STANDARDIZATION OF TISSUE CULTURE CONDITIONS FOR *VIOLA ODORATA* L.

ISLAMABAD

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DECLARATION

This is to certify that this dissertation entitled **"Standardization of tissue culture conditions for *Viola odorata L.*"** submitted by Muhammad Naeem Khan is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of M.Phil. in Plant Biochemistry and Molecular Biology.

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Dedicated

To

My loving parents

and

caring sisters

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ALL Praises to Almighty Allah, the omnipotent, the most compassionate and His Prophet Muhammad (P.B U.H) The Most perfect among all human beings ever born on the surface of earth, who is forever a source of guidance and knowledge for humanity as a whole.

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Muhammad Naeem Khan

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List of Abbreviations

2, 4-D: 2, 4-Dichlorophenoxyacetic acid

BA: 6-Benzylamino-purine

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

DPPH: α, α-diphenyl-β-picrylhydrazyl

GA_3: Gibberellic acid

IAA: Indole acetic acid

KIN: Kinetin

MS: Murashige and Skoog

NAA: 1-naphthaleneacetic acid

PGRs: Plant growth regulators

ROX: Reactive oxygen species

TDZ: Thidiazuron

ABSTRACT

Viola odorata L. is commonly known as banafsha belong to family Violaceae is an herbaceous perennial evergreen wild plant. Whole plant stem, leaves, roots and flowers are used for medicinal purposes and are very important for pharmaceuticals. Important secondary metabolites found in *Viola* are violin, volatile oil, salicylic acid, methyl ester, saponins, flavonoids and alkaloids which show different activities like antioxidant, antimicrobial, and antipyretic activity. *Viola odorata* is included in the list of endangered species because of its intensive use in medicine in local market but it is not being cultivated commercially to fulfill its market requirements. In order to conserve the plant and to overcome over exploitation of it in the local market for medicinal purposes an *in vitro* protocol for callogenesis and organogenesis was developed and optimized on half strength MS medium using 6-benzyladenine (BA) 2.5 mg/l and 2, 4-dichlorophenoxyacetic acid (2, 4-D) 0.15 mg/l growth hormones which can further be used for its micropropegation and commercial cultivation. Leaf, stem and petioles were used for callus induction in which leaf was the best explant for callus induction. The callus formed by different combination of plant growth hormones and different explant sources were studied comparatively in their colour, texture and efficiency for organogenesis. Incorporation of different types of plant growth regulators induced organogenesis and subsequently produced shoots. Optimum shooting occurred on medium having naphthalene- acetic acid (NAA) 0.5 mg/l, Gibberellic acid GA_3 1.05 mg/l, Silver nitrate $AgNo_3$ 0.42 mg/l and Thidiazuron (TDZ) 2.5 mg/l. The relative concentration of $AgNo_3$ and TDZ have very important role and have effect on the organogenic ability and potential of explant along with plant growth regulators. The different biochemical and physiological changes take place during callogenesis and organogenesis has direct effect on its secondary metabolites which were evaluated by determining their antioxidant activity by DPPH (α, α-diphenyl-β-picrylhydrazyl) method. The antioxidant activity of callus was higher than wild plants and had better performance which is medicinally very important and can be preserved and sub cultured for medicinal use on commercial scale.

Key words: *Viola odorata*, callogenesis, organogenesis, antioxidant activity.

Chapter No. 1

Introduction

Viola odorata L. is medicinally and economically important perennial plant of temperate forests, edge bushes and open habitats (Lambinon *et al.*, 1992) which have the ability of vegetative propagation. It belongs to family Violaceae, a medium sized family including violets or pansies having 22 genera and 900 species (Heywood, 1993). Among all these genera *Viola* is the largest genus having 525-600 species in the world (Ballard, 1999). *Viola odorata* is commonly known as Sweet Violet, English Violet, Common Violet, or Garden Violet. This herb is known as Banafsa, Banafsha or Banaksa in Pakistan and India. *Viola odorata* is considered as native plant of Southern Europe, North-Western Africa, and Western Asia. In Europe, it is found only in South of Alps and in parts of Western Europe, but it has spreaded much more because of extensive cultivation (Valentin *et al.*, 1968; Huetteman and Preece, 1986; Marcussen and Borgen, 2000).

Now *Viola odorata* is found in all the temperate zones of all continents and because of multiple introduction it is found in areas which are non-native of it, for instance in Scandinavia (Marcussen *et al.*, 2001), but inspite of these facts *Viola odorata* has very little morphological variation throughout its natural range. In Pakistan it is found in Southern hilly regions of Swat, Hazara, Muree and Kashmir represented by 17 species. It is frequently cultivated in gardens up to 1900 m, usually found as escapes (Qaiser and Omer, 1985). Violets grow mostly on alkaline soils (Henry and Malécot, 2005) but it can also be found on acidic soils with low calcium concentration (pH 4.1-7.8). Soils from natural habitats contain a significant amount of organic matter (5.8-10.86 %). Organic matter has a positive effect on plant growth because it contributes to the improvement of the physical, chemical and biological properties of soil.

1.1. Plant morphology

Viola odorata is an acaulescent perennial herb which propagates by stolons to form mats (Thomas, 2005). The Rhizomes are, creeping, densely articulate, 3-5 mm thick, branched, with terminal leaf rosettes and lateral stolons. Rosette leaves are several in number, short-hairy, stipules ovate or broadly lanceolate, 3-5 mm, acute, pale or sometimes greenish, densely fimbriate with fimbriae usually 0-1 mm but in some populations 1-2 mm; petiole 5-17 cm; blade ovate to orbicular, 4-10 × 3-7 cm, broadest near the middle; margin

convex, crenate with 20–26 teeth; base cordate; apex obtuse. Stolons creeping, 2 mm thick and up to 30 cm long, with 1–6 cm long internodes, rooting, in the second year with terminal and lateral leaf rosettes. Stolon leaves similar to the rosette leaves but smaller and blades sometimes wider than long, with a shallowly cordate to almost truncate base (Qaiser and Omer, 1985; Marcussen *et al.*, 2005).

1.1.1 Flower morphology

The wild type expresses seasonal cleistogamy, producing showy, potentially out crossing chasmogamous flowers in early spring and obligately self-pollinated, cleistogamous flowers in favourable periods throughout the summer. The chasmogamous flowers are heavily scented; arise from the leaf rosettes and stolons. Pedicels of chasmogamous flowers are up to 5 cm which exceeds the foliage at time of flowering; bracteoles are 4-5 mm present in the middle third of the pedicel. Sepals are ovate or obtuse and are 4-6 × 2-3 mm (including the appendage). Appendage is 2-4 mm, petals are lilac, sometimes white or sordid pink and obovate. Upper petals are 7-8 × 4-8 mm, lateral petals are usually with hyaline hairs at the throat and spurred petal is 15-21 × 5-8 mm excluding the spur which is variably violet, darker or lighter than the petal limbs and 3-4 mm (Qaiser and Omer, 1985).

Cleistogamous flowers are 1–3 mm and have decumbent pedicel. Capsule is globose, 8-12 mm and is non-explosive. Seeds are ellipsoid 2 × 3 mm. Some forms and cultivars have deviant corolla color, such as white, purple, or even yellow or apricot, and others produce chasmogamous flowers throughout the winter. However, many sweet smelling cultivars often assigned to *Viola odorata,* belong to other species, such as *Viola alba* (the famous Parma Violets) (Henry *et al.*, 2005), *Viola suavis, Viola rossica* (Gams, 1925) and *Viola sintenisii*, a species reported and cultivated in Central Asia (Yuzepchuk, 1949; Marcussen *et al.*, 2005).

1.1.2 Chromosome number

The chromosome number of *Viola odorata* is 2n = 20 and belongs to section *Viola Uncinatae*, a well-characterized Eurasian group of approximately 25 species (Marcussen and Borgen, 2000; Dinç and Yıldırımlı, 2002; Dinç *et al.*, 2003). Morphological and allozymic evidence reveals that its closest relatives are two species *Viola pyrenaica* Ram

($2n$ = 20), a nonstoloniferous disjunctive of the mountains of south Europe and *Viola suavis* ($2n$ = 40), an allopolyploid derivative of *Viola pyrenaica* and some unknown taxon (Marcussen and Borgen, 2000). Their hybrids are rare but have been reported to be semi-fertile, which is often the case with interspecific hybrids within the section.

1.1.3 Chemical constituents

The flowers of *Viola odorata* contain the odorous principle, blue coloring matter and sugar (glycoside). Its flowers also contain 4.0 % anthocyanins, 1.1 % flavonoids, 0.4 % outside, 18 % mucilage and 8.5 % ash (Lamaison *et al.*, 1991). Flowers along with leaves, stem and roots contain an alkaloid violin. *Viola*-quercitin is found throughout the plant and salicylic acid is also obtained from the plant extract (Jackson and Bergeron, 2005). Essential oil of *Viola odorata* contains ionine, saponins, glycoside, methyl salicylate, mucilage, vitamins A and C, and alkaloids (Kathi, 1991). The elemental investigation of sweet violet showed the presence of carbon, oxygen, sodium, magnesium, aluminum, silicon, chlorine, potassium, calcium and iron in different parts of plant in varying concentrations. It was established that higher concentration of nitrogen and potassium accumulates in vegetative plant parts, calcium in roots while phosphorus and sulphur in the flower. Moreover it was observed that potassium, magnesium and sulphur levels are the highest at the end of the flowering season (Bibi and Ghulam, 2006).

