark green calli with dense brownish root hairs.
12	**-**	**43.7 (1.2)[a]**	**5**	**1.0**	**Light green broad leaves, moderate brown calli and much lesser root hairs.**
15	-	25.0 (1.1)[c]	2	0.7	Lesser root hairs with smaller light green long and curly leaflets.
-	3	12.5 (1.1)[e]	1	0.9	Poor root hairs formation, high brown calli and smaller leaflets.
-	6	18.7 (1.2)[d]	3	1.2	Moderate light green calli and root hairs formation with curly smaller leaflets.
-	9	31.2 (2.1)[b]	4	1.0	Root hairs formation moderate and low brown calli.
-	12	25.0 (1.7)[c]	2	1.0	Long smaller curly leaves and moderate root hairs formation.
-	15	18.7 (1.4)[d]	1	0.5	Poor root hairs formed and smaller light greenish shoots with curly leaflets and stunted growth rate.

#±SE: Standard error from mean; Data with the same *letters* in the column are not significantly different at 5% level;

@ On MS medium supplemented with sucrose (3%, w/v), agar (0.8%, w/v)

Data represented the mean of five replicates**.**

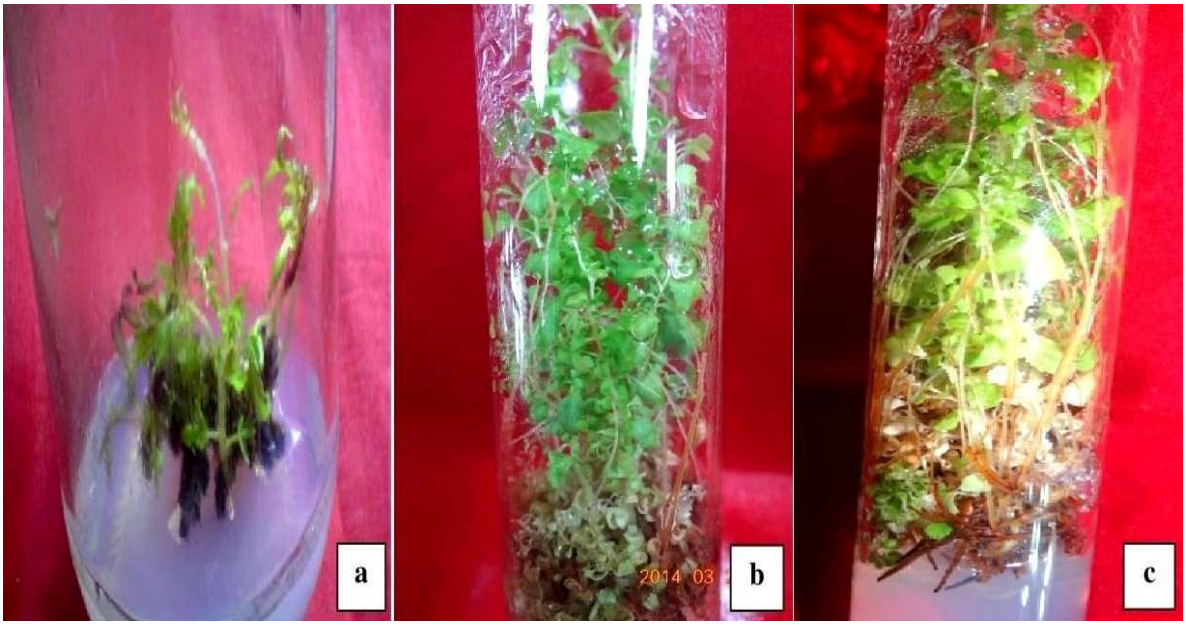

Figure – 12

Figure 12: *In vitro* rooting of micro shoots of *Saurauia punduana*. **a**. The isolated micro shoots cultured on rooting medium started producing roots after one wk of culture; **b**. Micro shoots developed rootlets with distinct secondary roots; **c.** Well rooted micro shoots ready for hardening.

Plant Regeneration and Culture Proliferation

The shoot buds micro shoots developed from both the nodal and foliar explants were transferred on MS regeneration and proliferation culture medium containing different organic carbons (sucrose, dextrose and glucose) at varied concentrations (0-4%). The cultured were conditioned under the influence of different concentrations (0-15 µM) of cytokinins (BA and KN) singly for another 2-3 passage from initiation cultured. The cultures in absence of growth regulators exhibited retarded growth and degenerated subsequently. The shoot buds proliferated in subsequent subcultures on MS medium containing sucrose (3%) and BA (9 µM) and converted into micro shoots with well expanded leaves (**Fig. 9 d, 10 e, 11 d**). These micro shoots were harvested from the regeneration medium and was transferred on rooting medium.

Rooting of Micro Shoots

Micro shoots developed on proliferation medium were transferred to rooting medium **(Fig. 12 a)**. For inducing rooting of micro shoots two different auxins (IBA and NAA) were incorporated singly at concentrations of 0-15 µM. Incorporation of one of the auxins was necessary for inducing roots. Of the two auxins tested in the present study, IBA was found to be more effective for inducing roots. Rooting was done on sets of foliated and defoliated micro shoots, and observed better responds in defoliated micro shoots. Of the different concentrations of IBA tested, optimum rooting was achieved on MS medium fortified with 12 µM from defoliated micro shoots, where as many as 5 roots per shoot developed in ~43% micro shoots (**Table 20 and Fig. 12 b**). At this concentration the plants were healthy with green broad leaves and healthy root hairs. In contrary NAA enriched medium supported fewer roots per shoot. The well rooted plantlets were selected for *in vitro* hardening (**Fig. 12 c**).

Figure – 13

Figure 13: *In vitro* hardening of *S. punduana* regenerates and transplantation to potting mix. **a.** Well rooted plantlets transferred in partially chopped coconut coir for hardening; **b.** Hardened plantlets transferred in potting mix and maintained in polyhouse (after 3 wk of transfer); **c.** Potted regenerates maintained in the natural day light ready for field transfer.

Hardening of Regenerates and Transplantation of Regenerates

The rooted plantlets were taken out from cultured vials, washed with luke warm water to remove any intact trace of agar remains. These plantlets were transferred to culture vials containing 1/4th MS liquid medium containing 1% sucrose without any PGRs. Sets of processed dry coconut coir, forest litter and saw-dust were used as substrata during hardening process, and maintained the plantlets for 5-6 wk in the normal laboratory conditions (**Fig. 13 a**). During the process of hardening new shoot buds sprouted. It was found that plants were dark green accompanied by formation of new roots and secondary/adventitious roots from the stem. Of the different substrata tested in the present study with *S. punduana* chopped coconut coir was found to be most suitable for hardening where ~72% regenerates survived under hardening condition and no new shoots developed during the hardening process but increase .8 cm in height. Compared to coconut coir, agar gell medium saw dust and forest letter were found to inferior for the purpose.

The suitably hardened plants were taken out from the culture vials and transferred to potting mix contained mixtures of soil, sand and decaying wood power in the ratio 1:1:1 ratio in poly bags (15x12 cm) (**Fig. 13 b**). The potted plants were maintained for 5-6 wk under partially controlled conditions of light Ca. 70% sunlight. Regular interval of water was feed, once a wk for 5-6 wk, followed by exposed for 2 hr interval a day under natural day light for 1 wk, where finally left under natural day light. Within 2-3 wk of transfer in the potting mix the new leaves started emerging**.** Established transplants were maintained for 2-3 months in the poly house where leaves turned dark green (**Fig. 13 c)** before transferring to the cultivated plot. About 225 plants were transferred to the field of which ~70% transplants survived after two months of transfer.

Discussion

Initiation of Culture and Culture Proliferation

Nodal explant

In most of the plant species the *in vitro* morphogenesis depends on the type of explants used for culture initiation. Thus it is very important to select the right explants type and source for culture initiation. The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators and the metabolic capabilities of the cells. Therefore, *in vitro* propagation of woody plants has always been difficult due to problems with establishment of aseptic cultures, microbial contamination and varied nutritional medium requirements (Agrawal *et. al*., 2002; Deb *et. al.,* 2014). The most commonly used tissue explants are the meristematic ends of the plants like the stem tip, auxiliary bud tip and root tip. These tissues have high rates of cell division. *In vitro* organogenesis from vegetative tissues by appropriate *in vitro* manipulations is the ultimate objective in micropropagation technique.

