IN VITRO CLONING OF *CISSUS QUADRANGULARIS* LINN. – AN IMPORTANT MEDICINAL PLANT

Thesis submitted in
partial fulfillment for the award of

Degree of
Master of Science in Biotechnology

BY

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CANDIDATE'S DECLARATION

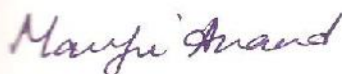
I, hereby declare that the work presented in thesis entitled, "***In vitro*** **cloning of** ***Cissus quadrangularis*** **Linn.- an important medicinal plant**" in partial fulfillment for the award of degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work done during the period of six months from January 2011 to June 2011, under the guidance of Dr. Manju Anand, Assistant Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

DATE: TEJINDER KAUR

PLACE: Patiala

CERTIFICATE

This is to certify that the thesis entitled, "***In vitro* cloning of *Cissus quadrangularis* Linn.- *an* important medicinal plant**" submitted by Tejinder Kaur in partial fulfillment of the requirement for the award of degree of Master of Science in Biotechnology, to Thapar University, Patiala, is an authentic record of her own work carried out by her during the period of six months from January 2011 to June 2011, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other university or institute.



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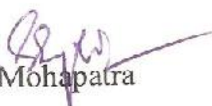

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ABBREVIATIONS

Å	Angstrom
BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog's medium
MS	Murashige and Skoog's medium
^{O}C	Degree Celsius
CM	Coconut Milk
NAA	Naphthalene acetic acid
2, 4-D	2, 4-dichlorophenoxy acetic acid
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
2-ip	2-isopentenyl adenine
Kn	kinetin
Zn	Zeatin
AS	Adenine sulphate
TDZ	Thidiazuron
NK	NAA (7.35µM) + Kn (2.325µM)
µM	micromolar
W	watt

CONTENTS

CHAPTER	PAGE NO.
1. ABSTRACT	1-2
2. INTRODUCTION	3-10
3. REVIEW OF LITERATURE	11-15
4. MATERIAL AND METHODS	16-21
5. RESULTS AND OBSERVATIONS	22-47
6. DISCUSSION	48-50
7. LITERATURE CITED	51-58

ABSTRACT

The present investigation was carried out on an important vulnerable medicinal plant *Cissus quadrangularis* Linn. belonging to family Vitaceae. This plant is commonly known as "Hadjod" in Hindi because of its ability to promote healing of the fractured bones. The different vegetative parts i.e. nodal explant, shoot apices, stem and leaves were excised from an elite field grown mature plant and thereafter planted on variously supplemented Murashige and Skoog's medium for multiple shoot proliferation, callus induction and organogenesis.

Cissus exhibits a good degree of propensity of multiple shoot proliferation from nodal and shoot apex segments. Multiple shoot proliferation from nodal explants was observed on MS medium supplemented with Zn (2.28µM-9.12µM) either alone or in combination with BAP (4.4µM-8.8µM). Best results were however obtained on MS medium supplemented with Zn (4.56µM), where 10-12 shoots were obtained after 8 weeks of culturing. Shoot apices also exhibited multiple shoot proliferation when cultured on MS medium supplemented with Zn (2.28µM-9.12µM) either alone or in supplement with BAP (4.4µM-8.8µM). BAP (8.8µM-26.4µM) in combination with Kn (4.65µM) or AS (1.35µM-21.9µM) also exhibited the formation of multiple shoots from shoot apices. Regenerated shoots were rooted on a separate root inducing medium which consisted of full strength basal MS medium. Well developed, long white roots were formed in about 100% of cultures after 3 weeks of culturing. The plantlets with elongated root and shoot systems were subjected to hardening and attempts are underway to establish these plantlets in the soil.

Callus formation occurred from stem and leaf segments when planted on different concentrations and combinations of auxins and cytokinins. Stem segments callused on MS medium supplemented with different concentrations of NAA (7.35µM-14.7µM) and Kn (2.325 µM -9.3µM). Murashige and Skoog's medium supplemented with NAA (7.35µM) and Kn (2.325µM), hereby, designated as NK medium turned out to be optimal medium for initiation and good growth of callus. Further, addition of 15% CM to the NK medium considerably enhanced the callus formation and extending its browning period. The callus formed from the stem explant was highly friable and heterogenous in nature being composed of cells of different sizes and shapes and showed the presence of significant amount of starch granules.

Formation of leaf callus was observed on MS supplemented with NAA (7.35 µM) and Kn (2.325µM). Root differentiation from leaf calli occurred on NK medium after 6-7 weeks of culturing. The roots were thick, small and white in colour. A low frequency of shoot differentiation from the leaf callus was observed in 2% of the cultures on NAA (14.7µM) and Kn (2.325µM) after 8 weeks of culturing.

INTRODUCTION

Plants have been used in traditional medicine globally across the various cultures for several thousand years (Abu-rabia 2005). The knowledge of medicinal plants has accumulated in the course of many centuries based on different medicinal systems such as Ayurveda, Chinese medicine, Unani, Siddha and the Japanese Kampo. Recent estimate suggests that over 9,000 plants have known medicinal applications in various cultures and countries. In India, it is reported that the traditional healers use 2500 plant species, out of which 100 species of plants serve as regular source of medicine (Pei, 2001).

According to the World Health Organization, 80% of the world's population, primarily those of developing countries rely on plant derived medicines for their health care needs (Gurib-Fakim, 2006). Medicinal plants are used at the household level by women taking care of their families, at the village level by medicine men or tribal Shamans and by the practitioners of classical traditional systems of medicine. Medicinal plants form the resource base for rapidly growing pharmaceutical and cosmetic industry. Allopathic medicine too owes a tremendous debt to medicinal plants and one in four prescriptions filled in a country like United States is either a synthesized form or derived from plant material (Srivastava *et al*. 1995).

Over the past few years, the medicinal plants especially those used in Ayurveda and other systems of medicine, have regained a wide recognition. Drugs obtained from plants are believed to be much safer and exhibit a remarkable efficacy in the treatment of various ailments (Siddhiqui *et al*. 1995). Hence, the herbal remedies are making a comeback as people rediscover the values of natural ingredients and natural cures and question the side effects of pharmaceutical drugs. In India, approximately 1,700 plant species are used in Ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi system of medicine with substantial overlap of common plants among these systems. The World Health Organization noted that out of 119 plant derived pharmaceutical medicines, about 74% are used in modern medicine in ways which are correlated directly with the traditional uses of plant medicines by native cultures.

The Richness of Indian Biodiversity

India is a 'gene-rich' country. Although its total land area is only 2.4% of the world, it accounts for 8% of the total global diversity. Out of the 1,26,188 spp/biota described from India,

plant species apportion around 45,000 including 15,000 flowering plants. It stands tenth among the plant genetic resource rich countries and one of the world's top twelve-mega diversity nations (Khoshoo, 1995).

The diversity of India's flora is spread across different bio-geographic zones of the country. India’s land surface has been grouped into ten distinct bio-geographic zones and these are further divided into 25 biotic provinces (Rodgers and Panwar, 1988). Medicinal plants constitute a very important natural resource of India because she has one of the richest plant based ethno-medicinal traditions in the world going back to over 3000 years old medicinal heritage (Rajasekharan and Ganeshan, 2002).

Present status of medicinal plants in India

There are about 45,000 plant species (nearly 20 per cent of the global species) found in the Indian Subcontinent. Of these, about 3,500 species of both higher and lower plant groups are of medicinal value. Of around 500 medicinal plant species used by the contemporary Ayurvedic industry, around 90 per cent are procured from wild areas, mostly notified as forest land. Medicinal plants procured from cultivated private fields account for 10% of the total medicinal plants in active trade. The forests of Himachal Pradesh and the Western Ghats are known to supply a very large proportion of the medicinal plant requirements of India. Cultivation of medicinal plants at the farm level is one of the interventions being focused and tried to meet their ever increasing demand. The crucial point is that all medicinal plants cannot be cultivated because of their agro-climatic requirement specificity. Further, the effect of agro-climatic conditions on the chemical composition and therapeutic properties of medicinal plant species are well recognized and documented in Ayurveda. Seasonal variations and age has a bearing on the composition of drugs. These factors limit the number of medicinal plants which are amenable for cultivation and extent to which it can be cultivated. On the other hand, technology and institutional arrangements influence which species are preferred for cultivation and who are going to grow them. Given these facts, there is an urgent need to assess priority species for future planning (Singh, 2006).

A high level exploitation over the recent past coupled with habitat loss and degradation as a result of various biotic pressures has led to a noticeable decline in the population levels of many of the valuable medicinal plant species. Over 70% of the plant collection involves

destructive harvesting because of the use of the parts like root, bark, wood, stem and whole plant in case of herbs. The growing demand is putting a heavy strain on the existing resources causing a number of species to be either threatened or included in endangered category. This posses a definite threat to the genetic stock and to the diversity of medicinal plants. The assessments done so far for the prioritized native medicinal species have resulted in the assignment of IUCN red list status to nearly 200 of India's medicinal plant species (Ved and kumar, 2000).

Raising demand

The World Health Organization (W.H.O.) has estimated that the present demand for medicinal plants is approximately US $14 billion per year. The demand for medicinal plant based raw material is growing at the rate of 15 to 25% annually and according to an estimate of WHO, the demand for medicinal plants is likely to increase more than US $5 trillion in 2050. In India, the medicinal plant related trade is estimated to be approximately US $1 billion per year.