Cyclotides are also common in *Viola odorata* which are mini proteins consist of 28-37 amino acids residues that have the remarkable feature of a head-to-tail cyclic backbone surrounding a cystine knot. This molecular structural design gives the cyclotides sharp resistance to thermal, chemical and enzymatic degradation and has encouraged investigations into their use as scaffolds in peptide therapeutics. These cyclotides show a range of biological activities; for example, uterotonic (Gran, 1973), hemolytic (Schopke *et al.*, 1993), antibacterial, antifungal (Tam, 1999), anti-HIV (Bokesch *et al.*, 2001, Gustafson *et al.*, 1994 and Hallock *et al.*, 2000), insecticidal (Jennings *et al.*, 2001) and cytotoxic activities (Lindholm *et al.*, 2002).

The antibacterial, antifungal and insecticidal properties of the cyclotides suggest that they are involved in plant host-defense (Craik, 2001). It has been proposed that the cyclotides represent the perfect host-defense proteins, since the cystine knot and the N- and C-

termini joined in a peptide bond represent a tremendously stable and stiff structure that enables them to coexist with various proteases (Trabi and Craik, 2002). It is recently reported that cyclotides are produced by genes which are found in different isoforms within each plant species (Jennings *et al.*, 2001). The production of several isoforms within a single species further supports the hypothesis that these peptides are involved in defense. To make possible the establishment of their structure-function relationship, it is necessary to carefully characterize the three-dimensional (3-D) structure of cyclotide isoforms.

1.1.4 Medicinal uses

Viola odorata is medicinally very important and has a long history in phytomedicine. The Greeks made use of this herb as a medicine from ancient time. Long accounts of its properties are found in most Arabic and Persians works in Materia Medica (Said, 1969). The different parts used in traditional and Unani medicine are roots (Bekh-e-banafsha), fruits (Pool-e-banafsha), flower (Gul-e-banafsha) and oil (Roghan-e-banafsha). In India and China its flower are used for the purification of blood and the plant is used as a pot herb. The plant extract is also mashed and applied to ulcer and foul sores (Kirtikar and Basu, 1918). Its flower are collected in March- April and mixed with powdered *Ziziyphus sativa* fruit which is then mixed with soup and is used in sore throat, kidney and liver disorder. Decoction and powder of whole plant is laxative, antimycotic, antibacterial, antipyretic, expectorant, diuretic, diaphoretic, bitter and pungent and employed against cough, jaundice, malaria, bronchitis, asthma, catarrhal and pulmonary troubles, eczema, earache, eye and skin diseases and also used as general body tonic (Ahmad and Sher 1997).

Syrup and decoction of flower (Gul-e-banafsha) is emollient, demulcent, laxative in nature and used for curing cough, sore throat, ague and harshness. Root extract of bekh-e-banafsha have emetic, diuretic, anti-inflammatory, purgative properties and used as a medicine for abdominal pain, skin disorders, upper-respiratory problems, and cough (Hansel *et al.*, 1993). Extract of leaves and seeds have analgesic properties and it has been used for the treatment of pain in throat and mouth cancer. In large doses, leaves as well as roots are cathartic and the seeds are poisonous (Khan, 1996). Although, the use of

Viola as herbal medicine goes back centuries, the biological activity of its main secondary metabolites has hardly been studied. Reports about *Viola* deal only with the cytotoxicity of its cyclic peptides (Svangard *et al.*, 2004) and its antimicrobial activity (Witkowska *et al.*, 2005).

1.1.5 Economic importance

The flowers, leaves and roots of various *Viola* species are used mostly for medicinal purposes on industrial scale, being rich in vitamins A, C and also contains a type of antioxidant called anthocyanins. Its flowers are used to make herbal tea which is used in Chinese herbal medicine. The flower and leaves contain volatile oil which contain 21 % eugenol (Sabetay and Traband, 1941) and other essential chemicals like Benzyl alcohol, 2, 6 Nonadien-1-ol, n-Hexanol, 2, 6 Nonadien-1-al and parmone which are used in chemical industry. Moreover Krocher described that it contain Me, salicylate in the form of glycoside and saponin which is much valued for production of high grade perfumes. Its flowers are used to flavor breath fresheners. Pigments extracted from the flowers are used as litmus to test for acid and alkaline. *Violet* flowers are also used in confectionery for the decoration of cakes and ice cream.

1.2. Tissue culture

Plant cell or tissue culture is the *in vitro* culture of sterilized plant cells, tissues or organs on a nutrient medium under defined physical and chemical conditions (Sharma, 2000). Unlike animal cells, many plant cells are totipotent, meaning that each cell has the capacity to regenerate the entire plant. This fact lies at the foundation of all tissue culture work. The concept of plant tissue culture is based on the principles of cellular totipotency and regeneration, which can be traced back to the Cell Theory of Matthias Jakob Schleiden and Theodor Schwann (Vasil, 2008). In 1839, Schleiden and Schwann proposed that cell is the basic unit of organisms. They visualized that cell is capable of autonomy and therefore it should be possible for each cell to regenerate and copy itself if given an environment to regenerate into whole plant.

Based on Schleiden and Schwann work, in 1902, a German physiologist, Gottlieb Haberlandt developed the concept of *in vitro* cell culture. Haberlandt isolated single fully differentiated individual plant cells from different plant species like palisade cells from

leaves of *Laminum purpureum*, glandular hair of Pulmonaria and pith cells from petioles of *Eicchornia crassiples* etc and was first to culture them in Knop's salt solution enriched with glucose. The cells increased in size in culture, accumulated starch but failed to divide. Therefore, Haberlandt's prediction failed that the cultured plant cells could grow, divide and develop into embryo and then to whole plant. Despite lack of success, Haberlandt made several predictions and suggestion about media and its ingredients which could induce cell division, regeneration, proliferation and embryo induction. Because of his pioneering work and experimentation in the field of tissue culture, Haberlandt was appropriately recognized as the father of plant tissue culture (Thrope, 1990).

The science of tissue culture progressed slowly for many years due to unavailability of nutrients solution that could support the growth of isolated plant cells and tissues. Molliard in France, Kotte in Germany, and Robbins in the United States, cultured fragments of embryos, and excised roots, on Knop's salt solution. Some growth was observed on this minimal medium, but none of the cultures could be maintained for more than few weeks (Vasil, 2008). The most detailed and systematic studies on the nutrient requirements of cultured plant tissues were carried out by Albert Hildebrandt at the University of Wisconsin. Albert made an attempt to develop an ideal nutrient solution (Hildebrandt *et al.*, 1946; Hildebrandt and Riker, 1949; Vasil and Hildebrandt, 1966) succeeded in the development of solutions that contained greatly elevated levels of mineral salts, such as the tobacco high salts medium (Vasil and Hildebrandt, 1966).

Toshio Murashige at the same time was trying to obtain optimum and expected growth of cultured tobacco pith tissues which Skoog needed for performing reliable bioassays of cytokinin activity. Murashige studied that by adding an aqueous extract of tobacco leaves to White's medium growth can be increased more than four times and this was due to largely by the inorganic constituents of the leaf extract. Similar results were obtained when the ash of tobacco leaf extracts, nitrate, large amounts of ammonium and potassium salts were added to the White's medium and this led to the formulation of a new and completely defined nutrient solution, the Murashige and Skoog or MS medium. To make the media more stable Murashige included chelated iron, myo-inositol, and a mixture of four vitamins. MS media is the most widely used media for plant tissue culturing

(Murashige and Skoog, 1962) and remains one of the most highly cited publications in plant biology.

Phytohormones or plant growth regulators (PGRs) are known to play an important role in the stimulation of grain germination (Akazawa, 1972) and its activity is enhanced at low external pH (Sinjorgo *et al.*, 1993). Therefore, changes in pH could be a mechanism by which processes during germination can be controlled by phytohormones. PGRs have been found to play a central role in the incorporation of the responses expressed by plants under stress conditions (Amzallag *et al.*, 1990). It has been found that water uptake is affected under stress conditions, either by increasing membrane permeability or by increasing the internal concentration of osmotically active solutes (Banyal *et al.*, 1983).

Went (1928) in Netherland for the first time isolated and quantified plant hormone which he called "wuchstoff" (hormone), and coined the phrase "Ohne Wuchstoff, kein Wachstum" (without hormone, no growth). The hormone later on identified as the naturally occurring auxin which regulates the amount, type, and direction of plant growth. Auxins include both naturally occurring substances and related synthetic compounds that have similar effects. Auxins are found in all members of the plant kingdom. They are most abundantly produced in growth areas (meristem), e.g., root and shoot tips, but are also produced elsewhere, e.g., in the stems and leaves. Similarly, Indole acetic acid (IAA) was first isolated in crystalline form from the urine of pregnant women by Kögl (Kögl *et al.*, 1934) in Copenhagen and from large scale cultures of the fungus, Rhizopus suinus, by Thimann (1935) at the California Institute of Technology (Caltech) in the United States. Thimann and his students James Bonner and Folk Skoog also studied auxin and contributed a lot in understanding the structure and function of auxin. They also contributed in the development of wide variety of synthetic auxin such as 2, 4-D which are now being used widely in the field of plant tissue culture (Vasil, 2008).