Several explants related factors influence the organ development potential of the cultured tissue (Benson, 2000). These include growth conditions, donar plant physiology and genotype of the source plant etc. However, this observation did not receive much attention until the late 1990s (Lakshmanan *et. al*., 1995, 1996).

Seasonal effect of Saurauia punduana nodal explants collection from in vivo sources: In the present study with *S. punduana* establishment of aseptic cultures was not possible round the year. Many plant species are found varied responses on *in vitro* culture with the seasons, length of day light, temperature, humidity, rainfall which shows impact on *in vitro* response. Explants collection season is one of the important factors in the establishment and growth of *in vitro* cultures (Bhatt and Dhar, 2004). In the present

study nodal explants collected after September exhibited better response but optimum *in vitro* morphogenetic response was achieved from the nodal segments collected during December where browning of medium was significantly lesser and cultures were healthy. It indicates the winter shows less secretion of endogenous phenolic compounds which results browning of medium and tissue necrosis. In other species few centimeter long shoots were found to be ideal for culture initiation such as litchi (Chandra and Padaria, 1999) and guava (Mishra *et. al*., 2007). While, nuinodal segment was ideal in Aonla micropropagation (Mishra *et. al*., 1998).

Effect of antioxidants on in vitro culture initiation: Most of the hardwood species produce phenolic compounds after wounding. Accumulation of phenolic compounds in medium adversely affects the growth and survival of *in vitro* explants. This problem has not been resolved satisfactorily and continues to be a bottle neck in development of efficient regeneration protocol. Browning of medium reduces or cease effectively with the use of antioxidants. Thomas (2008) reported that browning medium affects adsorption of nutrients like vitamins, metal ions, PGRs etc. In present study, browning of medium has been successfully control with the use of three different antioxidants AC, CA and PVP at different concentrations (0-700 mg L^{-1}). Optimum morphogenetic response was noted at a concentration of 300 mg L^{-1} in PVP in the initiation medium. In browning controlled medium cultured nodal explant developed dark green shoot. Elmore *et. al*. (1990) and Gautam *et. al*. (1993) suggested that pretreatment of explants with PVP could absorbs phenols and thus prevents from oxidation in neem. Use of antioxidants such as PVP and CA in combination at 200 mg L^{-1} and 100 mg L^{-1} respectively in combination could successfully control the effect of browning in *Pinus kesiya* (Deb and Tandon, 2004). Raghuvanshia and Srivastava (1995) cultured mango leaves on MS mediums supplemented with 0.05% PVP. Baker *et. al*. (1992) reported that

AC shows comparatively large surface area to volume which gives AC a unique adsorption capacity of inhibitory compounds results in decrease the toxic metabolites in the medium. However, incorporation of AC affects the medium p^H, alter the ratio of its components and subsequently influence plant regeneration (Druart and Wulf, 1993) and shows ineffective towards sugars like glucose, sucrose, dextrose, inositol etc from the medium (Yam *et. al*., 1990). Whereas, nodal explant of *brahylaena huillensis* was successfully cultured with ascorbic acid 200 mg L^{-1} as antioxidant (Ndakidemi *et. al*., 2014).

Effect of organic carbon source: Organic carbon plays vital role in plant as an energy sources for various metabolic activities. Quality and quantity of organic carbon brought about different affects on morphogenetic responses was observed in the present investigation. It was observed that organic carbon is a must for successful growth in the culture. Optimum morphogenetic response was found on nutrient medium fortified with sucrose (3%) where ~67% of cultured nodal segments responded positively within 6 days of culture initiation. Other two organic carbon sources (dextrose and glucose) across the concentrations found to be inferior for invocation of morphogenetic response from cultured nodal segments (**Table 16**). The least response was found on medium enriched with dextrose (1%) where only ~12% of nodal segments responded. Requirement of organic carbon for *in vitro* morphogenesis was reported by many workers in the past (Venkatachalam *et. al*., 2000; El-Bakry, 2002; Preethi *et. al*., 2011; Deb *et. al.*, 2014). In *Stevia rebaudiana,* Preethi *et al*. (2011); in pineapple Zuraida *et. al*. (2011) found sucrose (3%) was most effective for culture initiation from shoot and leaf explants. In orchid cultures were successfully initiated on sucrose fortified medium (Costa *et. al*., 2000; Temjensangba and Deb, 2005; Pongener and Deb, 2009).

***Effect of quality and quantity of cytokinins*:** Growth regulators plays essential role in determining the development pathway of plant cells and tissues in culture medium. The auxins, cytokinins and gibberellins are most commonly used plant growth regulators. Uses of quality and quantity of PGRs depend mainly on the species, organs, tissues and the objective(s) of the research. In the present study with *S. punduana* nodal segments from *in vivo* source, BA and KN (0-15 µM) were used singly. In general it was found that BA enriched medium morphogenesis was better over KN enriched medium. Under the given conditions optimum response was recorded on MS medium fortified with sucrose (3%) and BA (9 µM) where as many as 5 shoot buds sprouted from a single node and ~67% cultured explants responded positively. Conversely on KN (9 µM) fortified medium there was only 4 shoot buds formation per node from 41% cultured explants. There are many reports available in literatures on the effect of different cytokinins on *in vitro* culture initiation in different species. Puddephat *et. al*. (1997) successfully established cultures *Quercus robur* L on cytokinin rich medium. Abubacker and Ramanathan (2004) reported that MS medium fortified with BA and KN in combination was suitable for shoot regeneration. In pineapple culture could be successfully established from shoot buds on MS medium supplemented with 5 mg L^{-1} BA where ~ 86% explants produced shoots in culture (Zuraida *et. al*., 2011).

Foliar Explants Culture

Effects of cytokinins: Of the different factors evaluated by various workers in the past on organogenesis from various explants sources, the role of PGRs, especially cytokinins were widely regarded as an important supplement (Deb *et. al.,* 2014). The present study from leaf explants of *S. punduana* too revealed in agreement with the past reports. In the study, the foliar explants cultured on MS medium supplemented with BA and KN singly exhibited different responses. However, the cultured leaf explants responded optimally

on medium conjunct with sucrose (3%) and BA (6 µM) where ~56% leaf explants responded to organogenesis with slight callusing. While KN rich medium there was fewer shoot bud formation and mostly callused (**Table 18**).

In the past many researchers reported the superiority of BA over other cytokinins in many tree species (Pattnaik and Chand, 1997). Cytokinin alone in the culture medium induces shoot formation in many plants. In *Petunia hybrida*, massive shoot multiplication was achieved by using MS medium amended with 2.2 µM BA and 5.7 µM IAA within 4 weeks of culture (Sharma and Mitra, 1976). Besides these, the important role of BA for shoot proliferation was also reported for other species like *Artemisia annua* (Usha and Swamy, 1998) and in *Eclipta alba* (Dhaka and Kothari, 2005).

Besides the culturing leaf explants on cytokinin rich medium, leaves were also cultured on BAA-NAA and KN-NAA fortified medium. Findings indicate that the combined treatments of either BA-NAA or KN-NAA are inferior to singly treatment of either BA or KN for direct organogenesis. In all the combined treatments fewer shoot buds yielded but supported callus formation (**Table 19**).

Effects of cytokinins on culture proliferation: Cytokinin is an important agent to stimulate multiple shoot bud formation for *in vitro* propagation of plants. Many researchers have been demonstrated that organ formation occurs only with medium containing cytokinin. In the present investigation, cytokinin such as BA and KN incorporated singly at varied levels of concentrations (0-15 µM). It was found that incorporation of BA at 9 µM gives optimum shoot buds formation with healthy growth rate, affecting ~56.2 % of nodal explants with an average of ~5 micro shoots developed within 5 wk of cultured. Where KN could produce 4 shoot buds at 6 µM concentration affected ~50 % explants. This indicates that KN is less preferred which agreed with the reports of that in some plant species it may even respond with both embryogenesis and

organogenesis together depending on the culture conditions (Dornelas *et. al*. 1992; Dodsworth, 2009). In all the treatments range from 0-15 µM developed micro shoots however it increases its calli development with increase in concentration. But in absence of cytokinins, nodal explants failed to developed shoot bud.