This indicates that production, consumption and domestic and international trade in medicinal plants based products is going to grow at a significant rate. For making full use of this potential, India must develop scientific cultivation, post harvest technology, processing, manufacturing, research and extension, patenting and marketing for medicinal plants. The small and poor growers of these plants, mostly located in hills, mountains and inaccessible places must also be made more involved with the processes of commercial production and marketing of these products so that they can increase their earnings and are definitely not exploited. The state governments have to carry forward this task with great zeal. Though economic importance of medicinal plants is well known, it is considered as a forestry sub-sector (non timber forest products) in India. Till Medicinal Plants Board was constituted in year 2000, no nodal agency was there to look into medicinal plants as an ‘economic sector’ and different organizations were dabbling with different aspects of medicinal plants without any focus and co-ordination thereby leading to paradox of simultaneous existence of under-utilization and overexploitation. Further, the lack of co-ordination has also led to critical research gap, that is, there is a regrettable absence of any research community working on socio-economic and policy aspects of medicinal plants, such as that which exists with regard to agro-technology, biotechnology etc. (Kala *et al.* 2006).

The need of the hour is to re-plan India's participation in the global market and to collect all the available information regarding medicinal plant development in the country in order to obtain a comprehensive overview which will provide the necessary insight for the co-ordination and effective action.

Medicinal plants and Plant Tissue Culture

The rising demand of plant based drugs is creating heavy pressure on some selected high valued medicinal plants in wild due to overharvesting. Several of these medicinal plant species have slow growth rates, low population densities and narrow geographical range, therefore they are more prone to extinction. Most of the medicinal plants either do not produce seeds or their seed are too small and do not germinate in soil. Thus mass propagation of disease free planting material is generally a problem. Moreover the plants raised through seeds are highly heterozygous showing great variations in growth, habit and yield. Likewise majority of the medicinal plants are not amenable to vegetative propagation by cutting or grafting and the plants raised through vegetative propagation are invariably infested with one or more pathogens. It is therefore imperative to adopt alternative methods having high multiplication rates to produce large number of plants of improved quality and shortened rotation. In this regard micropropagation holds high potential for true to type, rapid and mass multiplication of high quality planting material under disease free conditions. *In vitro* propagation from very small plant part (0.2-10mm), also called micropropagation, is infact the miniature version of conventional propagation which is carried out under aseptic conditions. Micropropagation has number of advantages like:-

- Higher rate of multiplication.
- Environment can be controlled or altered to meet specific needs of the plant.
- Plants are available all year round (independent of regional or seasonal variations).
- Identification and production of clones with desired characteristics.
- Production of secondary metabolites.
- New and improved genetically engineered plants can be produced.
- Conservation of threatened plant species.
- Preservation of germplasm by cryopreservation.

In vitro techniques are being increasingly used for the multiplication and conservation of the germplasm of medicinally important plants threatened with extinction (Bhojwani and Arora, 1989). The successful and efficient protocols for micropropagation of many rare and endemic species of Western Ghats have been established in *Trichopus zeylanicus* (Krishnan *et al.* 1995), *Rauwolfia micrantha* (Sudha and Seeni, 1996), *Aristolochia indica* (Manjula *et al.* 1997), *Gymnema elegans* (Komalavalli and Rao, 1997), *Dictyospermum ovalifolium* (Thoyajaksha and Ravishankar, 2001) and *Calophyllum apetalum* (Lakshmi and Seeni, 2003).

Other endemic and endangered plants throughout India for which micropropagation protocols are available are *Woodfordia fruticosa* (Krishnan and Seeni, 1994), *Eremostachys superba* (Sunnichan and Shivanna, 1998), *Holostemma annulare* (Sudha *et al.* 1998), *Decalepis hamiltonii* (Anitha and Pullaiah, 2002), *Psoralea corylifolia* (Jeyakumar and Jayabalan, 2001), *Holostemma ada-kodien* (Martin, 2002), *Agave victoriae* (Alejandro *et al.* 2003), *Saussurea obvallata* (Joshi and Dhar, 2003) and *Anemopaegma arvense* (Pereira *et al.* 2003).

Techniques of Micropropagation

Micropropagation of plants can be achieved through three main techniques:-

1. **Enhanced axillary shoot proliferation:**

   Micropropagation through apical and axillary shoot proliferation is the most common, reliable and applicable method for *in vitro* mass multiplication. Cells of the meristems are uniformly diploid and least susceptible to genetic changes. Hence, it is the most reliable technique for mass propagation since it ensures genetic stability of the clones.

2. ***De novo* formation of adventitious shoots**: New adventitious shoots can develop either:
   Directly from the explants like root, stem, petiole, leaf lamina and flower parts etc.
   Or
   Indirectly from callus cultures obtained from these explants. Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it.

3. **Somatic embryogenesis:**

   It involves the formation of bipolar embryos from the somatic cells which can develop into fully functional plants under appropriate conditions.

Stages in micropropagation

Micropropagation involves 4 definite stages. These are as follows:

Stage 0: Selection of healthy disease-free elite mother plant for culture initiation.

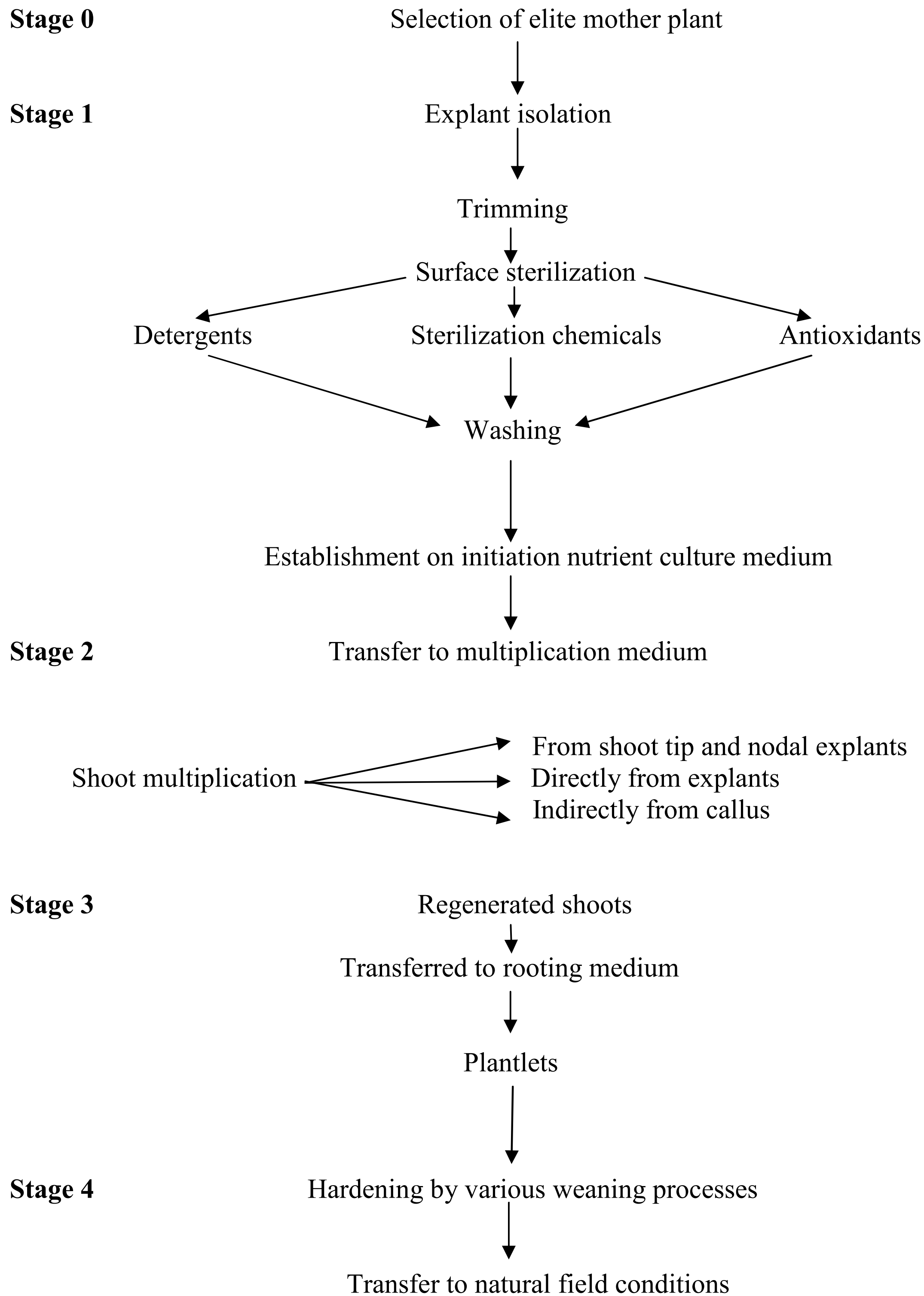
Stage I: Initiation and establishment of aseptic cultures. (Main steps: explants isolation, surface sterilization and establishment of explants on appropriate culture medium).

Stage II: Shoot multiplication or rapid somatic embryo formation using a defined culture medium.