Another important class of plant growth regulator includes cytokinin which is naturally occurring plant growth hormone that has had a profound impact on the culture of isolated plant cells. It was discovered by Folke Skoog and his colleagues at the University of Wisconsin. Only a few naturally occurring cytokinin have been identified, but it is due to

the work of Skoog and his colleagues, a number of cytokinins with varying levels of activity were synthesized. Soon after the discovery of cytokinins, Skoog and Miller (1957) made another significant contribution, by demonstrating the hormonal (auxin-cytokinin) regulation of morphogenesis in plants, which allowed the controlled formation of shoots or roots in cultured callus tissues, and is rightly considered an important milestone in understanding plant morphogenesis, and in micro propagation and regeneration of plants from cultured tissue. Nearly at the same time Carlos Miller isolated kinetin from aged autoclaved herring sperm DNA. Kinetin was identified as N6-furfurylaminopurine (Miller *et al.*, 1956) and was given the name kinetin because of its ability to induce cell division, provided that auxin was present in the medium.

Gibberellic acids also called Gibberellins (GA_3) have been considered to increase the extracellular acidification of mature barley aleuronic layers (Drozdowicz and Jones, 1992). It is also suggested that GA_3 stimulates phosphate and organic acid release by the aleurone layers. No stimulation of extracellular acidification was observed when aleurone layers of wheat were treated with GA_3 (Hamabata *et al.*, 1988). Involvement of GA_3 has been suspected for it because they are known to promote elongation of Avena internodes. Gibberellins have also been shown to mediate the stimulatory effect of ethylene on stem elongation in the semi-aquatic plant *Callitriche platycarpa*. It has been reported to promote cell division and cell elongation (Naylor, 1984).

PGRs like 2, 4-dichlorophenoxyacetic acid (2, 4-D) or 1-napthalene- acetic acid (NAA), and cytokinins such as 6-benzyladenine (BA) are prerequisites for plant tissue cultures, including liquid suspension cultures and solid agarose bed cultures. However, the relative growth rate of the plant cells in culture strictly depends on the initial cell density, even if sufficient amounts of auxin and cytokinins are present in the medium.

There have been studies about the cultivation, inheritance and breeding of *Viola* (Rupinder and Ramesh, 1998; Novotna, 1984), however, there was little information about callus induction and plant regeneration of this species. Babber and Kulbhushan (1991) obtained callus derived from root, hypocotyls and cotyledonary segments of *Viola tricolor* but failed to achieve shoot regeneration. In a report Sato *et al.* (1995) achieved regeneration of plantlets from petiole callus of wild *Viola* (*Viola patrinii* DC.). In yet

another report Wijowska *et al.* (1999) obtained callus, autonomous endosperm and roots *in vitro* by culturing unfertilized ovules of *Viola odorata*. Direct regeneration of *Viola odorata* was carried out by Hussain *et al* (2010) has better quality than parent plants. Regeneration is a useful tool for rapid propagation and for induction of somaclonal variation in plants (Snyman *et al.*, 2000). Although attempts have been made, successful regeneration of *Viola wittrockiana* has not made much progress. It seems that this species is one of the recalcitrant plants for *in vitro* regeneration.

Plant tissue and cell culture techniques have become very important as they increase the work in the area like biotechnology, genetic engineering and production of pathogen free plants. Now tissue culture has become an important tool for genetic engineering as plant regeneration is essential for stable transformation (Ishii, 1998). Plant tissue culture techniques have great potential in plant improvement if plants are regenerated in large number (Jain *et al.*, 1998). In regenerated plants, variation can be induced by tissue culturing called somaclonal variation (Larkin and Scowcroft, 1981) which can result genetically stable variation, useful in crop improvement (Skirvin *et al.*, 1993). Recently tissue culture techniques have been adopted for reliable commercial production of economically important plants (Honda *et al.*, 2001). The possible method to protect the endangered and rare plant species is to multiply and conserve the plants by *in vitro* cultures. Plant tissue culture technology has been successfully used for the commercial production of microbe free plants (Parmessur *et al.*, 2002; Liao *et al.*, 2004), for the conservation of germplasms of rare and endangered plant species to protect them from extinction (Milkulik, 1999; Chang *et al.*, 2000 and Jaime and da Silva, 2003).

Medicinal plants are the most exclusive source of life saving drugs for majority of the world's population. The utilization of plant cells for the production of natural or recombinant compounds of commercial significance has gained increasing concentration over the past decades (Canter *et al.*, 2005). These bioactive compounds currently extracted from plants are used as pharmaceuticals, additives, pesticides, agrochemicals, flavour and fragrance ingredients. The secondary metabolites play a major and important role in the adaptation of plants to their environment, but also represent an important source of pharmaceuticals (Ramachandra and Ravishankar, 2002). Cell suspension culture systems could be used for large scale culturing of plant cells from which

secondary metabolites could be extracted. The advantages of this method are that it can ultimately provide a constant reliable source of natural products.

The traditional system of medicine has become internationally very important topic in recent years. Although modern medicine may available in every country yet phytopharmaceuticals or herbal medicines have gained popularity because of historical and cultural reasons. Discoveries of cell cultures capable of producing particular medicinal compounds at a rate similar or superior to that of whole plants have been accelerated in the last few years (Vanisree and Tsay, 2004). The medicinal compounds which are localized in morphological specialized tissues or organs of native plants have been produced in culture system by inducing specific organized cultures and also by undifferentiated cell culture. For the specific biotransformation of natural compounds the uses of plant cell culture have been established (Ravishankar and Rao, 2000). Because of these advances, research in the area of tissue culture technology for production of plant based chemicals has bloomed beyond expectations.

1.3. Antioxidant activity

Molecules which inhibit the oxidation of other molecules are called antioxidants and its activity is called antioxidant activity. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. When oxygen is used to generate energy these free radicals are produced as by-product in living system. These free radicals are generally reactive oxygen species (ROS) such as super oxide anion, hydroxyl radical and hydrogen peroxide that result from the cellular redox process. ROS in low concentration has beneficial effects on cellular responses and immune function but at high levels free radicals are harmful and can damage cell structure (Pham-Huy *et al.*, 2008).

These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative diseases. Plants are rich sources of biologically active compounds called phytochemicals which possess a number of biological activities including antioxidant activities (Craig, 1999). These phytochemicals plays a vital role in the prevention of cancer, cardiovascular diseases (Gerber *et al.*, 2002) and neurodegenerative diseases,

including Alzheimer and Parkinson diseases (Di Matteo and Esposito, 2003) as well as inflammation and problems caused by continues aging (Ames *et al.*, 1993).

Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydrogen peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Epidemiological studies show that many of antioxidant activities possess anti-atherosclerotic, inflammatory, antimutagenic, antitumor, anticarcinogenic and antiviral or antibacterial activities to a greater or lesser extent (Halliwell, 1994). Moreover, it can be used as an alternative to the synthetic food additives such as Butylated hydroxytoluene (BHT) or Butylated hydroxyanisole (BHA).

Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical scavengers (Duh, 1998). Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids and phytoestrogens have been recognized as having the potential to reduce disease risk. The extracts of different native plants having antimicrobial and antioxidant properties have been reported for centuries, and indigenous plants have been used in herbal medicine to cure various diseases (Tepe *et al.*, 2005).

Herbal remedies are still being used widely in many countries of the world and therefore research on biologically active extracts and compounds from natural sources has been of great interest to scientists in order to discover new sources for drugs that may be useful in combating infectious diseases. About 80 % of the world population used medicinal plants for their essential health care needs (Zaidi and Crow, 2005). It is necessary to establish a relationship among biological, chemical and therapeutic activities in traditional medicine. Plants having antioxidant and antibacterial activities against various diseases are of great importance and a number of studies related to antioxidant and antibacterial screening of extracts of medicinal plants have been conducted.

For estimating the antioxidative potential of chemical components, several methods have been developed to assay free radical scavenging capacity and total antioxidant activity of plant extracts (Prior *et al.*, 2005). Most of them require a spectrophotometric

measurement and a certain reaction time in order to obtain reproducible results (Kulisic *et al.*, 2004). The most reliable and commonly used method involves the determination of the disappearance of free radicals, such as 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic) acid radical (ABTS+) (Miller *et al.*, 1993) and DPPH (1,1-diphenyl-2 picrylhydrazyl) radical (Williams *et al.*, 1995) using a spectrophotometer. Reduction of ABTS+ or DPPH by a radical species (R) (ABTS+ or DPPH+R. ABTS-H DPPH-R) or by an antioxidant (A) (ABTS+ or DPPH+A ABTS-H or DPPH-H + A) causes a loss of absorbance at 734 nm or 515 nm, respectively. These methods have been modified and improved in recent years (Re *et al.*, 1999).

The DPPH method is described as a useful, rapid and simple method independent of sample polarity for screening of many samples for radical scavenging activity (Koleva *et al.*, 2001). The use of DPPH for a radical scavenging measuring method is described by Yen and Duh (1994), Yordanov and Christova (1997), Masuda *et al.* (1999), Anderson and Padhye (2004), and Iwashima *et al.* (2005). DPPH is a stable free radical in a methanolic solution and in its oxidized form, the DPPH radical has an absorbance maximum centered at about 520 nm (Molyneux, 2004). Usually, the DPPH absorbance is measured at a wavelength of 515-520 nm (Bandoniene *et al.*, 2002; Pavlov *et al.*, 2002 and Gazi *et al.*, 2004). For its function as a measure of antioxidant potential, absorbance at the selected wavelength should be mainly influenced by the DPPH radical and not by interference as caused by antioxidants (Bondet *et al.*, 1997).

1.4. Aims of the present study

Viola odorata is not only plant of higher medicinal value but also have great economic importance. Keeping in view the importance of this valuable plant following objectives has been designed for this study.

- To establish and characterize an efficient callus culturing protocol for *Viola odorata*
- To determine the effects of different hormonal concentrations and type of explants on callus growth
- Standardization of an efficient protocol for organogenesis i.e shooting and rooting
- To determine organogenic potential of callus and effects of different hormones on it
- To determine antioxidant activity by DPPH method in wild plants and callus.