In line of similar finding with lower concentrations of BA favored high rates of shoot multiplication and better shoot elongation as in *Cinnamomum camphora* (Huang *et. al*., 1998); in the species *Holarrhena antidysenterica* (Kumar *et. al*., 2005). Higher number of micro shoots generations also correspondingly observed in KN 6 mg L^{-1} treatment but its growth rate are remarkably affect with much slower and much calli formation. This may be due to its preferential nature of species on its growth regulators affects as reported in several species by many earlier workers studies. However, Sharma and Ramamurthy (2000) reported a profuse callusing with *Eucalyptus tereticornis* on MS medium supplemented with TDZ (2.27 and 4.54 mg L^{-1}) with NAA (0.54 mg L^{-1}), but the production of 4–8 healthy shoots per explant on medium with BA and NAA (0.44 and 0.54 mg L^{-1}), respectively. However, used of growth regulators such as NAA and BA in combination were reported in propagation of *Ornithogalum* species from bulb scale explants (Nel, 1981; Suh *et. al*., 2005). Whereas the highest regeneration of *O. ulophyllum* was obtained with ~4.83 per explant in a medium containing 2.0 mg L^{-1} BAP combined with 0.50 mg L^{-1} NAA (Ozel *et. al*., 2008).

Rooting of Micro Shoots

For rooting of micro shoots, MS medium was supplemented with IBA and NAA singly (0-15 µM). In the past many workers tested different auxins like IAA, IBA, NAA as these growth regulators are known to influence the higher rate of root induction from micro shoots (Beck *et. al*., 2000; Nanda *et. al*., 2004; Deb *et. al.,* 2014). While, Davies

(1980) reported 100% rooting in cultivars of rose on MS medium devoid of growth regulators but supplemented with 4% sucrose.

In the present work, it was observed that, IBA treatment exhibited its superiority in rooting of micro shoots, where an average of 5 rootlets per micro shoot developed with 1 cm long accompanied by moderate calli formation. However, in presence of NAA an average of ~4 rootlets with moderate root hairs developed. Optimum rooting was recorded on medium conjunct with IBA at 12 µM level of concentration which affected in ~43.7 % cultured micro shoots.

Under different conditions, several workers have successfully manipulated auxins in induction of rooting in different species. Zia *et. al.* (2010) used IBA to induced *in vitro* rooting in many soybean genotypes, where (Vengadesan *et. al*., 2002) could achieved 55% rooting from micro shoots of *Acacia sinuate* on ½MS medium supplemented with 7.36 µM IBA. Likewise Monteuuis and Bon (2000) reported that exposing the micro shoots to 4–6 µM concentrations of IAA or IBA in the dark significantly increased rooting of the mature clone. However, it was reported that types of auxin preference depend on tissue and species which support the present work (Rao and Padmaja, 1996).

In another report, combination of IAA + NAA, IAA + IBA or IBA + NAA did not show any positive response on rooting, rather it formed callus at the cut end of the excised shoot and also the shoot became yellowish (Yadav *et. al*., 1990; Upreti and Dhar, 1996). But Shan *et. al.* (2005) reported that rooting was achieved even in the medium without PGRs which was contradict to present results, where micro shoots did not developed any root formation in auxin devoid medium.

Acclimatization and Transplantation of Regenerates

Hardening of plants

Most of the works are done on *in vitro* conditions where it control majority of the regeneration processes. However, efficiency of the *in vitro* raised plantlets to its survival in the natural conditions forms the basis of successful plant tissue culture. In fact at this stage, micro propagated plants are at crucial stage for its survival as it gradually starts experience the natural conditions in much higher light intensity with lower relative humidity. Besides readily available nutrients too are kept much lower, therefore much depends on their fate of their fully developed organs to its functionality. Different techniques have been employed by tissue culturists by modifying its nutrients supply with high stability substratum. The *in vitro* plants are generally photo synthetically incompetent (Preece and Sutter, 1991) and the plants are photo synthetically inactive. Lower chlorophyll levels of *in vitro* plants clearly suggest lower or negligible rate of photosynthesis due to heterotrophic mode of nutrition that ultimately limits its biosynthesis feasibility. Therefore it can be concluded that supplementation of essential nutrients during acclimatization and photoautotrophic development of micro propagated plant can increase survival rate. *In vitro* plants showed higher levels of reducing sugars. In micropropagation process, carbohydrate reserves are built up in leaves by manipulating sucrose in regeneration/shoot multiplication stage prior to acclimatization.

In addition to work on aspects of *in vitro* biology such as autotrophy and hormone physiology such as auxin regulated axillary growth (Reinhardt *et. al*., 2000). Light quality has a potentially environmental factor which often been overlooked but has affect on the direction of plant morphogenesis *in vitro* (Morini *et. al*., 2000) and the switch between gametophytic and sporophytic pathways. Tissue culture often involves much of cutting/injury of tissues in plants which causes stress in its physiological changes (Leon *et. al*., 2001).

The rooted plantlets were taken out from cultured vials, washed with luke warm water to remove any intact trace of agar substratum remains, whereby transferred it into liquid ½ MS medium containing 1% sucrose without any PGRs. Sets of processed dry coconut coir, forest litter and saw-dust were used as substrata, and maintained for 5-6 wk in the normal laboratory conditions. Further, the hardened plants were transferred to potting mix contained mixtures of soil, sand and decaying wood power in the ratio 2:1:2 respectively in poly bags (15x12 cm). The potted plants were maintained for 5-6 wk under partially controlled conditions of light Ca. 70% sunlight. Regular interval of water was feed, once a wk for 6 wk, followed by exposed for 2 hr interval a day under natural day light for 1 wk, where finally left under natural day light. About 70% transplants survived after two months of transfer. In this work, the well rooted plantlets raised from *in vitro* were taken out and transferred in the pre-filled and autoclaved coconut coir in culture vials. But regenerates were supply with less concentration nutrient medium ($1/4^{th}$ MS solution) with coconut coir as substratum for 5 wk in normal laboratory conditions and later transferred to fresh culture vial with same conditions except for 6-7 wk. Usually, micro propagated plantlets present higher transpiration rates than those grown in greenhouse, due to their abnormally functioning stomata and thin cuticle, which results in plant wilting and, in some cases, in leaves or plant death (Dias *et. al*., 2013; Osorio *et. al*., 2013). Therefore, the stabilization of the water status of micro propagated plants during *in vivo* acclimatization is very much adhering to its survival. Besides water stress, during *in vivo* acclimatization plants are exposed to light intensities higher than those used under *in vitro* conditions, resulting usually in photo inhibition (Osorio *et. al*., 2010; Dias *et. al*., 2013, 2014). Unfortunately, many *in vitro* raised plantlets while transfer to natural conditions restricted by high percentage of plants lost or damaged during the process of acclimatization (Pospisilova *et. al*., 2009; Loureiro *et. al*., 2007).

Conclusions

The species *S. punduana*, as a horticultural important as well as threaten in wild which need immediate attention for conservation and development of efficient protocol for propagation to avoid the further stress on the species in the natural habitat. Present work is the first ever attempt to develop protocol to produce clonal planting materials from different explants. Protocols for the plant regeneration were standardized and plants were regenerated with the supplements of many PGRs (Auxin and cytokinin) from nodal explants and leaf explants directly shoot buds regeneration and with and without callusing phase. The protocols developed would help in propagation of this species and reduce the stress on the natural population.

Chapter – 4

Somatic Embryogenesis and Plant Regeneration of *Saurauia punduana* Wallich

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Saurauia punduana Wallich (Actinidiaceae) is a small to medium size multipurpose medicinal tree of ~3-5 m tall and reaches sexual maturity in 4-5 years. The species grows predominantly in sub-tropical mixed forest (dense forest, forest edges and open forest). Therapeutic uses of the species are many; the paste made from young leaves and young twigs are applied on cuts and wounds to stop bleeding. The young leaves are used as vegetable and help in building immunity (Dobriyal and Dobriyal, 2014). However the species is under threat in the wild due to removal of forest cover for various anthropogenic activities and over-exploitation for herbal medicine.