Stage III: Rooting of regenerated shoots and germination of somatic embryos. Shoots are separated manually from clusters and transferred to a rooting medium supplemented with auxin or on a medium having low salt concentration and/or low reduced sugar level.

Stage IV: Transfer of plantlets to natural environment through acclimatization or hardening. Hardening of plantlets imparts some tolerance to moisture stress and a shift from heterotrophic to autotrophic nutrition. During hardening, plantlets develop cuticle and their stomata start functioning. Hardened plantlets are then transferred to glass or polyhouse under normal environmental conditions.

Major steps in micropropagation



OBJECTIVES

The present investigation deals with the development of an efficient and reproducible protocol for the rapid multiplication of *Cissus quadrangularis* having high medicinal value. Propagation of this plant is not possible through seeds as the flowers are sterile and the plant does not set seeds. It is propagated by vegetative means only.

This valuable plant has not been studied through tissue culture technique and no micropropagation protocol is available so far for its multiplication. Micropropagation can be used as an effective supplementation to conventional method of vegetative propagation with the objective of enhancing the multiplication rates.

The objectives of present studies were:-

- To develop an efficient protocol for rapid and mass scale propagation of plant in short duration of time and space.
- To obtain genetically pure elites rather than having indifferent populations under *in vitro* conditions.

REVIEW OF LITERATURE

The science of plant tissue culture takes its roots from path breaking research in botany like discovery of cell followed by propounding of cell theory (Schleiden, 1838 and Schwann, 1839). Based on this premise, in 1902, a German physiologist Gottlieb Haberlandt developed the concept of *in vitro* cell culture and gave the idea of totipotency. He isolated single fully differentiated individual plant cells from different plant species like palisade cells from leaves of *Laminum purpureum*, glandular hairs of *Pulmonaria* and pith cells from petioles of *Eicchornia crassipes* and was the first to culture them in Knop's salt solution enriched with glucose. In his cultures, cells increased in size, accumulated starch but failed to divide. Haberlandt's attempt to grow vegetative cells in an artificial medium did not succeed due to the non discovery of growth hormones at that time and unfortunate choice of highly specialized material, but it did open up new vistas in morphogenesis.

Haberlandt's hypothesis has by now flowered into a vigorous discipline- "Tissue Culture". Tissue culture refers to the technique of growing plant cells, tissues and organs in a sterile environment on nutrient medium. Today plant tissue culture has emerged as a potential technique and forms the backbone of biotechnology. Tissue culture techniques are widely applied for the improvement of field crops, forests, horticulture and plantation crops for increased agricultural and forestry production. The technique has been commercialized globally and has contributed significantly toward the enhanced production of high quality planting material.

During the last few years, micropropagation has emerged as a promising technique for rapid and large scale propagation of selected plants. Micropropagation is infact the miniature version of clonal propagation, which is carried out under aseptic conditions. The technique of micropropagation is based on the concept of totipotency as proposed by Harberlandt. Every cell of the plant body is totipotent i.e. capable of giving rise to a new plant under proper nurture conditions. Micropropagation is now a well established technique commercialized globally for rapid production of a number of commercially important plants.

Micropropagation is a complex process which can be achieved by any of the three approaches

- Multiplication by axillary and apical shoots
- Multiplication by adventitious buds
- Somatic embryogenesis

The first two approaches lead to plantlet formation via organogenesis through production of unipolar shoots which must then be further multiplied, followed by rooting in a multistage process. In contrast, somatic embryogenesis leads to the formation of bipolar embryos through steps that are often similar to zygotic embryogenesis. Shoot multiplication is widely used for the clonal propagation using the above approaches.

Multiplication by axillary and apical shoots

Axillary and apical shoots contain quiescent or active meristems depending upon the physiological state of the explants. Micropropagation through apical and axillary shoot proliferation is the most reliable technique for mass propagation since it ensures genetic stability of clones. A shoot tip and an axillary bud when grown under high cytokinin concentration, usually develop axillary shoots which can be subdivided into smaller clumps of shoots which in turn can develop similar clusters after subculturing on fresh medium. This process can go on indefinitely and millions of plants can be raised starting from a single shoot tip or axillary bud. This method ensures genetic stability as the cells of meristems are uniformly diploid and are least susceptible to genotypic changes.

The multiplication rate through this technique varies with genotype and cytokinin requirement has been extremely variable. Das *et al*. (2011) reported multiple shoot proliferation (more than 11 shoots) in *Stevia rebaudiana* from a single shoot apice on MS medium supplemented with 3% sucrose and 2mg/l kinetin. Ananthi *et al.* (2011) reported successful multiple shoot formation from nodal explant (42 shoots) and shoot tip explant (37 shoots) in *Rorippa indica* on MS medium supplemented with 3mg/l BAP. Multiple shoot formation (11 shoots) was also induced from nodal explant on MS with 2.0 mg/l BAP in *Clitoria ternatea* by Pandeya *et al*. (2010).

In *Ceropegia spiralis,* Murthy *et al*. (2010) successfully achieved multiple shoot production (14 in number) using nodal explants on MS medium supplemented with 2.22µM BAP. Kalimuthu *et al*. (2010) established a rapid, simple, two step protocol for the

micropropagation of *Aloe vera* through enhanced axillary branching on MS medium supplemented with 1.5 mg/l BAP and 50 mg/l AS. In *Baliospermum montanum,* an effective micropropagation protocol was established with maximum shoot bud induction and shoot multiplication (22 in number) from nodal explant on MS medium supplemented with 2.0 mg/l BAP (Sasikumar *et al*. 2009). Murashige and Skoog's medium supplemented with 2.0 mg/l BAP and 1.13 mg/l IAA was found to be most effective in inducing bud break and growth and initiating multiple shoot proliferation at the rate of 39 microshoots per nodal explant after 30 days of culture in *Stevia rebaudiana* (Debnath, 2008).

Parabia *et al.* (2007) successfully achieved multiple shoot proliferation (6 shoots per node) from nodal explants on MS medium supplemented with BAP (4mg/l) and NAA (0.5mg/l) in *Leptadenia reticulata*. In *Cissus sicyoides,* highest bud induction from the nodal explants was achieved on MS medium supplemented with 4.64 mM of kinetin and 2.7 mM of NAA (Abreu *et al.* 2003).

Multiplication by adventitious buds

Many medicinal plant species have been successfully propagated *in vitro* by adventitious shoot initiation. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf lamina and flower parts or indirectly from the calli obtained from these explants. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots.

In *Solanum nigrum,* the regeneration of shoots from leaf was found to vary with varying concentrations of BAP. In *in vitro* derived leaf explants, maximum number of shoots (38.0) was obtained on MS medium supplemented with BAP (3.0 mg/l) and IAA (0.5 mg/l) (Sridhar and Naidu, 2011a). In *Digitalis trojana,* adventitious shoots were regenerated from leaf explants cultured on MS medium supplemented with 0.1 mg/ml NAA + 3.0 mg/ml BAP (Corduk and Aki, 2010).

Baskaran and Jayabalan (2010) reported *in vitro* regeneration of *Psoralea corylifolia* through adventitious shoot regeneration from hypocotyl explants cultured on MS medium supplemented with 3.0µM BA, 1.0µM NAA, 5µM ascorbic acid and 100mg/l casein hydrolysate. In *Tanacetum cinerariifolium*, multiple shoots were induced from leaf and petiole explants. Leaf

segments were more responsive than petiole cuttings and produced maximum shoot regeneration on MS medium supplemented with 4 mg/l BA and 0.2 mg/l 2, 4-D (Hedayat *et al.* 2009).

Beegum *et al.* (2007) reported direct organogenesis from leaf and internodal explants of *Ophirrhiza prostrate* on MS medium containing BAP alone and also reported indirect organogenesis on MS medium fortified with NAA alone or in combination with BAP or Kn. Uddin *et al.* (2005) established a protocol for rapid multiplication of shoots from cotyledonary node of *Peltophorum pterocarpum*.

Sridhar and Naidu (2011b) devised an efficient protocol for plant regeneration from *in vitro* derived callus of young leaves of *Solanum nigrum.* BAP or Kn alone or in combination with NAA and IAA was used for regeneration of plantlets from callus culture. Siddiqui *et al.* (2010) proposed an effective protocol for *in vitro* regeneration of the *Cassia angustifolia* via indirect organogenesis from petiole explants. Organogenic callus were induced on Murashige and Skoog's medium supplemented with 5.0 µM 2,4-D and 2.5 µM TDZ. Singh *et al.* (2009) reported *in vitro* shoot regeneration of *Rauvolfia serpentina* from leaf callus with highest shoot regeneration on MS medium supplemented with BAP (2.5mg/l) and IAA (0.4 mg/l). Fasial and Anis (2005) developed a protocol for high frequency shoot organogenesis and plant establishment from stem derived callus of *Tylophora indica.* Callus was developed on MS medium supplemented with 2,4,5-T (10µM) and multiple shoot induction was achieved on MS medium containing Kn (5µM).

As far as clonal propagation is concerned, the plants obtained from calli may not be true elites because of considerable morphological, physiological, and genetic variations found within the callus and there are relatively high incidence of aneuploidy and polyploidy associated with callus cells and plants obtained from it. Another disadvantage of the method of shoot multiplication involving a callus phase is that it is not applicable to many important crop species. Where applicable, the initial plant regeneration capacity of the tissues may decline with the passage of time and is eventually lost. But still callus constitutes one of the unique materials for rapid multiplication of plants, since thousands of plants can be obtained from a small tissue.