Chapter No. 2

Materials and Methods

The present research work was carried out in Plant Biochemistry and Molecular Biology laboratory, Quaid-i-Azam University, Islamabad. In the present study experiments were carried out to optimize condition for callogenesis and organogenesis of *Viola odorata* and to determine and compare the antioxidant activity of callus and wild plant extracts. The experimental work was carried out in three steps. First, conditions for callus induction or callogenesis were optimized on half strength MS medium using different plant growth hormones (BA and 2, 4-D) with different concentrations. In the second step, the calluses obtained were transferred to regeneration medium for checking their organogenic ability with respect to the explant origin and the media type used. Thirdly, the antioxidant activity of wild plant and callus extracts were determined by DPPH method. A brief account of material and methods used and all procedures adopted are given below.

2.1. Collection of plant material

The samples of *Viola odorata* were taken from green house of National Agriculture Research Council (NARC) Islamabad, Pakistan. The leaves, stem and petioles were used as explants for callus induction.

2.2. Preparation of glassware

All glass ware used in the experiments were washed thoroughly using a commercially available detergent. These were then washed three times with distilled water prior to sterilization. The forceps, scissor, beakers and media were sterilized by autoclaving at 15 Ib pressure/square inch, at 121°C for 15 minutes prior to use in experiments.

2.3. Sterilization and inoculation of explants

Leaves, stem and petioles were washed with distilled water to remove the dust and soil particles. Then these were kept in 70 % ethanol for one minute and in 50 % clorox (bleach) for 8-10 minutes. The bleach was removed by washing thrice with autoclaved distilled water under aseptic conditions. The explants were kept on autoclaved filter paper in a petri plate for drying. The leaves and petiole were cut into small pieces of 10-15 mm

in size rectangular or square in shape with the help of a sterilized surgical blade and were inoculated on half strength MS basal medium.

2.4. Media preparation

Callus is a coherent but unorganized and amorphous tissue, formed by the vigorous division of plant cells. For callus culture of *Viola odorata* half and full strength MS medium was used supplemented with twenty different concentrations and combinations of hormones (Table 1). The pH of media was adjusted at 5.8 and 2 g/l gelling gum powder was also used to solidify the media.

2.5. Aseptic transfer of explants

Aseptic transfer of explants were accomplished in Laminar flow hood. Before transferring the explants, the laminar flow hood was sprayed with 70 % ethanol and was treated with UV light for 15 minutes prior to use. Then explants were transferred on to the medium for callus induction.

2.6. Culture conditions

The callus culturing was done on media present in petri plates. These cultures were kept under 16 hrs photoperiod, 55±5 % relative humidity at 25-26 °C and the light intensity was maintained at 1000 lux inside growth chamber for callus induction.

2.7. Maintenance of *in vitro* cultures

In order to increase the callus induction rate, the explants were sub-cultured on the similar medium having same concentration of hormones after a regular interval of 25-30 days. The callus initiated on different media was also maintained by sub-culturing in the same way. Colour, size and texture of callus were noted and photographs were taken every week.

Table 1. Media with different combinations of hormones used for callus induction of *Viola odorata*.

Media Code(C*)	Half(H)/Full(F) MS	PGR's	Concentration mg/l
C1**	H	BA+2, 4-D	2+0.11
C2	H	BA+2, 4-D	2.2+0.12
C3	H	BA+2, 4-D	2.3+0.13
C4	H	BA+2, 4-D	2.5+0.15
C5	H	BA+2, 4-D	2+1.9
C6	H	BA+2, 4-D+NAA	2.5+0.1+0.15
C7	F	BA+2, 4-D	2+0.11
C8	F	BA+2, 4-D	2.3+0.13
C9	F	BA+2, 4-D	2.5+0.15
C10	H	BA+2,4-D+Kin	1.5+1+0.1
C11	H	BA+2,4-D+Kin	0.5+1+0.4
C12***	H	BA+IAA+Kin	1.5+1+.0.1
C13	H	BA+IAA+Kin	3+2+0.4
C14	H	BA+NAA	2.2+0.54
C15	H	Kin+NAA	2+0.8
C16	F	Kin+2,4 D	2+0.15
C17	F	Kin+IAA	1.5+0.2
C18	H	BA+2, 4-D	4+0.22
C19	H	BA+2, 4-D	4.4+0.26
C20	H	BA+2, 4-D	5+0.3

C*: Callus media

**Media stated by Wang and Man (2006)

*** Media stated by Hussain *et al.* (2010)

2.8. Shoot formation

After callus induction it was transferred to another half strength MS media called shooting media supplemented with different combinations and concentration of Thidiazuron (TDZ), Silver nitrate ($AgNo_3$), Gibberellic acid (GA_3) and 1-naphthaleneacetic acid (NAA) (Table 2) adopted from Wang and Man (2006) previously used for shoot initiation of *Viola wittrockiana*. The shooting media were poured in glass bottles inside the Laminar flow hood. After inoculation of callus on shooting media the bottles were kept inside the growth chamber for shoot initiation.

2.9. Root formation

After shoots formation, it was transferred to rooting medium for root initiation. The rooting media were supplied with different concentration of 6-Benzylaminopurine (BA) and NAA (Table 3).

2.10. Antioxidant activity

Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. In the present study the free radical scavenger activity of methanolic extract of *Viola odorata* and its callus is evaluated.

2.11. Preparation of extract

The plant material was ground into fine powder by using pestle and mortar. It was then soaked in 98 % methanol for 5-7 days with regular stirring. It was then filtered with Whatman filter paper No.1 and was further concentrated by evaporating the methanol either by using rotary evaporator or simply by pouring it into large petri plate and placed it in open air for some time. After evaporation of methanol fine crystals like dried plant material were scratched and collected in a falcon tube (Khalaf *et al.*, 2007). The same method was followed for the preparation of callus extract.

Table 2. Shooting media with different hormonal combinations.

Media code(S*)	PGRs	Concentration mg/l
S1	NAA+GA_3+$AgNo_3$+TDZ	0.2+0.99+0.4+2
S2	NAA+GA_3+$AgNo_3$+TDZ	0.5+1.05+0.42+2.5
S3	GA_3+$AgNo_3$+TDZ	1.2+0.45+1
S4	NAA+GA_3+$AgNo_3$	0.5+1.5+ 0.2

S*: Shooting media

Table 3. Rooting media with different hormonal concentration.

Media code (R*)	PGRs	Concentration mg/l
R1	NAA+BA	0.1+0.8
R2	NAA+BA	0.204+0.248
R3	NAA+BA	0.3+2.5

R*: Rooting media

2.12. Determination of antioxidant activity

The capability of prepared extracts to scavenge free radical α, α-diphenyl-β-picrylhydrazyl (DPPH) was then determined by the method of Amarowicz *et al.* (2004). Briefly, 2 mg of plant tissue extracts were dissolved in 4 ml of methanol and then added to methanolic solution of DPPH (0.5 ml). The resulting mixture was vortexed for 15 seconds and then left to stand for 30 minutes at room temperature. The absorbance of the resulting solution was examined spectrophotometrically at 517 nm. A methanolic solution of DPPH that had decayed and hence no longer exhibited purple colour was chosen for background correction, instead of pure methanol. Finally, the radical scavenging activity was calculated as percentage of DPPH discoloration using the following formula (Yen and Chen, 1995).

% scavenging DPPH free radical = $100 \times (1-A_E/A_D)$

Where AE is absorbance of the solution, when extract has been added at a particular level and AD is the absorbance of the DPPH solution with nothing added i.e no plant extract.

Chapter No. 3

Results

3.1. Callogenesis

For callus induction of *Viola odorata* twenty types of media with different hormonal combinations were used (Table 1). However, callus was induced on nine different media i.e C1, C2, C3, C4, C8, C9, C10, C11 and C12 media (Table 4) with different induction percentage. Among these media half strength MS medium along with plant growth regulators BA and 2, 4-D was more effective and significant for callus induction as compared to full strength MS medium. It was observed that both full and half strength MS medium without any growth hormone did not promote callus induction. Full strength MS medium along with BA and 2, 4-D induced callus as in C8 having BA 2.3 mg/l, 2, 4-D 0.13 mg/l and C9 having BA 2.5 mg/l and 2, 4-D 0.15 mg/l, however the media were not so much effective for callus induction as compared to half strength MS media like C2 having BA 2.2 mg/l, 2, 4-D 0.12 mg/l, C3 having BA 2.3 mg/l, 2, 4-D 0.13 mg/l and C4 having BA 2.5 mg/l, 2, 4-D 0.15 mg/l media.

The effect of different plant growth hormones alone and in combination was also checked for callus induction. All the hormones alone did not induce callus induction however in combination with each other were more effective for example, in C3, C4, C8, C9 and C10 media, the hormones in different combination with each other induced optimum callus with higher induction percentage. The explant response, growth rate, colour and size of callus was studied for all the plant growth regulators and their different combinations (Table 4). Among all the hormones BA 2.5 mg/l and 2, 4-D 0.15 mg/l proved to be more effective in combination with each other and callus induction was optimized efficiently and with greater percentage as C4 compared to other media. Callus induction occurred after 65 days of incubation and up to 50-55 % on medium C1 and C2 but by increasing concentration of BA and 2, 4-D, callus response was quick and induction occurred after 55 days of incubation and up to 70 % on C3 medium and were even more quick on medium C4 occurred after 40-45 days of incubation up to 85 %. By increasing further concentration of these hormones callus induction was observed to be decreased. The callus was however more or less green in colour and compact in all the media.