For propagation and conservation of threatened and economically important plants, biotechnological tools like plant tissue culture has proved to be effective (Rai *et. al.*, 2007). Realizing the threat of the species under question, it was necessary to develop an efficient regeneration system for large scale propagation of the species.

During the recent past many successful attempts have been made for *in vitro* regeneration of plants of economically important and threatened species via somatic embryogenesis (Deb, 2001; Chalupa, 2005; Jaiswal and Jaiswal, 2005; Rai *et. al.*, 2007; Lincy *et. al.*, 2009; Shi *et. al.*, 2010; Asghar *et. al.*, 2013). Plant regeneration through somatic embryogenesis has been considered the preferred method for woody plants because somatic embryogenesis leads to simultaneous formation of bipolar structures containing both shoot and root meristem (that develops into the tap roots of trees) while having a single cell origin. The power of somatic embryogenesis as a propagation tool becomes especially apparent when the goal is to generate large number of propagules of a woody plant species.

Somatic embryogenic cultures of economically important plants are increasingly finding application as a source of clonal planting material and/or propagules amenable for genetic transformation. Besides this, secondary somatic embryogenesis offers additional advantages to primary somatic embryogenesis, viz., a higher multiplication rate, greater independence from explant source and a higher level of uniformity, encouraging reproducibility of technique (Rai *et. al.*, 2007; Shi *et. al.*, 2010; Gholami *et. al.*, 2013). This present study is the first ever successful attempt to develop a reproducible regeneration protocol for efficient plant recovery via somatic embryogenesis of *Saurauia punduana* which is characterized by well defined stages of embryo development, maturation, germination and plant regeneration.

Materials and Methods

Plant Materials

Seeds of *Saurauia punduana* at different seed developmental age (3-8 week old) were collected from plants growing near Nagaland University, Lumami, India at an altitude of 1,250 m MSL. For the present study, three different small populations were selected and from each population, five plants were selected randomly for harvest of the fruits to ensure that the results are representative of the species. Fruits of various developmental ages were collected from the plants, washed with 'Labolene' (0.1%, v/v, a commercial laboratory detergent) and then rinsed under running tap water for 10 min. The fruits were surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride for 3 min and then washed 4-5 times with sterilized distilled water. The seeds were removed from the sterilized fruits and cultured on MS medium fortified with sucrose (3%, w/v), and BA (0 or 2 µM). They were maintained in dark or full laboratory illumination (40 µmol m^{-2} s^{-1}) for seed germination and seedling formation. In each culture vial (~55 mm diameter), ~100 seeds were cultured. The cotyledonary leaves from the *in vitro* germinated seeds were used for the experimental purpose.

Initiation of Embryogenic Culture

The full cotyledonary leaves from the *in vitro* germinated seedlings on MS medium fortified with 2 µM BAP were harvested after four weeks of culture initiation for seed germination (**Table 21**). The cotyledonary leaves were cultured on agar gelled MS medium fortified with sucrose (0-5%), varying concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), BA and kinetin (KN) (0-4 µM) either singly or in combination (EC Initiation Medium) (**Table 22**). It was ensured that lower epidermis of the cotyledonary leaves were in full contact with the medium. Two *in vitro* produced cotyledonary leaves were cultured in each test tube and

the cultures were maintained at 40 µmol m^{-2} s^{-1} illumination (12/12, D/L photoperiod) provided with white cool fluorescent light and 25±2^{o}C temperature.

Proliferation of Cultures and Embryogenesis

The calli developed from excised cotyledonary leaves were maintained for another 2-3 passages on different strengths of MS medium (½MS, MS and 2MS) supplemented with different concentrations of sucrose (2-5%) for embryogenic callus proliferation (EC Proliferation Media). For each treatment, ~200 mg of embryogenic callus was used as inoculum and biomass production was checked after 4 weeks of culture.

The cultures with proembryonal masses were maintained on ½MS and MS medium supplemented with sucrose (0-5%), NAA and BA (0-5 µM) either singly or in combination for embryo formation and maturation (SE Formation Media). The somatic embryos so developed were transferred onto different strengths of MS medium (0 to full strength of inorganic salts but with full strength organic supplements) containing BA (2 µM) and sucrose (4%) and maintained at a higher illumination (60 µmol m^{-2} s^{-1}) for 7-8 weeks for maturation and germination (SE Maturation Media). The germinated embryos/seedlings were hardened by transfer onto MS hormone-free medium enriched with sucrose (3%) at a higher illumination (60 µmol m^{-2} s^{-1}) for three-four weeks (SE Germination Media) before transferring to a potting mix (sand, soil, decayed wood powder and chopped coconut coir at a 1:1:1:1 ratio with a layer of moss topping). For the initial two weeks, the pots were watered twice in a week and covered with transparent polybags to maintain the humidity. The transplants were maintained in the poly house with 75% shade for 5-6 weeks followed by transplanting to the field.

Experimental Design and Statistical Analysis

A completely random experimental design was followed. In each test tube, two cotyledonary leaves were cultured and for each treatment, 20 test tubes were maintained. All cultures were sub-cultured at 3-4 week interval unless mentioned otherwise. All the experiments were repeated at least thrice. Cultures were monitored on alternate days and data collected on a weekly basis. Data was analyzed by one way ANOVA using the General Linear Model procedure in SAS Statistical Package (SAS Ins.) and standard deviation from mean was worked out and compared using Least Significant Difference (LSD) test at $p \leq 0.05$.

Results

Seed germination: For the present study seeds were collected from three different populations and 5 plants from each population were selected randomly. Fruits of different developmental ages were collected from all the plants to ensure that the results in the present study are not an individual plant specific. In the present study, seeds were cultured on MS medium fortified with 0 or 2 µM BA under two different light regimes (**Table 21**). Seedlings on BA-free and BA fortified (at 2 µM) medium exhibited differential seedling morphology (data not shown). Cotyledons and hypocotyls of the seedlings were thinner on BA-free medium against dark green and thick on BA fortified medium.

Seeds on MS medium with sucrose (3%) and 2 µM BA and cultured in the dark exhibited better germination rates compared to the cultures in the light. Within three wk of culture, seeds started germinating and released their first set of leaves within 4 wk. Of the various fruit age, seeds harvested from 5-7 wk old fruits yielded better germination and subsequent embryogenic response. The other seed ages were found to be unsuitable for germination. Under the given conditions, within 3 wk of culture in the dark, ~84%

seed germination was achieved from 6 wk old seeds; the percentage germination from 5, 6 and 7 week old seeds were not significantly different but were significantly higher than both younger and older seeds (**Table 21**). The cotyledonary leaves were collected from the seedling resulted both from light and dark cultured seeds and cultured separately the leaves harvested from the seedlings of various developmental age of seeds on MS medium fortified differently for embryogenic calli formation. Interestingly, *per cent* calli formation was significantly highest from cotyledonary leaves harvested from 6 weeks old light grown seedlings. Seeds cultured in the light, on the other hand, either failed to germinate or were delayed in response.

Initiation of Embryogenic Culture

In the second experiment, the cotyledonary leaves were harvested from the seedlings germinated *in vitro* and cultured on MS medium fortified with sucrose (0-5%) and different plant growth regulators (PGRs) (**Table 22**). Within one week the leaves started swelling (**Fig. 14 a**) and within three weeks, calli developed throughout the cotyledonary leaves followed by proembryonal masses (**Fig. 14 b**). Amongst the different concentrations of sucrose tested for callus induction, sucrose at 3% supported healthy callus formation. There was healthy calli formation on medium supplemented with BA+NAA, and BA+2,4-D but cultures maintained on KN fortified medium either singly or in combination with 2,4-D or NAA resulted in poor embryogenic cultures. Of the different PGR combinations, BA+NAA supported healthy culture formation.