Somatic embryogenesis

Somatic embryogenesis involves the formation of a bipolar structure containing both shoot and root axis and developing in a same manner similar to zygotic embryo.

These embryo like structures have been variously designated as accessory embryos, adventives embryos and embryoids. These embryoids can developed into fully functional plants under appropriate conditions. Somatic embrogenesis was first reported by Steward *et al.* (1958) and Reinert (1959) in tissue cultures obtained from secondary phloem of carrot roots. Embryoid formation has been reported in tissue and organ cultures of a number of plant species derived from leaf, petiole, root, floral parts and nucellar tissue of *Chrysanthemum* (Mani and Senthil, 2011), *Withania sominfera* (Sharma *et al*. 2010), *Datura stramonium* (Sundar and Jawahar, 2010), *Rauvolfia serpentina* (Singh *et al*. 2009), *Podophyllum peltatum* (Kim *et al*. 2007) *Tylophora indica* (Chandrasekhar *et al,* 2006), *Syngonium podophyllum* (Zhang *et al.* 2005), *Ceropegia candelabrum* (Beena and Martin, 2003), *Podophyllum hexandrum* (Pandey *et al*. 2002), *Pimpinella tirupatiensis* (Prakash *et al*. 2001) and many more medicinal plants.

As far as *Cissus quadrangularis* is concerned, no investigation on micropropagation through any of the techniques has been reported so far.

MATERIAL AND METHODS

Choice of material

Cissus quadrangularis Linn. (Family Vitaceae) commonly known as the "bone setter" was selected as an experimental material. The plant is referred to as "**Asthisamdhani**" in Sanskrit and "**Hadjod**" in Hindi because of its ability to join bones. It is a succulent, perennial climber having a thick, quadrangularis jointed cactus like stem. The thick stem is glabrous and fleshy and sharply 4 angled with constrictions at its nodes. Internodes are 8-10 cm long and 1.2-1.5 cm wide (Fig.1).Its alternate, simple leaves are also thick and ovate with serrated margins. Numerous tendrils grow out of the plant's nodes. Flowers are small, bisexual, tetramerous occurring in umbellate cymes opposite to the leaves. The flowers have lobed cup shaped calyx and greenish yellow petals with red tips (Fig.2). The fruits are small, round berries ±0.7 cm in diameter, red on ripening and one seeded (Panda and Das, 2004 and Wealth of India 2005). Flowering and fruiting time is May- June. Plant does not set seeds as flowers are sterile.

Habitat and distribution

General habitat of *Cissus quadrangularis* is lowland and medium altitude dry grassland, deciduous bushland and woodland often associated with termite mounds.

It grows natively in hotter and dried regions of India including the Deccan Peninsula extending west to lower eastern slope of Western Ghats. It is also found in the drier areas of the world such as Srilanka, Malaya, java, Thailand, and South Africa.

Medicinal importance

Cissus quadrangularis is an ancient valuable medicinal plant and the use of this plant by common folk for promoting fracture healing process is an old practice. The plant has been documented in Ayurveda and Siddha systems of medicine for the treatment of various ailments like gouts, syphilis, venereal diseases, piles, leucorrhoea, diarrhoea and dysentery. The entire plant is of medicinal value and is known to have a number of pharmacological effects like bone healing, anti-inflammatory, analgesic, antiosteoporotic, antimicrobial, antiviral, antiulcer, antioxidant and antiobestity properties (Singh *et al*. 1984, Deka *et al.* 1994, Murthy *et al.* 2003,

Figure 1. A plant of *Cissus quadrangularis*

Figure 2. Cissus plant with fruit and flowers

Jainu *et al.* 2006, Oben *et al.* 2006, Panthong *et al.* 2007, Austin and Jegadeesan, 2009, Balasubramanian *et al.* 2010).

Marketed product

Cissus quadrangularis is an ingredient of an Ayurvedic preparation "Laksha Gogglu" which is used for alleviating pain, reducing swelling and promoting healing of simple factures (Mishra *et al.* 2010). Plant has been prescribed in ancient Ayurvedic text by Bhava Prakash and Chakra Dutta as a general tonic especially for the patients with fractured bones. It is an ingredient of 'Bonton' capsules (Vasu Healthcare) used for osteoporosis and 'Cissus Capsules' (PrimaForce).

Ethnoveterinary usage

Cissus quadrangularis is fed to cattle as a galatologue to induce flow of milk. The whole plant is used in cases of fractures, sprains, rheumatism, irregular growth of teeth, broken horn, anthrax, haematuria, elephantiasis, dislocation of hip, various wounds and cracked tail.

Safety profile

Safety studies in rat shows no toxic effects at dosages as high as 2000 mg/kg of body weight. The fresh juice of the plant may irritate the skin and cause itching.

Chemical constituents

- Phytochemical analysis has revealed that the plant contain high amount of dietary antioxidants that include vitamin C, beta-carotene, and polyphenols.
- Stilbene derivatives: Stem has unique stilbene derivatives which are Quadrangularins A, B, and C (Adesanya *et al*. 1999), resveratrol, piceatannol, pallidol and parthenocissin are also present in the stem.

- Phytosterols and lipids: ketosteroids, tetracyclic triterpenoid (7-oxo, oncer-8-ene-3 β, 21 α-diol), pentacyclic triterpenoids (8-amyrin and β-amyrone), β-sitosterols and lipids have been isolated from aerial parts especially stems (Gupta and Verma 1990, Adesanya *et al*. 1999, Mehta *et al.* 2001, Jain *et al.* 2009)

- The plant extract serves as a rich source of calcium ions which when treated with CO_2 leads to formation of calcite crystals of highly irregular morphology (Sanyal *et al.* 2005)

Glassware

The glassware used for culture work comprised of Corning test tubes (25×125mm), conical flasks (150 ml, 250 ml, 500 ml, and 1000 ml), culture bottles (25 × 150 ml), measuring cylinders (100 ml, 1000 ml), Petri dishes, beakers and a range of pipettes (200ul, 1ml, 2ml, and 10ml). Before use, the glassware was subjected to chromic acid solution (mixture of $K_2Cr_2O_7$ + H_2SO_4 + H_2O) followed by thorough washing with tap water. All the vessels were then washed thoroughly with teepol (1% v/v) detergent solution. Then they were cleaned under running tap water. The glassware was then inverted in a clean tray and left to dry in the oven. Plugs for the tubes and flasks were made out of absorbent surgical cotton wrapped in muslin. 5 - 10 ml water was then poured into each culture vessel which was tightly plugged. The glassware was then steam sterilized in an autoclave at a pressure of 15 lb/in^2 at 121°C for 15 - 20 minutes.

Culture medium

The media formulation described as Murashige and Skoog (1962) referred as MS medium was selected as the optimal culture medium. Stock solutions of generally 4 times major elements, 100 times minor elements, 10 times organic constituents were prepared. These stock solutions were stored in a freeze chest at 4°C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days. Stocks of the hormones were also made either 2X or 4X. They were also kept at 4°C.

Defined amount of all ingredients were mixed and volume was adjusted by distilled water. After incorporation of all the ingredients, 2% sucrose was also added along with all these supplements and various concentrations of plant growth regulators. pH of the medium was adjusted to 5.6-5.7 with 0.1N HCl or NaOH. The prepared medium was then gelled with 0.7-0.8% w/v agar- agar.

Definite aliquots of the medium were then added depending upon the capacity of the culture vessels. Generally 25ml of medium was distributed into the test-tubes and 40ml in culture bottles. Tubes were plugged with cotton plugs, steam sterilized at 15lb/in^2 (121◦C) for 15

minutes. After autoclaving, tubes were placed in slanting stands to give slanted surface to agar media. These were then left to cool and solidify.

Table 1: Composition of Murashige and Skoog's medium (1962)

Ingredients	Concentration(mg/l)
Major elements:	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor elements:	
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.4H_2O$*	27.8
Na_2EDTA*	37.3
Organic constituents:	
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
PyridoxineHCl	0.5
Thiamine HCl	0.1
Sucrose	20,000

*ferric Na EDTA is the alternative to the use of these two salts and is added freshly to the medium (i.e. 0.04 gm/l).

Inoculations

All the experimental manipulations were carried out under strictly aseptic conditions in a laminar air flow bench fitted with a bactericidal U. V. tube (15 W, peak emission 2637 A^o). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, scalpels, blade etc.), gas burner, lighter, tube containing absolute alcohol etc. were also cleaned with alcohol. The fresh material to be inoculated was kept in a Petridish covered with a piece of black paper in order to

protect it from the harmful effects of U. V. rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with U.V. rays continuously on for one hour.

Surface sterilization of explants

The explants like leaves, nodal and internodal segments and shoot apices were taken from the healthy plant growing under the *in vivo* conditions. These were placed in bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (1% v/v) for another 10-15 minutes and then washed properly to remove the detergent. The explants were then treated with Bavistin (0.1 % w/v) for another 10-14 minutes to remove the fungal contamination and then washed properly to remove the fungicide.