Table 4. Data of callus induction on MS media with different combinations of hormones.

S. No.	Media code	Half(H)/Full(F) MS media	PGRs	Concentration mg/ml	Percentage	Colour	Texture	Size	Days
1	C1	H	BA+2, 4-D	2+0.11	50	Light green	compact	Small	65
2	C2	H	BA+2, 4-D	2.2+0.12	55	Light green	compact	Small	65
3	C3	H	BA+2, 4-D	2.3+0.13	70	Light green	compact	Medium	55
4	C4	H	BA+2, 4-D	2.5+0.15	85	Dark green	compact	Large	40-45
5	C8	F	BA+2, 4-D	2.3+0.13	25	Light brown	crystalline	Small	70
6	C9	F	BA+2, 4-D	2.5+0.15	30	whitish brown	crystalline	Medium	65-70
7	C10	H	BA+2, 4-D+Kin	1.5+1+0.1	60	Greenish brown	Granulated	Large	50-60
8	C11	H	BA+2, 4-D+Kin	0.5+1+0.4	20	Yellow	Granulated	Small	70
9	C12	H	BA+IAA+Kin	1.5+1+.0.1	25	Greenish brown	Granulated	Medium	60-65

BA was proved more important and effective than 2, 4-D for callus induction and had greater concentration than 2, 4-D i.e BA is 1 mg/l greater than 2, 4-D as in C4 medium, however BA without 2, 4-D did not induce callus (Fig. 1, A-D). Explants also did not have any response to medium having BA and 2, 4-D more or less in equal concentration as in C5 medium BA 2 mg/l and 2, 4-D 1.9 mg/l. 1-Naphthaleneacetic acid (NAA) was also checked along with BA and 2, 4-D but explant did not respond and no callus induction occurred as in C6 medium. Full strength MS media e.g C8 having BA 2.3 mg/l, 2, 4-D 0.13 mg/l and C9 having BA 2.5 mg/l and 2, 4-D 0.15 mg/l induced callus but at very slow rate smaller in size and whitish brown in colour (Fig. 1, E & F).

Explant response to C10 medium having kinetin 0.1 mg/l, BA 1.5 mg/l and 2, 4-D 1 mg/l were good and callus inductions occurred up to 50-60 % after 50-60 days but however the callus formed were greenish brown or yellow in colour and smaller in size. C11 have small concentration of BA i.e 0.15 mg/l due to which callus induction was slow with lower growth rate. In C12 medium IAA hormone was used which is a naturally auxin instead of 2, 4-D and the media induced callus having greenish brown colour, medium size, granulated texture and callus induction percentage was 25 % (Fig. 1, G-I). The hormonal concentration in C12 media was IAA 1 mg/l, BA 1.5 mg/l and kinetin 0.1 mg/l. Another medium C13 having double concentration of hormones than C11 i.e BA 3 mg/l, IAA 2 mg/l and Kinetin 0.4 mg/l did not induce callus. The over all percent response of callus to different PGRs are represented graphically (Fig. 2).

3.1.1 Effect of explant on callogenesis

The callus induction potential varied in different parts of plant used as an explant i.e stem, petioles and leaves. Efficient and frequent callus induction occurred at the cut edges of the midrib of wounded and cut leaves as compared to stem and petiole in which callus induction frequency was slow (Fig. 3). The response of different parts of plants

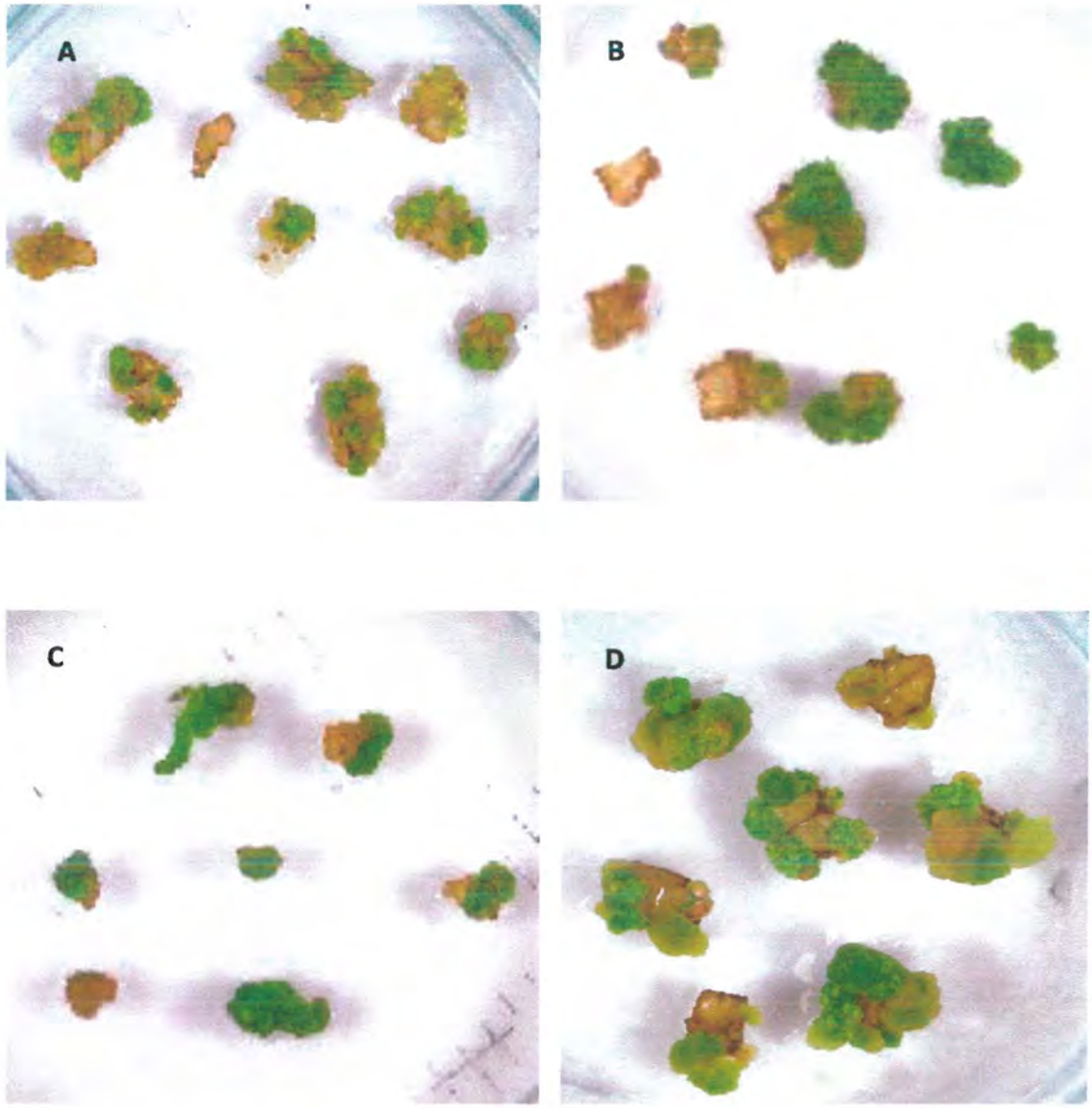

Fig. 1 Callus of *Viola odorata* formed on different MS media having varied hormonal concentration.

A: C1: BA 2 mg/l and 2, 4-D 0.11 mg/l (callus age 65 days), **B:** C2: BA 2.2 mg/l and 2, 4-D 0.12 mg/l (callus age 65 days), **C:** C3: BA 2.3 mg/l and 2, 4-D 0.13mg/l. Callus age 55 days. **D:** C4: BA 2.5 mg/l and 2, 4-D 0.15 mg/l (callus age 45 days).