In the present study, two different types of calli (designated Class I and II) developed. In most cases, callus was initially yellowish-white but grew to light-yellow, friable callus from the third week of culture. A part of the callus was yellowish green, non-embryogenic (Class I) or whitish green, embryogenic callus (Class II). Class-I callus was formed at higher concentrations of 2,4-D (1.0-2.0 µM). Besides this, combinations

of 2,4-D and KN resulted in non-embryogenic calli accompanied with retarded culture proliferation. In the present study, significantly more embryogenic cultures (68.2%) developed on MS medium supplemented with sucrose (3%), NAA (1.5 µM) + BA (4.0 µM) than on any other combination tested, followed by 1.0 µM NAA + 4.0 µM BA (51.1%) then 0.2 µM NAA + 3.0 µM BA (45.4%) supplemented media (**Table 22 , Fig. 14 c**).

Table 21: Effect of seed age of *Saurauia punduana* on germination of seeds and formation of embryogenic calli from their excised cotyledonary leaves

Seed age (wk)	Germination rate (%) (±SE)*		Average time for germination (days)	
	Dark	Light	Dark	Light
3	0	0	0	0
4	30.2 (0.3)^{c}	12.1 (0.4)^{c}	35^{c}	38^{c}
5	81.3 (1.1)^{a}	18.3 (0.4)^{b}	30^{b}	35^{b}
6	84.2 (0.7)^{a}	25.2 (0.5)^{a}	22^{a}	30^{a}
7	83.4 (0.6)^{a}	24.2 (0.3)^{a}	23^{a}	30^{a}
8	64.3 (1.0)^{b}	14.2 (0.2)^{c}	23^{a}	30^{a}

* ±SE: Standard error from the mean. Data with the same *letters* in the column are not significantly different at 5% level; The seed age was calculated from the day of anthesis.
Seeds were cultured on MS medium supplemented with sucrose (3%) and BA (2 µM).
N: 100, repeated thrice.

Table 22: Effect of PGRs on embryogenic culture induction of *Saurauia punduana* from excised cotyledonary leaves

PGRs Conc. (µM)*				% explants with embryogenic calli (±SE)**	Type of response
NAA	**2,4-D**	**BA**	**KN**		
0	0	0	0	0	No response
0.5	-	1.0	-	-	Slight callusing towards the cut ends
0.5	-	2.0	-	28.1 (0.2)^{f}	Callusing with 2-4 roots
0.5	-	3.0	-	45.4 (0.4)^{c}	Soft, gelatinous yellowish green callus with few roots
0.5	-	4.0	-	32.0 (0.3)^{e}	Slight green callusing
0.5	-	5.0	-	14.3 (0.3)^{h}	Yellowish hard callus
1.0	-	2.0	-	22.0 (0.5)^{g}	Yellowish gelatinous callus with few roots
1.0	-	3.0	-	38.5 (0.7)^{d}	Soft yellowish green callus with few roots
1.0	-	4.0	-	51.1 (0.3)^{b}	Soft, yellowish, friable callus with 1-2 roots
1.0	-	5.0	-	20.0 (0.3)^{g}	Yellowish brown callus towards the cut end
1.5	-	3.0	-	36.5 (0.7)^{d}	Soft yellowish fast proliferating callus with few roots
1.5	**-**	***4.0***	**-**	***68.2 (0.3)^{a}***	***Soft, friable yellowish green callus with few roots***
2.0	-	3	-	21.0 (0.4)^{g}	Yellowish brown soft callus with 5-6 roots
2.0	-	4	-	38.3 (0.5)^{d}	As above
2.0	-	5	-	25.1 (0.6)^{f}	As above
-	0.5	2.0	-	20.4 (0.3)g	Callusing from the cut end, soft, yellowish and translucent
-	0.5	3.0	-	26.0 (0.5)^{f}	As above
-	0.5	4.0	-	21.1 (0.2)^{g}	As above
-	1.0	3.0	-	28.0 (0.3)f	Callus soft, yellowish and translucent
-	1.0	4.0	-	25.4 (0.2)^{f}	Callus soft, yellowish white and translucent
-	1.5	2.0	-	12.2 (0.4)^{h}	Callus at the cut ends, soft and white
-	1.5	3.0	-	26.3 (0.6)^{f}	As above
-	1.5	4.0	-	25.7 (0.3)^{f}	As above
-	2.0	3.0	-	08.0 (0.2)^{i}	Callus soft, white and gelatinous
-	2.0	4.0	-	08.2 (0.3)^{i}	As above

* Only responsive treatments are computed. There was no embryogenic response on medium fortified with kinetin and mostly calli were hard, hence are not computed in the table.

** ±SE: Standard error from mean; N: 40, repeated thrice. BM + 3% sucrose, in the light 12/12; Data with the same *letters* in the column are not significantly different at 5% level. Cotyledonary leaves were harvested from the seedlings developed from 6 wk old seeds.

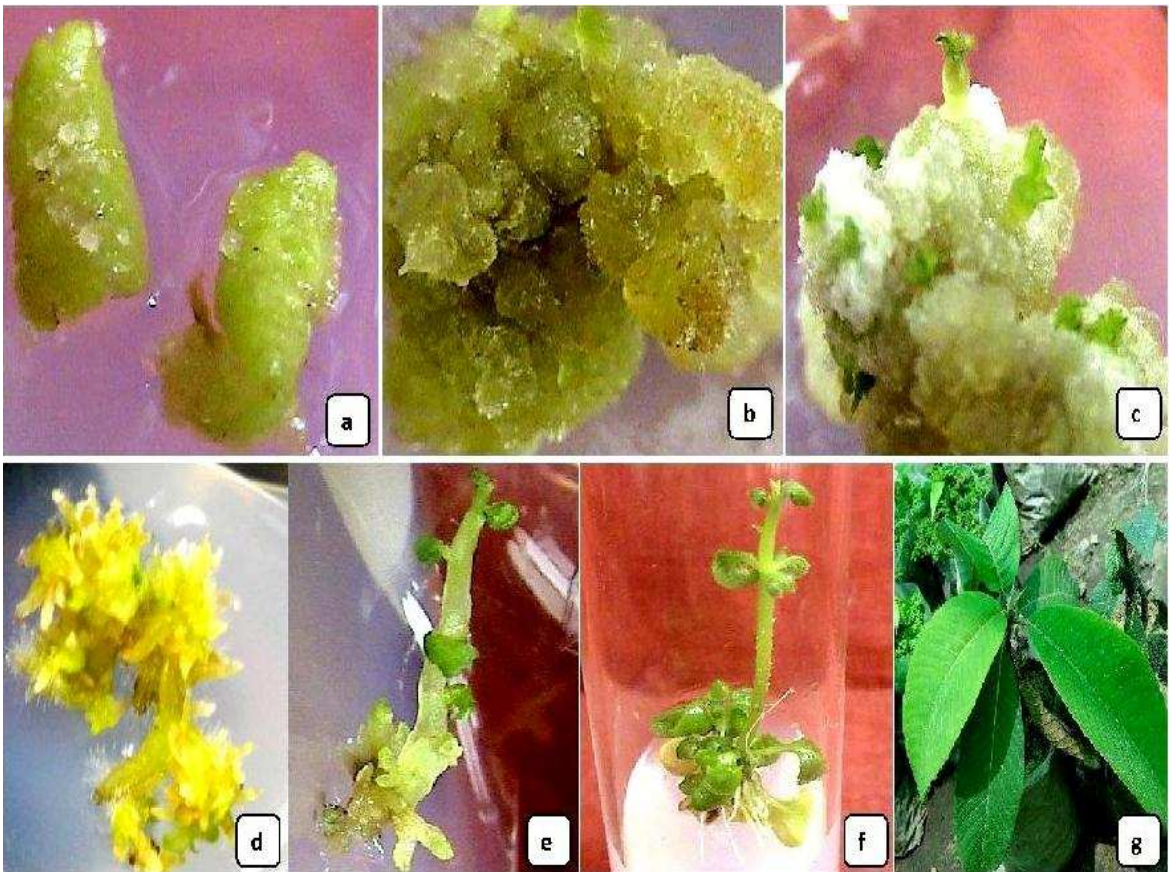

Figure – 14

Figure 14: Different stages of induction of somatic embryogenesis from cotyledonary leaves of *Suarauia punduana*. **a**. Cultured cotyledonary leaf segments showing initiation culture within one week of culture; **b**. Callus with proembryonal masses developed within 3-4 week of culture; **c**. Callus with distinct somatic embryos; **d**. Cotyledonary and torpedo shaped somatic embryos developed throughout the callus mass; **e.** Germinating somatic embryo with elongated hypocotyls with formation of recurrent/secondary somatic embryos at the base; **f**. Germinated somatic embryo converted into rooted seedling/embling and **g**. Potted seedling established in the poly-house.