Hands and arms which were to be used inside the inoculation chamber were scrubbed with alcohol before inoculation. The rims of the test tubes and the sides of the plugs were flame sterilized. Instruments (like forceps, scalpels, spatula etc.) were all sterilized by dipping in the alcohol and flaming a number of times. Care was taken to cool the instruments before putting into operation. The explants taken from field borne plants were treated with 0.1% mercuric chloride solution for 3- 5 minutes respectively depending upon the explants. Shoot apices and leaves of *Cissus quadrangularis* were treated with 0.1% mercuric chloride for 2 minutes and nodal and internodal segments were treated with 0.1% $HgCl_2$ for 3-4 minutes. The explants were then thoroughly washed (4 - 5 washings) with sterilized distilled water to remove the traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions. The explants were then planted on variously augmented MS medium.

Cultural conditions

All the cultures were maintained in an air conditioned culture room at a temperature of 25 $\pm$ 4°C. The source of illumination consisted of 4 feet wide fluorescent tubes (40 watt) and incandescent bulb (25 watt). The intensity of illumination was 50 μ mol $m^{-2}s^{-1}$ at the level of cultures and 12 hour light regime was followed by 12 hours of darkness.

RESULTS AND OBSERVATIONS

Nodal explant culture

Fresh nodal explants were collected from healthy, field grown mature plant of *Cissus quadrangularis*. 5mm long single node cutting having one dormant lateral bud was excised and then surface sterilized with 0.1% mercuric chloride. Damaged internodal tissue on both sides of the sterilized segment was cut off. The nodal segment 3-4 mm in size was then cultured on MS medium supplemented with various growth regulators. The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used.

Multiple shoot formation was induced from nodal explants on MS medium supplemented with different cytokinins i.e. Zn, BAP, Kn, Adenine sulphate either alone or in combination with each other and the auxin 2, 4-D.

When nodal explant was planted on zeatin (2.28µM-9.12µM), 4-12 shoots were formed depending upon the concentration of zeatin. On the lower concentration of zeatin i.e. 2.28µM, 3-4 shoots arose from the axillary position after 10 days of inoculation (Fig. 3). These shoots grew further forming well developed leaves (Figures 4 and 5). Out of various concentrations of zeatin used, 4.56µM zeatin gave the best results. Initially 3-4 shoots arose from the nodal position after 10- 15 days of culturing (Fig. 6) and with passage of time many groups of shoot initials were formed at the basal portion (Fig. 7). Many of these shoot initials sprouted into green leafy shoots later on. In this way nearly 10-12 shoots were formed from a single nodal explant after 6-8 weeks of culturing. Figure 8 shows a 10-weeks-old culture showing numerous well developed shoots and clusters of shoot initials. When nodal segments along with clusters of shoot initial were planted on fresh zeatin supplemented medium, the initials proliferated further and formed many shoots.

Axillary bud break was also observed on TDZ (4.5µM) supplemented medium, where 6 shoots were formed after 2-3 weeks of culturing (Fig. 9). The leaves were much larger in size, less green in colour and leaf incisions were much deeper (Fig. 10) as compared to the leaves of shoots formed on other cytokinins. Multiple shoot formation was also observed on MS medium supplemented with BAP (17.6-35.2µM), where 2-3 shoots were regenerated from single axillary

bud after 2 weeks of culturing (Fig. 11). BAP (4.4µM) when used in conjunction with Zn (4.56µM) also promoted the formation of 4-5 shoots from the axillary position (Fig. 12).

Out of the various auxins tested, 2, 4 –D (9.74µM) was able to induce multiple shoot proliferation (5 shoots) from the nodal segment. A number of roots also arose from the nodes of the newly formed shoots (Fig. 13). On subsequent subculturing on the same medium, more shoots proliferated. Response of various growth regulators on shoot proliferation from nodal segment is depicted in table 2 and figure 14.

Table 2:

S.no.	Plant growth regulators	No. of shoots
1	MS+BAP (8.8µM)	1
2	MS+BAP (17.6µM)	3
3	MS+BAP (35.6µM)	2
4	MS +BAP(26.4µM)+Kn(4.65µM)	3
5	MS+2, 4-D (9.74µM)	5
6	MS+2, 4-D (19.48µM)	1
7	MS+Zn(2.28µM)	4
8	MS+Zn(4.56µM)	10-12
9	MS+Zn(9.12µM)	2
10	MS+Zn(4.56µM)+BAP(4.2µM)	4-5
11	MS+TDZ(4.5µM)	6

Shoot tip culture

Shoot apices (2-3mm) were excised from the field grown plant and were surface sterilized and planted on MS medium supplemented with different growth regulators. Multiple shoots were initiated from shoot apex on MS medium supplemented with different concentrations of Zn and BAP used alone or in combination with Kn, AS or Zn.

Shoot apex when cultured on MS medium supplemented with Zn (2.28µM-18.2µM) showed the formation of 3 shoots initially (Fig. 16) and after subculturing on the same medium, clusters of shoot initials were formed after 8 weeks (Fig. 17). Many of these shoot initials developed into green leafy shoots subsequently.

Equally good results were obtained when shoot apex was cultured on BAP (4.4µM-26.4µM) along with either Zn (4.56µM) or AS (1.35µM) where 5-6 shoots were formed per

culture (Figures 18 and 19). Effect of various concentrations of plant growth regulators on multiple shoot formation from shoot apex is shown in table 3 and figure 15.

Table 3:

S.No.	Plant growth regulators	No. of shoots
1	MS+BAP(26.4µM)+Kn(4.65µM)	2
2	MS+BAP(8.8µM)+AS(21.9µM)	4
3	MS+BAP(22.0µM)+AS(1.35µM)	4
4	MS+BAP(4.4µM)+Zn(4.56µM)	5
5	MS+Zn(2.28µM)	6
6	MS+Zn(4.56µM)	6
7	MS+Zn(9.12µM)	5

Rooting of microshoots

Regenerated microshoots were carefully rescued from the bottles in laminar air flow on sterile glass plate. Then each of these shoots was carefully inoculated upright in the MS medium supplemented with or without auxins for root initiation. Shoots were inoculated on BMS medium and MS media with different concentration of IBA for root initiation. On IBA (4.98µM) supplemented medium, 3-4 root primodia were formed at the base of stem, but they did not grew further into roots (Fig. 20). Best results were however, observed on BMS medium where 100% shoots formed roots after 10 days (Fig. 21). The roots were long, white and devoid of root hairs. Complete plantlets with elongated shoot and root systems were formed after 30 days (Fig. 22).

Acclimatization and transfer of plantlets to the soil:

The rooted plantlets were successfully transferred to the field conditions through successive hardening stages. The rooted plantlets were gently removed from the cultures tubes keeping the roots intact by using forceps with extreme care to avoid any mechanical injury to the plantlets. Roots were thoroughly washed with tap water to remove any remaining agar sticking to them. Plantlets were then transferred to plastic pots containing sterile vermiculite (A mixture of soil and vermicompost 1:1), then covered with plastic bags having holes and kept under the culture room condition for 15 days (Fig. 23). The plants were thoroughly watered. The plants with newly formed leaves were shifted to green house for 2 weeks (Fig. 24) and attempts are underway to establish these plantlets in the natural environment.

Stem culture

Stem segments, 4-5 mm in length were excised from field grown plant and cultured on MS medium supplemented with different plant growth regulators.

Direct root induction:

Direct root induction from the internodal segment occurred on MS medium supplemented with IBA (4.98µM) within 20 days of inoculation (Fig. 25). 2-3 roots which were long and white developed from each segment. Direct root formation was also observed on MS medium supplemented with 2, 4-D (19.48µM).

Direct adventitious shoot formation:

Direct shoot regeneration from the stem segment occurred on MS medium supplemented with 2, 4-D (19.48µM) after 20 days of culturing in about 1% of cultures (Fig. 26).

Callusing

Callus induction took place on MS media supplemented with various concentrations and combinations of auxins and cytokinin. The best results were obtained on MS + NAA (7.35µM) + Kn (2.325µM), hereby, designated as NK medium. Callusing started at the cut ends after 7-8 days of culturing (Fig. 27) and after 3-4 weeks the entire segment turned into a mass of yellowish white and friable callus (Fig. 28).

Synergistic action of NAA (7.35µM), Kn (2.325µM) and CM (15%) was demonstrated for the prolific callusing from the stem segment. On this medium callusing initiated earlier and the explant turned into mass of callus within 3 weeks (Fig. 29). The callus however turned brown on subsequent subculturing. In order to delay or stop browning, callusing was induced on the above medium with 3% glucose instead of 2% sucrose in the medium. Good quantity of callus was formed on NAA (7.35µM)+Kn (2.325µM)+15% CM+3% glucose (Figures 30 and 31). The callus was whitish in colour and showed sustained growth on subculturing and a marked decrease in browning was observed with subsequent passage of time. Effect of different growth hormones on callus induction from stem segment after 4 weeks of culturing is shown in table 4.