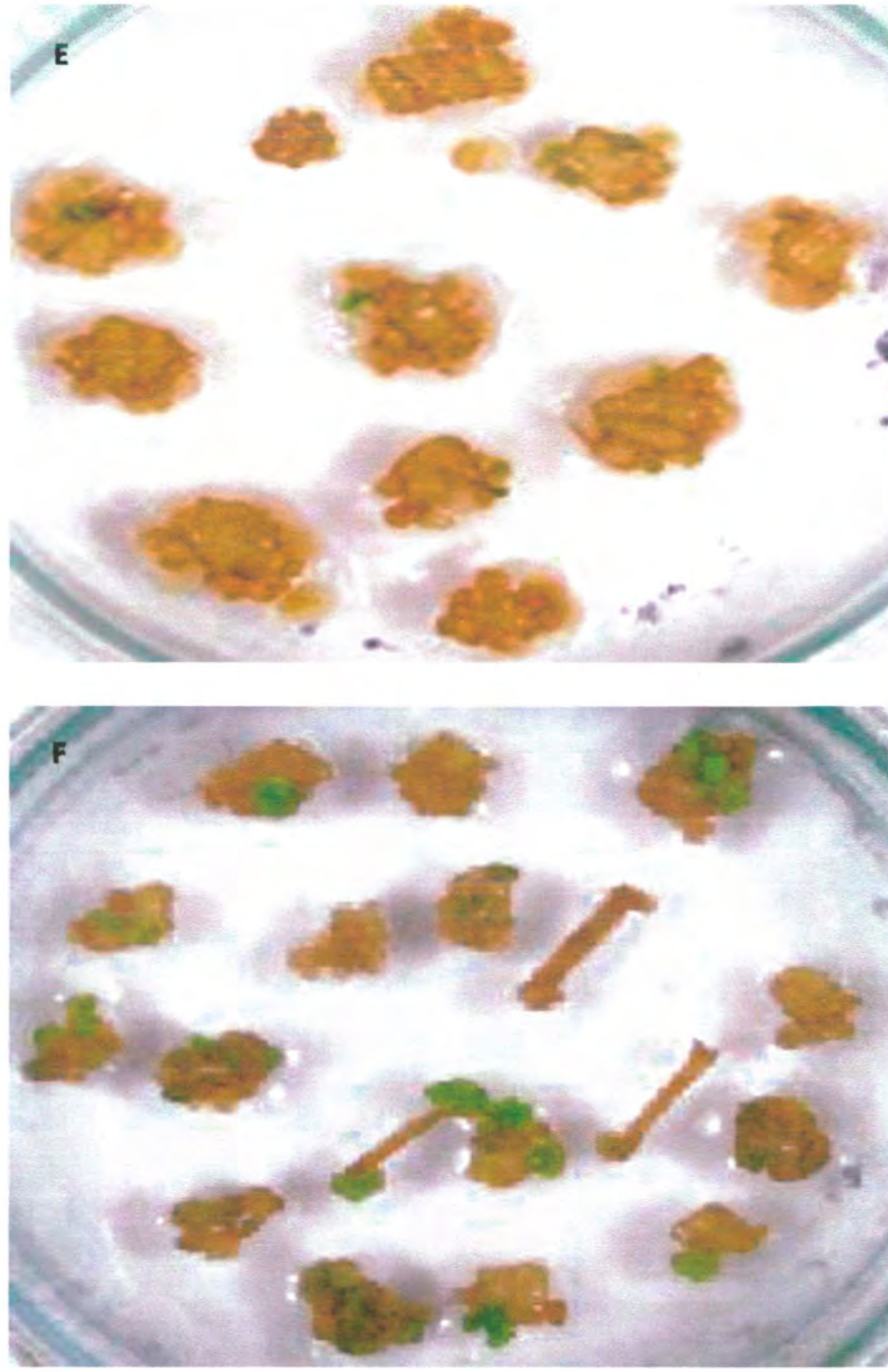

Fig. 1 Callus of *Viola odorata* formed on different MS media having varied hormonal concentration.

E: C8: BA 2.5 mg/l and 2, 4-D 0.13 mg/l (callus age 70 days), **F:** C9: BA 2.5 mg/l and 2, 4-D 0.15 mg/l (callus age 65 days).

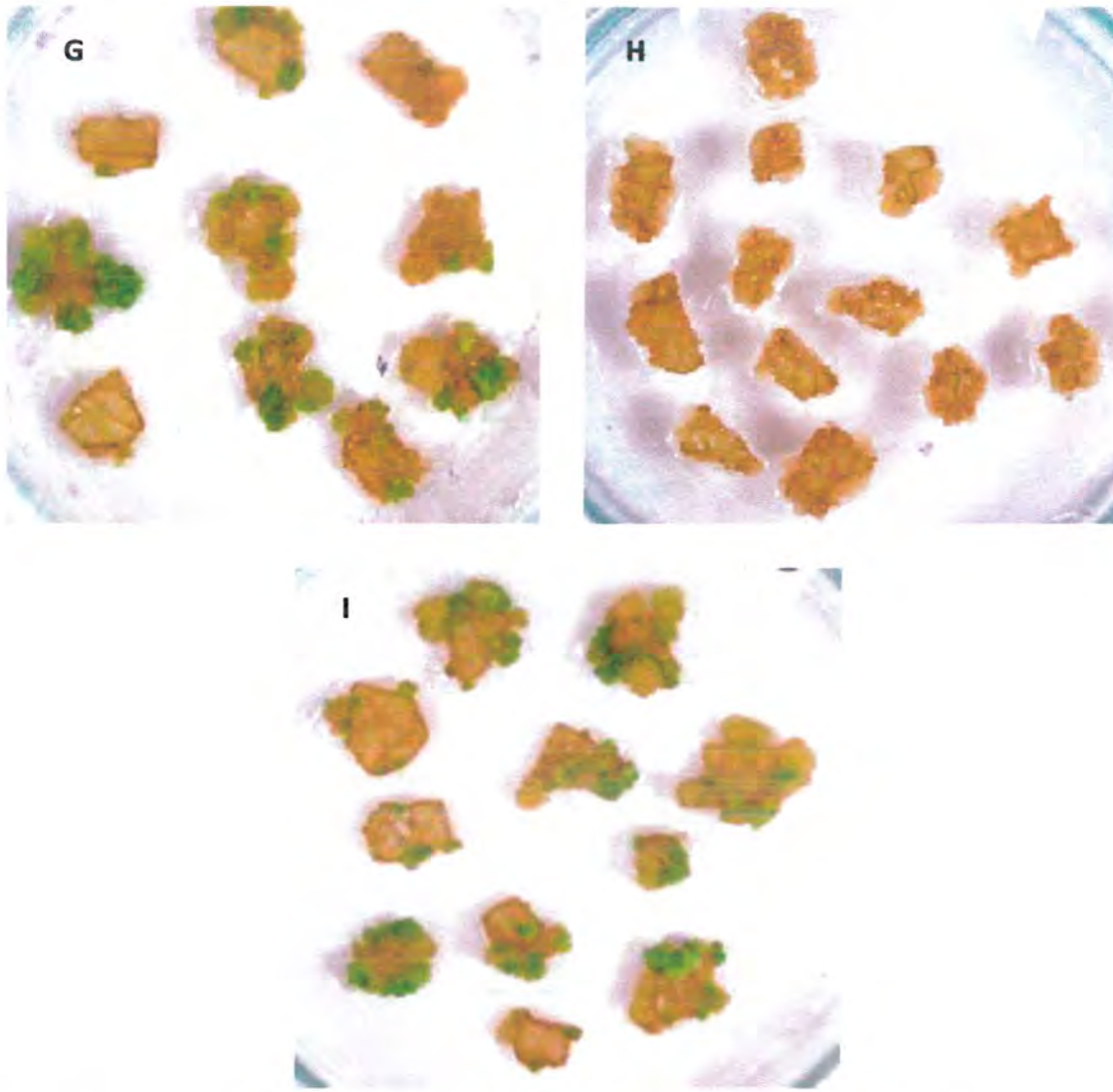

Fig. 1 Callus of *Viola odorata* formed on different MS media having varied hormonal concentration.

G: C10: BA 1.5 mg/l, 2, 4-D 1 mg/l and kinetin 0.1 mg/l (callus age 50 days), **H:** C11: BA 0.5 mg/l, 2, 4-D 1 mg/l and kinetin 0.4 mg/l (callus age 70 days), **I:** BA 3 mg/l, IAA 1 mg/l and 0.2 mg/l (callus age 60 days)

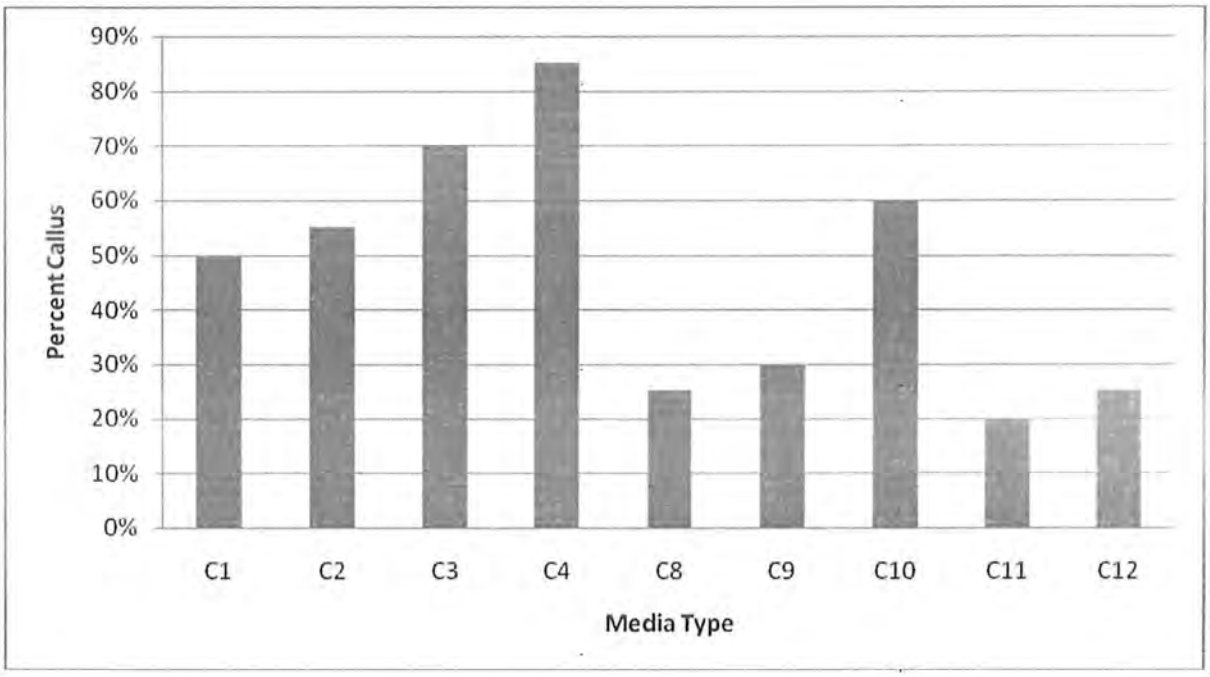

Fig. 2 Percentage callus induction of *Viola odorata* on media having different hormonal combinations. C1: 50 %; C2: 55 %; C3: 70 %; C: 85 %; C8: 25 %; C9: 30 %; C10: 60 %; C11: 20 %; C12: 25 %.