Culture Proliferation and Induction of Somatic Embryogenesis

The calli with embryogenic masses were transferred onto different strengths of MS medium fortified with different sucrose concentrations for culture proliferation (**Table 23**). Of the three media strengths tested, full strength MS medium was found to be superior over 2MS and ½MS media. For culture proliferation, sucrose was used at a concentration of 2-5%. Both at lower (2%) and higher concentrations (>3%) proliferation rate was significantly poorer than media supplemented with 3% sucrose and in all media strengths. Within 4 wk of culture, as high as 348.3% (3.483 times more callus) proliferation was registered on MS medium enriched with 3% sucrose, NAA (1.5 µM) and BA (4 µM). In contrast, there were ~234.4 and ~270.9% proliferation on half MS and double MS medium respectively on sucrose (3%) enriched medium (**Table 23**). During the proliferation of culture, proembryonal masses, globular embryos and secondary embryos formed (**Fig. 14 c**).

Embryo Development, Maturation and Germination

Cultures from the proliferation stage leads to various developmental stages but not necessarily proceeds to direct embryo maturation and germination. In general, auxins and cytokinins are the principal growth regulators which control culture proliferation, differentiation and or maturation of somatic embryos in culture (Feher *et. al.*, 2003). In the present study, maintenance of culture on proliferation medium for longer period (>6-7 weeks) did not ensure embryo development and maturation of embryos but rather led to culture degeneration and browning of culture. To avoid this, the cultures from the proliferation stage were incubated in two media strengths (half and full strength MS medium) fortified with low concentrations of NAA (0-1 µM) and BA (0-5 µM) and various levels of sucrose (0-5%) in order to promote embryo development and maturation (**Table 24 & 25**).

Table 23: Effects of nutrient medium strength and sucrose concentration on embryogenic calli proliferation

Nutrient medium proliferation strength	Sucrose Conc. (%, w/v)	Initial biomass of calli (±10 mg)	Biomass of calli after 4 wk (±10 mg)	%
½xMS medium	2	200	380.6^{e}	190.30^{e}
	3	200	468.8^{d}	234.4^{d}
	4	200	395.3^{e}	197.7^{e}
	5	200	344.5^{f}	172.3^{f}
1xMS medium	2	200	481.2^{d}	240.6^{d}
	3	***200***	***696.6^{a}***	***348.3^{a}***
	4	200	642.3^{b}	321.2^{b}
	5	200	558.6^{c}	279.3^{c}
2xMS medium	2	200	433.3^{d}	216.7^{d}
	3	200	541.7^{c}	270.9^{c}
	4	200	468.6^{d}	234.3^{d}
	5	200	336.1^{f}	168.1^{f}

Nutrient media fortified with NAA (1.5 µM) and BA (4 µM).
Data scored after 4 week of culture initiation and from the mean value of three repeated experiments.
Data with the same *letters* in the column are not significantly different at 5% level.
N: 10, repeated thrice.

Table 24: Effect of sucrose concentration on development and maturation of somatic embryos on MS and ½MS media

Sucrose Conc. (%)	% culture forming SE*!(±SE)#		No. of embryos per 200 mg culture	
	MS medium	½MS medium	MS medium	½MS medium
0	0	0	0	0
1	0	0	0	0
2	24.6 (0.4)^{c}	12.2 (0.3)^{d}	8.6 (0.2)^{c}	3.4 (0.3)^{c}
3	45.5 (0.7)^{b}	15.7 (0.3)^{c}	11.2 (0.4)^{c}	4.3 (0.3)^{b}
4	***62.5 (0.6)^{a}***	***21.1 (0.3)^{a}***	***38.1 (0.2)^{a}***	***8.3 (0.4)^{a}***
5	48.2 (0.4)^{b}	17.4 (0.3)^{b}	19.7 (0.4)^{b}	4.4 (0.2)^{b}

***** SE: Somatic embryos (globular, heart shaped and cotyledonary embryos); # ±SE: Standard error from mean; ! Per callus mass of 200 mg (±10 mg); Nutrient media fortified with BA (4 µM); Data scored after 4 weeks of culture initiation and from the mean value of three repeated experiments; Data with the same *letters* in the column are not significantly different at 5% level. N: 10; repeated thrice.

Table 25: Effects of strengths of MS medium and PGRs (NAA and BA) on embryo development

Quality & quantity of PGRs (µM)!		1xMS medium		½xMS medium	
NAA	**BA**	**% callus forming SE*#**	**No. of embryos per callus mass****	**% callus forming SE***	**No. of embryos per callus mass****
0	0	0	0	0	0
0	3.0	40.3 (0.4)[b]	31.5 (0.5)[b]	18.4 (0.4)[b]	6.5 (0.3)[b]
0	***4.0***	***62.5 (0.6)[a]***	***38.1 (0.2)[a]***	***21.1 (0.3)[a]***	***8.3 (0.4)[a]***
0	5.0	44.4 (0.6)[b]	28.6 (0.5)[c]	20.2 (0.6)[a]	8.1 (0.4)[a]
0.5	4.0	25.4 (0.3)[c]	18.2 (0.4)[e]	-	-
0.5	5.0	28.6 (0.4)[c]	21.0 (0.7)[d]	-	-

! Only responding treatments are computed; ***** SE: Somatic embryos (globular, heart shaped and cotyledonary embryos); # ±SE (Standard error from mean); ** Callus mass of 200 mg (±10 mg); Nutrient media fortified with sucrose (4%, w/v); Data scored after 4 weeks of culture initiation and from the mean value of three repeated experiments; Data with the same *letters* in the column are not significantly different at 5% level; N: 10, repeated thrice.

In the present study, embryonal masses that were cultured on full strength MS medium fortified with BA (4 µM) and sucrose (4%) supported optimum globular and cotyledonary stage embryo formation (62.5 %), which was significantly more than the other sucrose concentrations tested (**Fig. 14 d, e; Table 24**), where as many as 38 embryo formed against 8 embryos on ½MS medium from similar embryogenic culture within three week of culture.

Embryogenic calli transferred onto NAA and BA fortified medium did not support cotyledonary embryo formation (**Table 25**). Again, significantly higher percentages of cultures, and more embryos were produced on MS fortified with 4 µM BA. In the responsive cultures, embryos were unequally distributed on the culture masses and they developed as clumps of cotyledonary embryos (**Fig. 14 c, d**). The cotyledonary embryos turned green followed by germination with distinct hypocotyls and roots on MS medium fortified with BA (4 µM) and sucrose (4%) (**Table 25, Fig. 14 e, f**). It was observed that in most of the cases 5-7 secondary embryos formed at the base and axis of mature embryos on prolonged culture and repeated sub-culture led to repeated secondary somatic embryogenesis (**Fig. 14 e).**

Maturation and germination of somatic embryos were achieved on the same culture medium without subculture, albeit with some occurrence of secondary somatic embryos. The germinated embryos could be easily separated from the clump and transferred to potting mix as mentioned in the materials and methods and maintained in polyhouse for 5-6 weeks. A total of 350 seedling of uniform morphology were selected for the transplantation. About ~75% seedlings survived after 6 weeks in the potting mix (**Fig. 14 g**).