Table 4:

Plant growth regulators	% of explants forming callus	Growth
MS+NAA(7.35µM)	20	+
MS+NAA(14.7µM)	20	+
MS+NAA(14.7µM)+Kn(4.65µM)	80	+++
MS+NAA(14.7µM)+Kn(2.325µM)	80	+++
MS+NAA(14.7µM)+Kn(2.325µM)+BAP(2.2µM)	80	+++
MS+NAA(7.35µM)+Kn(9.3µM)	60	++
MS+NAA(7.35µM)+Kn(2.325µM)	90	++++
MS+NAA(7.35µM)+Kn(2.325µM)+15%CM	100	+++++
MS+NAA(7.35µM)+Kn(2.325µM)+15%CM+3%Glucose	100	+++++

"+" very less growth

"++" less growth

"+++" average growth

"++++" good growth

"+++++'' very good growth

Study of callus

The stem callus formed on NK was highly friable, breaking up into single cells or group of cells when placed in water. The callus was heterozygous comprising of cells of different shapes and sizes. Cells were spherical, ovoid and elongated having prominent nuclei and numerous starch grains (Fig. 32). A magnified cell is shown in Figure 33.

Leaf culture

Leaves were excised from field grown plants, were surface sterilized and cut into pieces of 3-4 mm size and planted on MS medium supplemented with different growth regulators used either alone or in conjugation with each other.

Callusing

Leaf explants were cultured on MS medium supplemented with various concentrations and combinations of NAA, Kn and 2, 4-D for callus induction.

Callusing was observed on MS + NAA (7.35-14.7µM) + Kn (2.325-4.65µM) from the cut ends of the leaf segment. However, best results were obtained on NAA (7.35µM) and Kn (2.325µM), where callusing initiated from the cut ends of the leaf segment after 2-3 weeks of culturing (Fig. 34) and within 4-5 weeks the whole leaf segment transformed into callus (Fig. 35). Leaf explant also showed callus formation on MS medium supplemented with NAA (14.7µM) and Kn (2.325µM) but the growth of callus was slow and leaf segment was transformed into callus after 6-7 weeks of inoculation. Effect of different concentrations and combination of growth regulators on callus induction from leaf explants is depicted in table 5.

Table 5:

Plant growth regulators	% explants forming callus	Growth
MS+NAA(7.35µM)+Kn(9.37µM)	60	+
MS+NAA(14.7µM)+Kn(4.64µM)	80	++
MS+NAA(7.35µM)+Kn(2.325µM)	90	+++
MS+NAA(14.7µM)+Kn(2.325µM)	60	+

"+" less growth

"++" average growth

"+++" good growth

Organogenesis from callus

Rhizogenesis:

Root differentiation occurred on NK medium after 6-7 weeks of culturing (Fig. 36). Initially a few roots were formed but with further proliferation of callus more roots appeared. These roots were small in size and whitish in colour.

Caulogenesis:

Differentiation of shoot initial from the leaf callus was observed only in 2% of the cultures on NAA (14.7µM) and Kn(2.325µM) after 8 weeks of culturing. Figure 37 shows the differentiation of a single shoot initial from the leaf callus which is further shown on magnified scale (Fig. 38).

Nodal explant culture

Figure 3. Formation of 3 shoots from the axillary position on MS + Zn (2.28µM) after 10 days of culturing.

Figures 4&5. Proliferation of more shoots from nodal explant on MS + Zn (2.28µM) after 4 and 6 weeks respectively.

Figure 6. Multiple shoot proliferation from nodal explant on MS + Zn (4.56µM) after 15 days of culturing.

Figure 7. Group of shoot initials (arrows) formed at the basal region on MS + Zn (4.56µM) after 7 weeks.

3

4

5

6

7

Figure 8. A 10-weeks-old nodal culture showing multiple shoot (10-12 shoots) proliferation from shoot initials (indicated with arrows) on MS + Zn (4.56µM).

Figures 9&10. Axillary bud break on MS + TDZ (4.5µM) showing multiple shoot proliferation after 2 and 4-weeks of culturing respectively.

8

9

10

Figure 11. 3 shoots formed from axillary position on MS + BAP (17.6µM) after 2-weeks of culturing.

Figure 12. A 4-week-old culture showing multiple shoots proliferation (5 shoots) on MS + BAP (4.4µM) + Zn (4.56µM).

Figure 13. Multiple shoots proliferated from nodal position on MS + 2, 4 D (9.74µM). A number of roots regenerated from the nodes of the newly formed shoots.

11

12

13

Figure 14. Histogram showing frequency of shoot formation from nodal explants on different growth regulators.

Figure 15. Histograms showing effects of different concentrations of growth regulators on shoot proliferation from shoot apex.

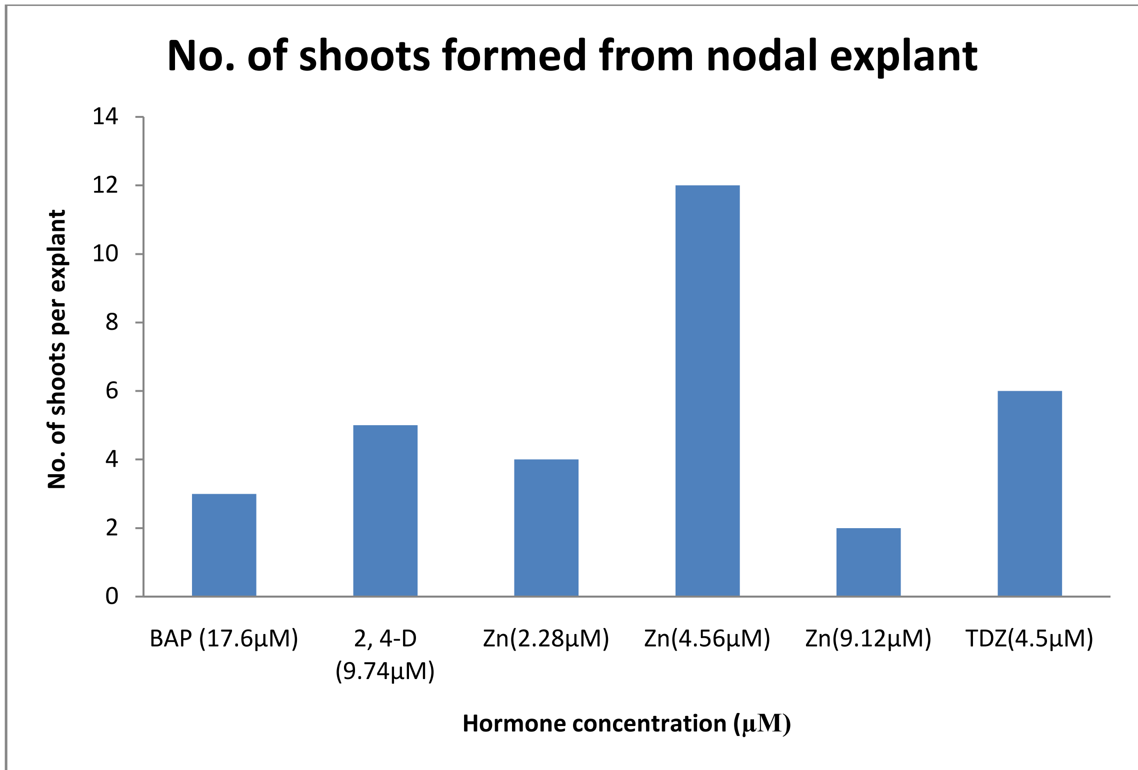


Figure 14

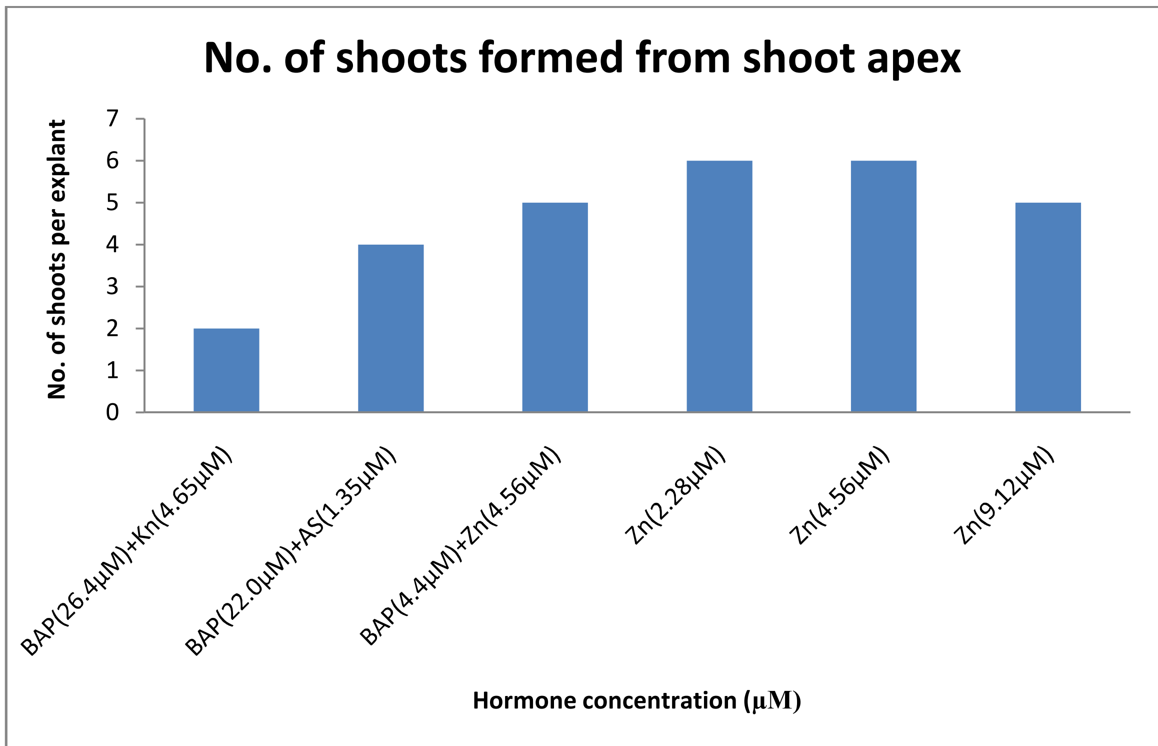


Figure 15

Shoot tip culture

Figures 16&17. Multiple shoot proliferation from the Shoot apex on MS + Zn (9.12µM) after 4 and 8-weeks of culturing respectively. Fig 17 shows cluster of shoot initials (arrow) formed at the basal end.