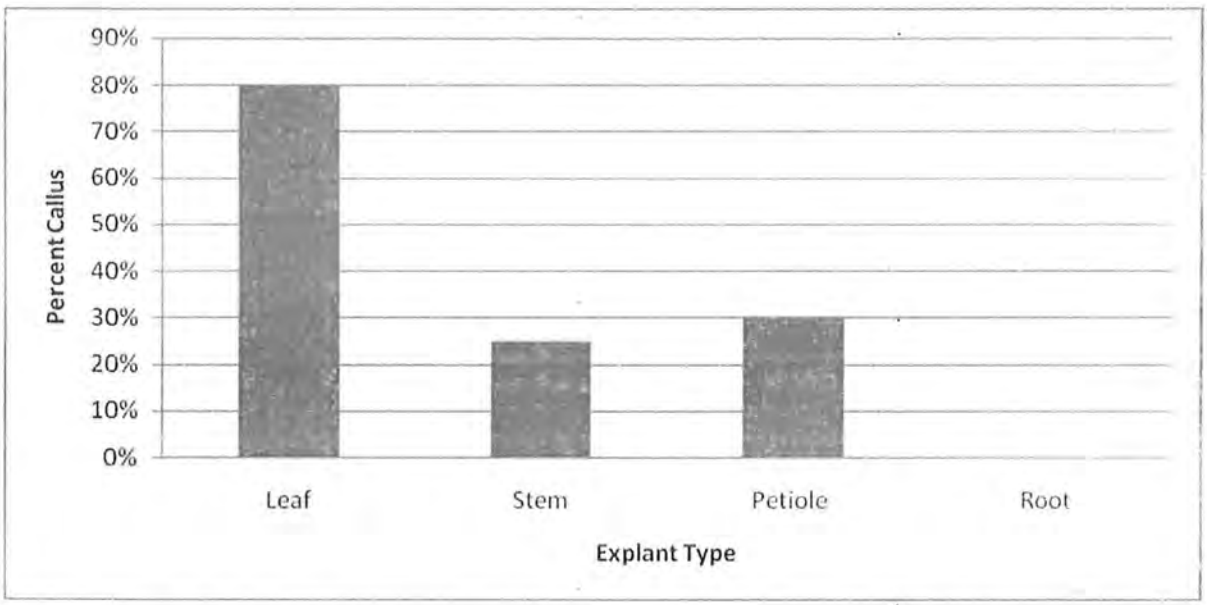

Fig. 3 Over all percent callus response of different explants. Leaf, 80 %; stem, 25 %; petiole, 30 %; root, 0 %.

used was dissimilar to different hormonal concentration. The young and green explants used were more efficient and frequent in callus formation as compared to old, diseased and spotted explants, particularly leaves. The spotted and old explants became decolorized after few days of incubation. It was observed that the small callus formed on stem and petiole was localized only to their tips and terminal parts, different from that of leaves which was formed through out the leaf disc. In case of petioles and stem contamination was also frequent as compared to leaves. No callus induction occurred in the case of roots when used as an explant. The calluses formed from explants and on all media were compact, green and soft initially but with the passage of time became hard, granulated and whitish brown or yellow in colour.

3.2. Shooting

The callus 60-70 days old, from callus induction media particularly from C2, C3 and C4 when became granulated, semi-compact and its colour changed to yellow green or brown were transferred to shooting MS medium supplemented with GA_3 and NAA hormones along with $AgNo_3$ and TDZ for shooting. Four media with different concentration of hormones were used for shooting each induced shooting with different percentage (Fig. 4). Gibberellic acid is simple gibberellins, promoting growth and elongation of cells caused shoot formation. NAA promote cell division and growth. It was observed that $AgNo_3$ and TDZ triggered shoot formation along with GA_3. S1 medium containing NAA 0.2 mg/l, GA_3 0.99 mg/l, $AgNo_3$ 0.4 mg/l and TDZ 2 mg/l has induced 65 % shooting after 35-40 days having 1-2 branches and 2-3 cm length. Best shooting occurred on S2 medium, containing NAA 0.5 mg/l, GA_3 1.05 mg/l, $AgNo_3$ 0.42 mg/l and TDZ 2.5 mg/l, indicating 80 % shooting after 30 days of incubation having 2-3 branches which reached 4-5 cm in length. No or very slow shooting occurred with less number of branches on media not supplied with $AgNo_3$ and TDZ. S3 medium having NAA 0.3 mg/l, GA_3 1.2 mg/l and TDZ 1.5 mg/l, not containing $AgNo_3$, induced 25 % shooting after 45 days with only one branch of 2 cm long. In another medium (S4) having NAA 0.5 mg/l, GA_3 1.5 mg/l and $AgNo_3$ 0.2 mg/l, induced 15 % shooting after 50 days having 2 cm long single shoot (Table 5). Moreover, it was observed that callus taken from C1, C3 and C4 medium showed greater number of shoots (Fig. 5 to Fig. 8).

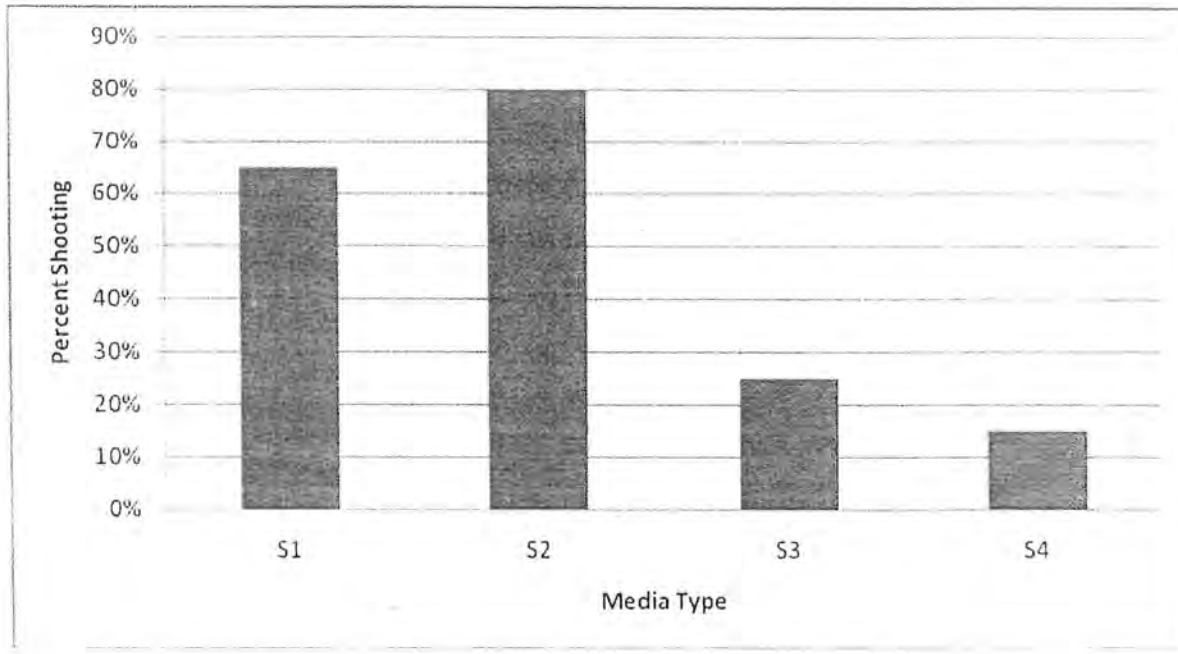

Fig. 4 Percent shooting (total shoot/total callus used) on media having different hormonal combinations. S1: (12/20) 65 %; S2: 80 %; S3: 25 %; S4: 15 %.

Table 5. Data of shooting on MS media containing different combinations of hormones.

Media code	PGRs	Concentration (mg/l)	Percent shooting	Days	Length (cm)	No. of shoots
S1	$NAA+GA_3+AgNo_3+TDZ$	0.2+0.99+0.4+2	65	35-40	2-3	1-2
S2	$NAA+GA_3+AgNo_3+TDZ$	0.5+1.05+0.42+2.5	80	30	4-5	2-3
S3	$NAA+GA_3+TDZ$	0.3+1.2+1.5	25	45	2	1
S4	$NAA+GA_3+AgNo_3$	0.5+1.5+ 0.2	15	50	2	1

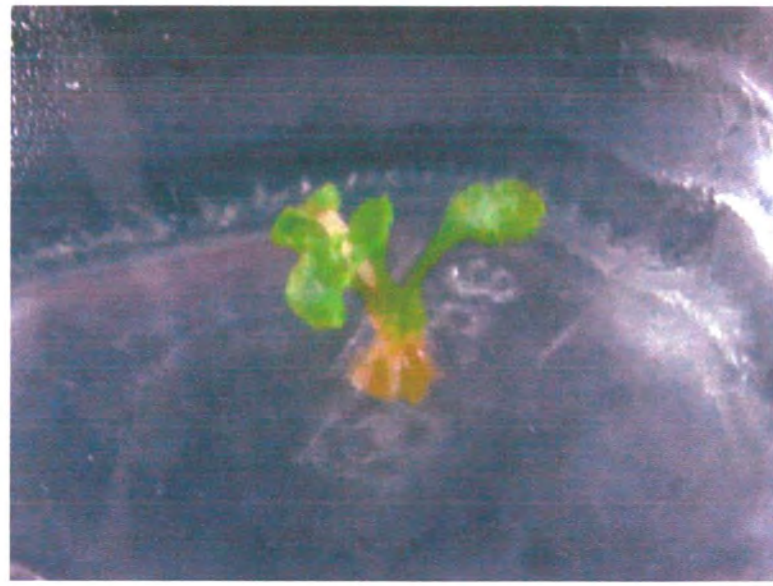

Fig. 5 Differentiated plant having more than two shoots after 35 days of culturing on S2 media supplemented with NAA 0.5 mg/l and GA_3 1.05 mg/l.