Discussion

Fruits were collected randomly from different population ti avoid the biasness in the experimental design. Fruits of different developmental ages were collected from all the plants to ensure that the results in the present study are not an individual plant specific. Seeds were cultured on MS medium fortified with 0 or 2 µM BA under two different light regimes. Seedlings on BA-free and BA fortified (at 2 µM) medium exhibited differential seedling morphology. Cotyledons and hypocotyls of the seedlings were thinner on BA-free medium against dark green and thick on BA fortified medium. The cotyledonary leaves were collected from the seedling resulted both from light and dark cultured seeds and cultured separately the leaves harvested from the seedlings of various developmental age of seeds on MS medium fortified differently for embryogenic calli formation. Interestingly, *per cent* calli formation was significantly highest from cotyledonary leaves harvested from 6 weeks old light grown seedlings. Seeds cultured in the light, on the other hand, either failed to germinate or were delayed in response. This study indicates that the seeds of *S. punduana* prefer the dark for their germination. But due to anthropogenic activities, forest covers are being removed and then seeds do not get the required dark regime for germination, which could possibly be one of the reasons their population is decreasing.

In the present study, seeds were germinated *in vitro*. It was observed that selection of the right developmental age of the fruits/seeds was very important for germination and callus formation. The seeds up to 3 weeks did not support any *in vitro* germination. Seed germination rate was enhanced significantly from the fourth week of seed development with the highest rate of germination as well as callusing being registered from seeds 6 wks old. For seeds older than 6 weeks, the germination rate declined gradually though the response was not very high. The cotyledonary leaves were

harvested from the seedlings developed from seeds of various developmental ages and cultured separately for callus induction. The cultured leaves also exhibited a similar response with reference to callus formation, i.e., highest callus formation was registered from cotyledonary leaves harvested from the seedlings developed from seeds of 6 week old. These results suggest that seed age plays an important role in germination and callus formation of *S. punduana*. After 4-5 weeks of culture, the cotyledonary leaves from the seedlings were harvested and cultured on differentially fortified MS medium for culture initiation.

The cotyledons were harvested and cultured on nutrient medium fortified with different concentrations of sucrose and PGRs for embryogenic culture initiation. Amongst the different concentrations of sucrose tested for callus induction, sucrose at 3% supported healthy callus formation. At lower concentrations, there was poor callus formation while at higher concentrations cultures turned brown. For induction of embryogenic calli, different PGRs were used at various concentrations. There was healthy calli formation on medium supplemented with BA+NAA, and BA+2,4-D but cultures maintained on KN fortified medium either singly or in combination with 2,4-D or NAA resulted in poor embryogenic cultures. Of the different PGR combinations, BA+NAA supported healthy culture formation (**Table 22**).

For callus initiation, cotyledonary leaves were harvested from germinated seeds of various ages. Induction of somatic embryogenesis from any explants is regulated by many factors like type and quantity of PGRs (Zavattieri *et. al.*, 2010; Al-Taha *et. al.*, 2012). In certain plant species, somatic embryogenesis was achieved on hormone free medium. However, in the present study, incorporation of PGRs in the medium was a prerequisite and somatic embryogenesis was achieved on MS medium fortified with NAA and BA in combination (**Table 22**). The findings of the present study are

concurrent with the report on *Citrus limon* where NAA and BA fortified medium was found to be productive for direct somatic embryogenesis (Gholami *et. al.*, 2013). In contrast, induction of somatic embryogenesis was reported in *Euterpe edulis* (Guerra and Handro, 1998), in *Dianthus* species (Pareek and Kothari, 2003), and in oil palm (Jayanthi *et. al.*, 2011) on 2,4-D rich media; in *Coffea arabica* (Gatica-Arias *et. al*., 2008) on 2,4-D and KN enriched medium; in *Melia azedarach* (Deb, 2001) on 2,4-D and NAA fortified medium.

The calli with embryogenic masses were transferred onto different strengths of MS medium fortified with different sucrose concentrations for culture proliferation. Of the three media strengths tested, MS medium was found to be most effective over 1/2MS and 2MS media for culture proliferation. At lower and higher sucrose concentrations proliferation rate was significantly poorer than media supplemented with 3% sucrose and in all media strengths. Within four weeks of culture, as high as 348.3% (3.483 times more callus) proliferation was registered on MS medium enriched with 3% sucrose, NAA (1.5 µM) and BA (4 µM). In contrast, there were ~234.4% and 270.9% proliferation on half MS and double MS medium respectively on sucrose (3%) enriched medium (**Table 23**). During the proliferation of culture, proembryonal masses, globular embryos and secondary embryos formed. Similar responses in line with the present study were also found with Carnation (Shi *et. al.*, 2010, Karami *et. al.*, 2008).

Cultures from the proliferation stage leads to various developmental stages but not necessarily proceeds to direct embryo maturation and germination. In general, auxins and cytokinins are the principal growth regulators which control culture proliferation, differentiation and or maturation of somatic embryos in culture (Feher *et. al.*, 2003). In the present study, maintenance of culture on proliferation medium for longer period (>6-7 weeks) did not ensure embryo development and maturation of embryos but rather led

to culture degeneration and browning of culture. To avoid this, the cultures from the proliferation stage were incubated in two media strengths (half and full strength MS medium) fortified with low concentrations of NAA (0-1 µM) and BA (0-5 µM) and various levels of sucrose (0-5%) in order to promote embryo development and maturation.

Embryogenic calli transferred onto NAA and BA fortified medium did not support cotyledonary embryo formation. Again, significantly higher percentages of cultures, and more embryos were produced on MS fortified with 4 µM BA. In the responsive cultures, embryos were unequally distributed on the culture masses and they developed as clumps of cotyledonary embryos. The cotyledonary embryos turned green followed by germination with distinct hypocotyls and roots on MS medium fortified with BA (4 µM) and sucrose (4%). It was observed that in most of the cases 5-7 secondary embryos formed at the base and axis of mature embryos on prolonged culture and repeated sub-culture led to repeated secondary somatic embryogenesis**.**

For successful somatic embryogenesis protocol development and routine integration of somatic embryogenesis in clonal propagation and plant improvement programmes of economically important species, somatic embryo maturation and germination are the most critical steps to be achieved. Poor maturation and conversion of somatic embryos is one of most important bottleneck which has hampered the routine application of this technology in tree improvement programmes (Asghar *et. al.*, 2013). In studies where somatic embryos had undergone proper maturation and germination treatment, this resulted in a higher conversion of somatic embryos into emblings. In *Myrica rubra* it was necessary to incorporate IBA for germination (Asghar *et. al.*, 2013). In the past some studies revealed that incorporation of ABA in the maturation medium helped by inhibiting precocious germination and improved the conversion frequency of

somatic embryos (Capuana and Debergh, 1997; Linossier *et. al.*, 1997; Langhansova *et. al.*, 2004). In other species, incorporation of TDZ improved the conversion (Khan *et. al.*, 2006). In *Citrus macroptera* (Miah *et. al.*, 2002) and date palm (Bhargara *et. al.*, 2003), addition of BA and 2,4-D in combination was beneficial for germination of embryos.

In the present study the mature somatic embryos did not germinate spontaneously on hormone-free medium and incorporation of PGRs were found necessary to trigger maturation of somatic embryos. In the present study with *S. punduana,* healthy germination (62.5%) was achieved on MS medium fortified with sucrose (4%) and BA (4 µM). Though in the present study, we could achieve a significant conversion, this still warrants further work to improve the conversion frequency and inhibit precocious germination.

Conclusions

The protocol developed is simple but reproducible one for somatic embryogenesis and secondary embryogenesis of *Saurauia punduana* and their conversion into embling. Further it was estimated that from the seed cotyledonary leaves of varied population, about 190 somatic embryos could be produced within 17-18 week time. The protocol developed will help in production of clonal planting materials and propagation of this species.

Chapter – 5

Summary

North Eastern region of India comprising the eight states viz. Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura is endowed with vast natural resources and has enormous potential for improvement. Nagaland forms the easternmost state of Indian Republic, located between 25°26′- 27°40′N latitudes and 93°20′ - 95°15′E longitudes. The state has an area of 16,579 km^2 mostly hills with varied climate where tropical wet evergreen forests, semi evergreen forests and sub-tropical broad-leaf wet hill forests distributed throughout the state. It constitutes a part of Indo-Burmese biodiversity hotspot. The region is very rich in different economically important plants but many of them are under severe threat in their natural habitats due to various anthropogenic activities. Biotechnological tools like plant tissue culture technique can be effectively used for propagation and conservation of these commercially important threatened species.