Figures 18&19. Multiple shoots formed (5-6 in number) from shoot apices on MS + BAP (4.4µM) + Zn (4.56µM) and MS + BAP (4.4µM) + AS (1.35µM).

16

17

18

19

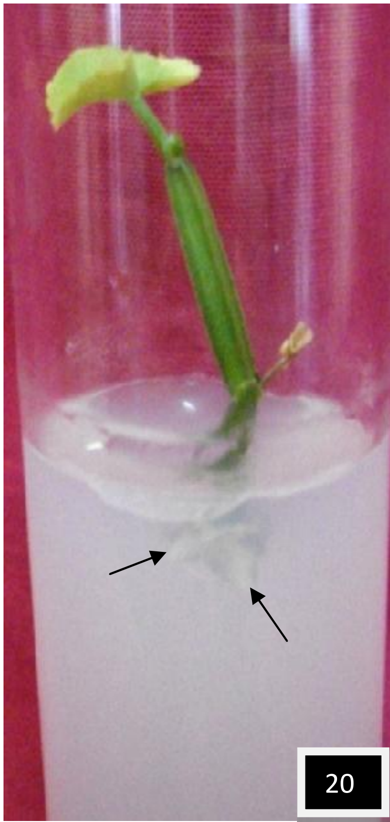
Figure 20. Initiation of root primodia (arrows) from the basal end of regenerated shoot on MS + IBA (4.9µM) after 10 days of inoculation.

Figure 21. Well developed roots formed from the basal end of regenerated shoot on BMS after 10 days.

Figure 22. Complete plantlets formed on BMS medium after 3 weeks.

Figure 23. Plantlets transferred to plastic pot and kept under the culture room conditions.

Figure 24. Plant with newly formed leaves after 30 days of hardening.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Apsara
DELUXE
22

23

24

Stem culture

Figure 25. Direct root induction from stem segment on MS + IBA (4.98µM) within 20 days of inoculation.

Figure 26. Shoot regeneration directly from stem explant after 20 days on MS + 2,4-D (19.48µM).

Figure 27. Callus initiation at cut ends of stem segment on NK medium after 1 week of culturing.

Figure 28. Formation of yellowish white and friable callus after 3-4 weeks of culturing on MS+NAA (7.35µM) + Kn (2.325µM).

25

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27

28

Figure 29. 3-4 week old stem culture showing callusing at the cut ends on NK medium + 15% CM after 3 weeks of culturing.

Figure 30. Callus initiation from stem segment on NK medium + 15% CM + 3% glucose after 2 weeks of culturing.

Figure 31. Whole stem segment transformed into mass of callus on NK medium + 15% CM + 3% glucose after 3-4 weeks of culturing.

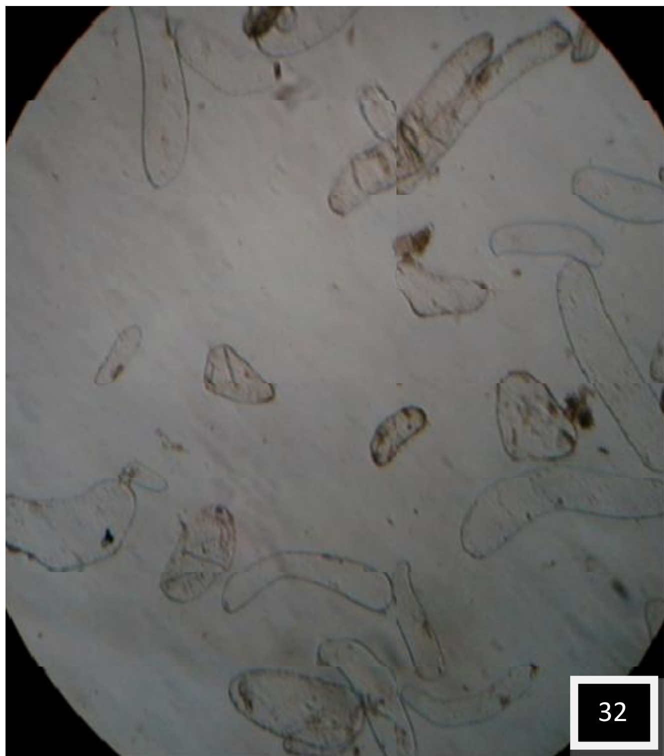
Figure 32. Group of cells of different shapes and sizes isolated from stem callus.

Figure 33. A magnified callus cell showing presence of starch granules.

29

30

31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Leaf culture

Figure 34. Leaf explant callusing at the cut ends on NK medium after 2-3 weeks of culturing.

Figure 35. Whole leaf segment transformed into mass of callus on NK medium after 4-5 weeks of culturing.

Figure 36. Root differentiation from leaf callus on NK medium after 6-7 weeks of culturing.

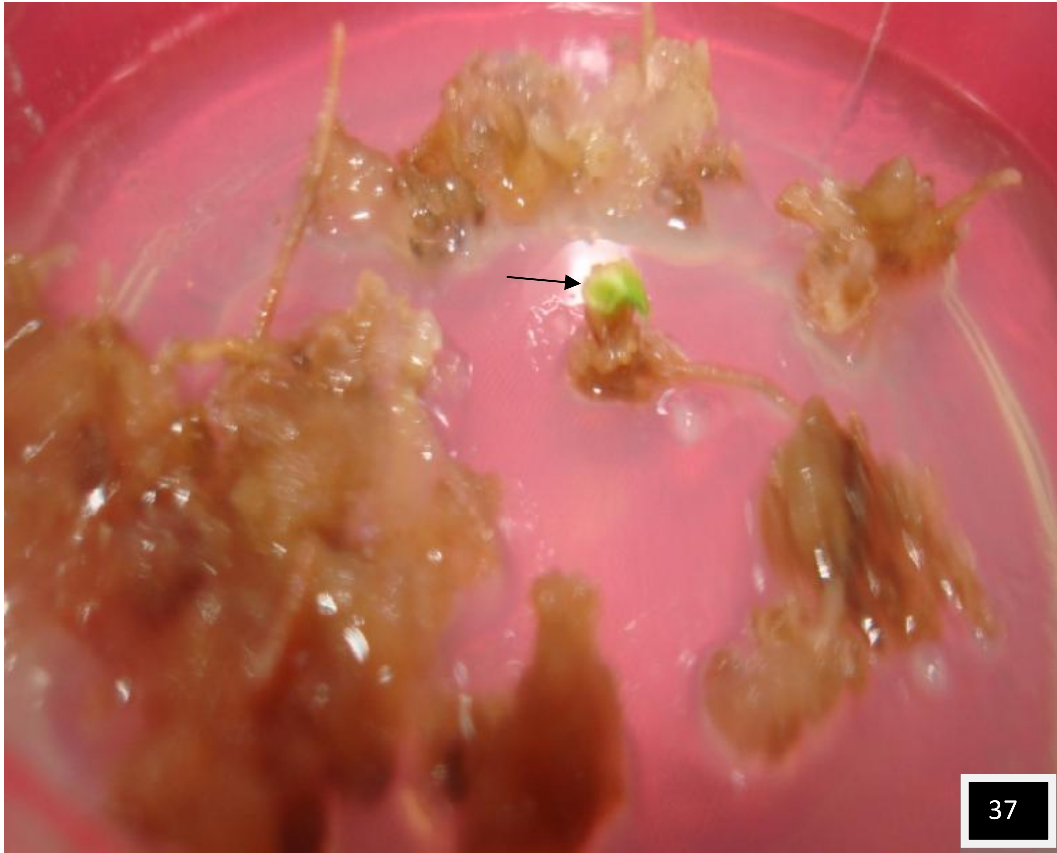
34

35

36

Figure 37. A single shoot (arrow) differentiating from leaf callus on MS + NAA (14.7µM) + Kn (2.325µM) after 8 weeks of culturing.