Actinidia deliciosa A. Chev. (Actinidiaceae) and *Saurauia punduana* Wallich (Actinidiaceae) are two horticultural important plant species, of which *A. deliciosa* is

propagated commercially in different parts of the world including Nagaland, India while *S. punduana* is a threatened species grows in the open edges of secondary forest. Present study '*In Vitro* Propagation of Two Economically Important Plants: *Actinidia deliciosa* A. Chev. (Actinidiaceae) and *Saurauia punduana* Wallich (Actinidiaceae)' presents the development of protocols of *in vitro* production of clonal planting materials of these two economically important species.

Micropropagation

During the study different factors like effect of seasons of explants collection, quality and quantity of antioxidant(s), organic carbon sources, plant growth regulators on culture initiation different explants, culture proliferation, rooting and hardening of regenerates, transplantation of regenerates to the potting mix are optimized. Effort was put into to use certain low cost substrata as agar alternative for *in vitro* hardening regenerates.

For initiation of *in vitro* culture from *in vivo* source nodal explants of both the species, newly flushed shoots were collected round the year at monthly interval. Of the different seasons, explants collected during November and December registered optimum morphogenetic response in *A.deliciosa* and *S. punduana* respectively where ~75 and 66% cultured nodal explants respectively responded positively and yielded shoot buds from nodal zone. The nodal explants of both the species exuded phenolics in the culture media and tissue turned necrotic. To stop/reduce the browning of medium, three different antioxidants (activated charcoal, citric acid and PVP) at different concentration was incorporated in the initiation medium. In general PVP was found to be superior over other two antioxidants incorporated in both the species. Under the given conditions, PVP at a concentration of 300 mg L^{-1} reduced the browning of culture media significantly without compromising the morphogenesis.

For invocation of morphogenetic response from the cultured nodal explants presence of one of the organic carbon source was prerequisite. In the present study dextrose, fructose and glucose at concentration of 0-4% (w/v) was incorporated for initiation of culture in both the species. Of the three organic carbon sources, dextrose and fructose were found to be far inferior compared to glucose for invocation of *in vitro* response. Sucrose (3%, w/v) supplemented initiation media supported early invocation of *in vitro* response from cultured nodal explants of both the species where within 5-6 days of culture explants exhibited morphogenetic response.

Besides above adjuncts in the initiation medium, presence of growth regulators was necessary for morphogenesis. Of the two cytokins (BA and KN) incorporated in the initiation medium, BA at a concentration of 6 µM supported optimum response in *A. deliciosa* nodal segments where 75% explants responded within 5 days of culture initiation and as many as 4 meristematic loci invoked per node. While a similar response was recorded from the cultured nodal segments of *S. punduana* on MS medium fortified with sucrose (3%) and BA (9 µM) where an average of 5 shoot buds formed per node in ~66% cultured nodal segments. In both the species, KN fortified media supported fewer shoot buds and more callus. Besides singly treatment of cytokinins, effort was also put into to test the combine treatment of BA-NAA and KN-NAA for culture initiation from nodal segments of kiwi. The combined treatments of neither BA-NAA nor KN-NAA supported direct organogenesis from cultured nodal segments. All the treatments mostly supported callus formation and very few shoot buds formation.

Foliar explants from *in vitro* raised micro shoots were harvested for *in vitro* culture initiation from both the species in the present study. Kiwi leaves were cultured in three conditions viz. intact leaf, segmented leaf and scrubbed leaf. Of the three conditions, *per cent* response was higher from intact leaves (~50%) while the leaf segments and scrubbed

leaves registered a similar response (~41%) but, higher shoot buds formation was registered from scrubbed leaves and segmented leaves where as many as 7 shoot buds developed from each explants compared to only 5 shoot buds from intact leaf. In all the three conditions optimal morphogenesis was achieved on MS medium fortified with sucrose (3%) and BA (9 µM). From the cultured leavers of *S. punduana* KN fortified medium was found to inferior for inducing morphogenesis and optimum response was registered on MS medium enriched with sucrose (3%) and BA (6 µM) where ~56% of cultured leaves responded positively and developed shoot buds, callus and fewer somatic embryos.

The meristematic loci/shoot buds developed from cultured nodal segments and leaf explants were maintained for two more passages on optimal initiation medium for culture differentiation. The distinct shoot buds from the initiation medium and developed from the callus were transferred on different strengths of MS medium fortified with different concentrations of BA and KN for shoot proliferation and differentiation. On the different strengths of MS medium tested, full strength MS medium fortified with sucrose (3%) supported optimum shoot differentiation and culture proliferation. At lower concentrations of MS salt solutions shoot buds exhibited stunted growth with fewer shoot buds formation. In kiwi, better culture proliferation was achieved on MS medium enriched with BA (3 µM) where 1:6 proliferation ratio was achieved. While, for *S. punduana* better proliferation was achieved on MS medium fortified with 9 µM BA.

In order to generate complete plantlets, root induction was initiated in rooting medium containing different auxins (NAA & IBA) at varied levels in both the species. For inducing roots, two techniques were followed viz. 1. Plus treatment of micro shoots with IBA and NAA (0-50 µM) singly for 0-24 hr; 2. Directly incorporating IBA and NAA inn the nutrient medium at a concentration of 0-15 µM singly. In general, plus treatment of micro shoots was found to be inferior of invoking roots in micro shoots of both the species.

In *A. deliciosa* defoliated micro shoots registered better rooting compared to foliated shoots. Of the different concentrations of IBA and NAA tested, better rooting was achieved on MS medium fortified with IBA (9 µM). At his concentration as many as 7 roots per micro shoot developed from single defoliated micro shoot compared to 5 roots in normal micro shoots. But in *S. punduana*, better rooting (5 roots per micro shoot) was achieved on MS medium fortified with 12 µM IBA where ~43% micro shoots responded to rooting treatment.

Somatic Embryogenesis of *S. punduana*

Saurauia punduana seeds were extracted from 5-7 weeks old fruits and cultured on MS medium fortified BA (2µM) for germination. The cotyledonary leaves were harvested from *in vitro* raised seedlings and cultured on MS fortified medium. Embryogenic callus was maintained on MS medium fortified with sucrose (3%), NAA (1.5 µM) and BA (4 µM) for callus proliferation. The callus was subsequently maintained for embryo development and maturation. About 68% of the leaf pieces formed embryogenic callus on MS medium fortified with sucrose (3%, w/v), NAA (1.5 µM) and BA (4 µM). The embryogenic cultures registered ~348% proliferation of inoculum callus after four weeks on subsequent subculture onto proliferation medium. The proembryos converted into cotyledonary embryos and torpedo shaped embryos when transferred onto MS medium fortified with sucrose (4%) and BA (4 µM); ~62% globular embryos converted to cotyledonary embryos. In addition as many as 38 embryos per 200 mg of embryogenic cultures were formed. The somatic embryos germinated on the conversion medium accompanied by secondary somatic embryo formation. The germinated embryos could be easily separated from the clump and transferred to potting mix and maintained in a polyhouse for 5-6 weeks, where ~75% seedlings survived after 6 weeks in the potting mix. This is a reproducible protocol for somatic embryogenesis and secondary embryogenesis of *Saurauia punduana* and their

conversion into embling. The protocol developed will help in production of clonal planting materials and propagation of this species.

Conclusions

Successfully acclimatized plantlets was produced through successive stages of *in vitro* hardening in non-agar substratum such as partially chopped coconut coir, forest litter and saw dust. Of the different substrata tested, partially chopped coconut coir favored the firm establishment of roots on substratum. The partially hardened plantlets were transferred to community potting mix prepared by mixing soil, sand and decayed wood powder at ratio 2:1:2. The potted plants were manually exposed in natural sun light for 0-2 hr a day with regular interval for a wk followed by transferring to the natural light conditions. Surviving plantlets after transferred into natural conditions reads 62 % in *A. deliciosa* and 70 % in *S. punduana*.

The protocols developed for micropropagation of two species from nodal segments as well as from leaf explants and somatic embryogenesis will help in production of clonal planting materials of this two economically important species. Horticultural Department may adopt the protocols developed in the present study for propagation of these two species and provide the planting materials to the farmers who are associated with cultivation of these species.

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