Figure 38. A magnified shoot initial differentiated from leaf callus.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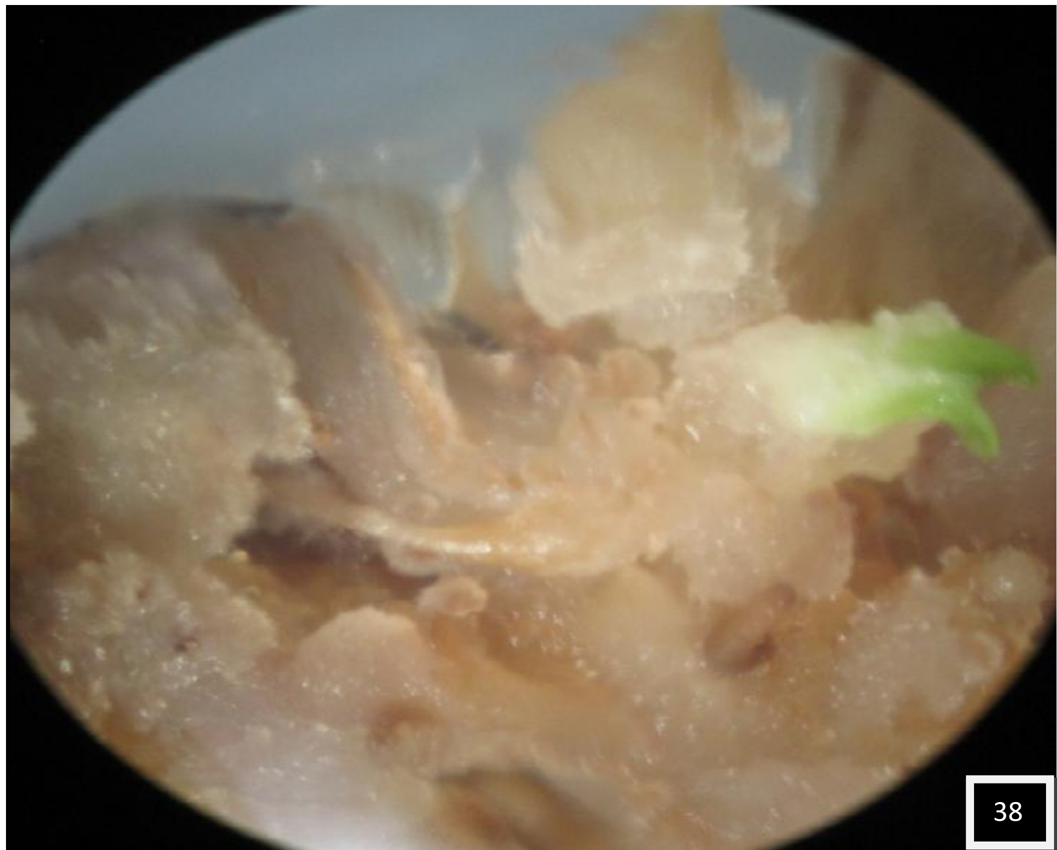
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DISCUSSION

The present investigation was undertaken on an important medicinal plant *Cissus quadrangularis* Linn. with a view to develop an efficient and reliable protocol for its clonal propagation under *in vitro* conditions.

Cissus quadrangularis is a vulnerable medicinal plant with diverse medicinal properties. Propagation of *Cissus quadrangularis* is not possible through seeds as the flowers are sterile and the plant does not set seeds. In the forests also this plant is not present in bulk and over exploitation of this plant from wild creates a need to develop an efficient protocol for micropropagation of *Cissus quadrangularis* to meet the demand of its commercial cultivation.

Micropropagation protocols have been developed for a wide range of medicinal plants which includes endangered, rare and threatened plant species. Some of these medicinal plants are *Saussurea lappa* (Johnson *et al.* 1977), *Oroxylum indicum* (Dalal and Rai, 2004), *Vanasushava pedata* (Karuppusamy *et al.* 2006), *Tylophora indica* (Fasial *et al.* 2007), *Swertia chirata* (Balaraju *et al*. 2009), *Ceropegia spiralis* (Murthy *et al.* 2010), *Picrorhiza kurroa* (Jan *et al*. 2010).

Micropropagation through apical and axillary shoot proliferation is the most reliable technique for mass propagation since it ensures genetic stability of clones. In the present investigation, axillary shoots were induced from the nodal segments and shoot apices on MS medium supplemented with different concentrations of Zn (2.28µM-18.24µM) alone or in combination with BAP (8.8µM-26.4µM). For nodal explants, best results occurred on MS medium supplemented with 4.56µM of zeatin where 10-12 shoots could be regenerated from a single nodal segment. Initially 4-5 shoots arose from the nodal position but the number gradually increased upto 10-12 after 8 weeks of culturing. Multiple shoot proliferation from shoot tip explant were observed on MS medium supplemented with 4.56µM Zn either alone or in combination with BAP (4.4µM-8.8µM). In present work BAP alone or in combination with adenine sulphate was also effective in multiple shoot proliferation from nodal explants and shoot apices.

For shoot proliferation, cytokinins are one of the most important factors affecting the response (Lane 1979 and Bhojwani 1980). Cytokinins are generally used in plant cell culture at a

concentration range of 0.1-10.0 mg/l. when added in appropriate concentrations, they may regulate cell division, stimulate axillary and adventitious shoot proliferation, regulate differentiation and stimulate protein and enzyme activity (Gross and Parthier, 1994). A wide range of cytokinins like kinetin, BAP, 2-iP, and zeatin have been employed for shoot proliferation (Bhojwani and Razdan 1983). However a wider survey of literature suggested that BAP is the most reliable and effective cytokinin for shoot proliferation. A number of plants such as *Stevia rebaudiana* (Debnath 2008*), Gynura procumbens* (Keng *et al.* 2009) and *Rorippa indica* (Ananthi *et al.* 2011) have been successfully multiplied using BAP.

In *Cissus sicyoides,* highest bud induction from the nodal explants was achieved on MS medium supplemented with 4.64 mM of kinetin and 2.7 mM of NAA (Abreu *et al.* 2003). Nath and Buragohain (2005) reported multiple shoot proliferation in *Adhatoda vasica* from shoot tip on MS medium supplemented with 2mg/l BAP. In *Ceropegia spiralis* multiple shoots induction was more successful using nodes as explants on MS medium supplemented with 2.22 µM of BAP (Murthy *et al.* 2010). High frequency and maximum number of multiple shoots were induced on MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l NAA in *Solanum nigrum* (Sridhar and Naidu 2011a).

In *Lilium nepalense* multiple shoots proliferated on MS medium supplemented with 20 µ*M* zeatin using longitudinally split shoot halves, where more than seven shoots were obtained from one explant in a 4-week culture period (Wawrosch *et al.* 2001). Higher frequency of shoot formation was observed on MS medium supplemented with 0.5 -1 mg/l BAP (31-49 shoots) and 0.25 mg/l of zeatin (33 shoots) in both shoot tip and nodal explant in *Mentha piperita* (Ghanti *et al.* 2004).

In the present work, callusing were induced from leaves and stem segments on Murashige and Skoog's medium supplemented with various concentrations of NAA (7.35µM-14.7µM) and Kn (2.325 µM -9.3µM). Good callusing, however, was observed on MS + NAA (7.35µM) + Kn (2.325µM), hereby, designated as NK medium. In the present investigation callus formed on NK medium showed browning of callus on subsequent subculturing but addition of 15% CM and 3% glucose (instead of 2% sucrose) to the medium considerably enhanced the callus growth and delayed its browning period.

Robinson and Almeida (2010), reported callus formation from leaf explants of *Cissus sicyoides on* MT medium supplemented with 1.0 mg/l NAA and varying concentrations of BAP. Likewise in *Solanum nigrum*, Sridhar and Naidu (2010b) reported the formation of greenish compact callus from the leaf explants cultured on MS medium supplemented with 3.0 mg/l NAA and 0.5 mg/l BAP. I*n Tylophora indica* callusing was observed on MS supplemented with NAA (9.0 µM) and Kn (4.65 µM) where callusing initiated within 7 to 8 days in almost 100% cultures (kaur *et al.* 2011). *In Gloriosa superba* best callus was obtained on B5 medium in combination with NAA (2 ppm) and Kinetin (0.5 ppm) as reported by Anirudha Rishi (2011). The addition of liquid endosperm of green coconuts to the nutrient medium has been found to promote the growth of many plant tissues. In *Solanum trilobatum,* combination of CM with NAA favored callus growth (Alagumanian *et al.* 2004). In *Adhatoda vasica* development of callus tissue and browning was eliminated by culturing shoot tip explant on MS medium supplemented with TDZ (0.30mg/l) and 15% CM (Nath and Buragohain 2005).

Root differentiation from the leaf callus was observed on MS + NAA (7.35µM) + Kn (2.325µM) and a low frequency of shoot differentiation was also observed on NAA (14.7µM) and Kn (2.325µM) in 1-2% of cultures.

Initiation and development of roots from the basal end of regenerated shoots is an important and indispensable step to establish tissue culture derived plantlets to the soil. In present investigation, basal Murashige and Skoog's medium was the most effective medium for root induction. Similarly high frequency of rooting was recorded on basal MS medium in *Adhatoda vasica* (Nath and Buragohain 2005) and in *Tylophora indica* (kaur *et al*. 2011).

It is concluded that in the present investigation, a reliable and reproducible protocol has been established for the micropropagation of *Cissus quadrangularis* via axillary shoot proliferation from shoot apices and nodal segments. It has not been possible to induce high frequency shoot organogenesis from callus cultures. It is opined that the cells in plant are undoubtedly totipotent but some vital hormonal and/or nutritional factor or their combination for differentiation could not be discovered by us during the stipulated period of this project.

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