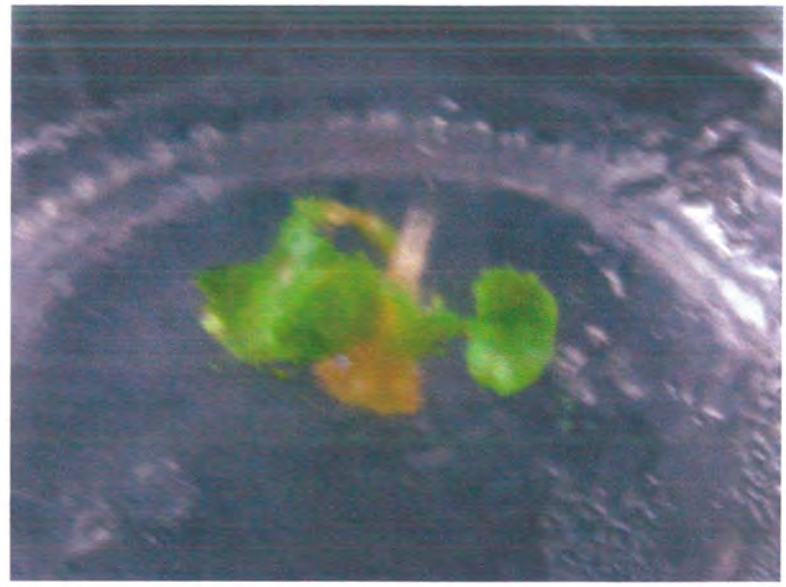

Fig. 6 Differentiated plant having two shoots after 30 days of culturing on S1 media supplemented with NAA 0.2 mg/l and GA_3 0.99 mg/l.

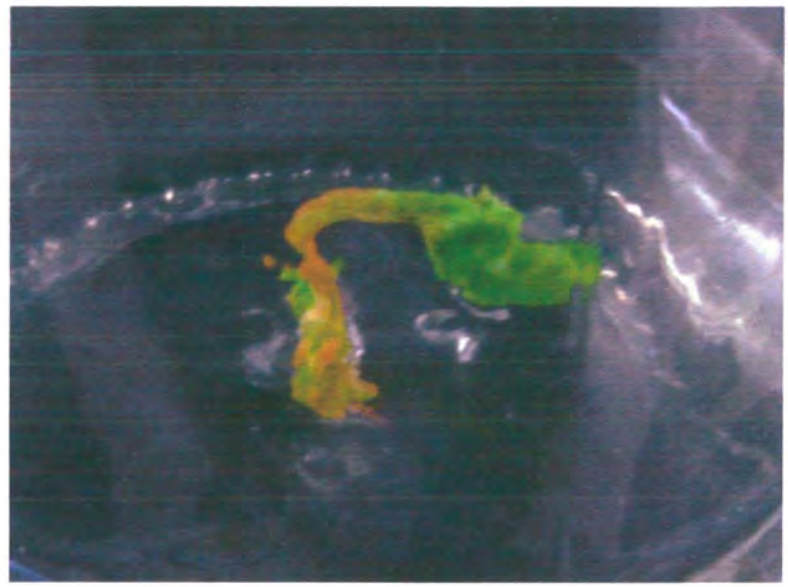

Fig. 7 Differentiated plant having one shoots after 45 days of culturing supplemented with NAA 0.3 mg/l, GA 1.2mg/l and TDZ 1.5 mg/l with out $AgNo_3$.

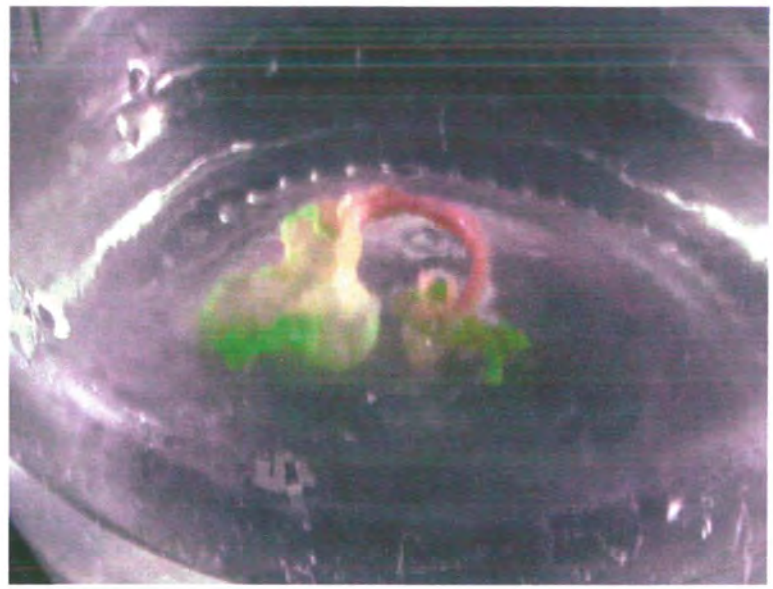

Fig. 8 Differentiated plant having one shoot after 50 days of culturing supplemented with NAA 0.5 mg/l, GA_3 1.5 mg/l and $AgNo_3$ 0.2 mg/l.

It is noted that organogenesis is depended on both PGRs concentration and also on size, colour and origin of callus. It is also studied that the callus which was greenish in colour and large in size showed rapid rate of organogenesis as compared to small sized and whitish or brownish calluses because greenish callus have greater organogenic potential as compared to brown or yellow callus.

3.3. Rooting

The differentiated shoots were transferred to another MS medium supplemented with various concentrations of NAA and BA. Three types of media R1 were having NAA 0.1 mg/l, BA 0.8 mg/l, R2 having NAA 0.204 mg/l, BA 0.248 mg/l and R3 having NAA 0.3 mg/l and BA 2.5 mg/l were used for rooting (Table 3). Roots are still under study and in the process of development.

3.4. Antioxidant activity

Antioxidant activity or radical scavenging activity (RSA) was determined by using DPPH method. The DPPH method is commonly used for checking antioxidant activity. The RSA was determined to elevate the antioxidant potential of regenerated tissue and these were compared with levels in wild grown plants. *Viola odorata* over all is a medicinal plant show higher antioxidant activity against free radicals and medicinally very important used in medicine for curing different diseases. In the present study the *in vitro* callus has formed showed greater antioxidant activity and has significantly higher capacity to detoxify DPPH free radicals than wild plants collected from wild condition. The antioxidant activity of callus extract determined by DPPH method was 38 %, while that of wild plant extract was 33 %. Wild plants have 5 % less antioxidant activity than callus.

Chapter No. 4

Discussion

4.1. Callus induction

The effect of various plant growth hormones such as BA, 2, 4-D, kinetin, NAA, IAA etc on explant response in preliminary experiments on callus induction from leaf, stem and petiole were evaluated. Callus production may be important for studies of indirect morphogenesis (Radice and Caso, 1997) or for studies of production of secondary metabolites in medicinal plants (Mertan *et al.*, 2009). In the present experiments, BA was proved more effective and important than kinetin for high percentage of callus induction while among auxin 2, 4-D proved effective than NAA for callus induction. Both PGRs BA and 2, 4-D in combination with each other showed good and optimum results and best callus induction occurred on media containing both these hormones as in media C1, C2 and C4 (Table 4). Previously Wijowska *et al.* (1999) obtained callus, autonomous endosperm and roots *in vitro* by culturing unfertilized ovules of *Viola odorata* incubated on medium supplemented with BA or kinetin and 2, 4-D with more or less equal concentration which agree with our observation that these PGRs are important for callus induction of *Viola odorata*. Both these hormones have synergistic effect for each other in combination and enhanced callus induction. It is also proved by our results that both these hormones used alone was not effective and did not induce callus as compared to in combination with each other. These observations are in contrast with that of Bhaskaran and Smith (1990) who demonstrated that 2, 4-D in many cases is usually the choice of auxin for callus induction and subculture of grasses and herbs. Because of this synergistic effect of BA and 2, 4-D the explant response is inclined towards the relative concentration of both BA and 2, 4-D. Similar response towards BA and 2, 4-D was found in case of *Viola wittrockiana*, in which callus was induced using leaves and petioles as an explant (Wang and Man, 2006).

The MS basal media used for callus induction in the experiments have half strength proved more effective and optimum for callus induction as compared to full strength MS media as shown by the results in C8 and C9 media (Table 4). These results are in agreement with that of *Viola tricolor* (Wang and Man, 2006) and *Hybanthus floribundus* (Bidwell *et al.*, 2001) in which callus induction optimized on MS media having half strength. However in case of ovule callus induction of *Viola odorata* (Wijowska *et al.*,

1999) and *Hybanthus enneaspermus* which belong to other genus of Violaceae (Parksh *et al.*, 1999) the MS media used have full strength on which callus has been optimized. The reason may be due to different explant origin, used for callus induction as in the present experiment leaves, petiole and stem are used, which respond differently than that of ovule. Secondly, it also explains that the amount and concentration of micro and macronutrients of MS basal media have effect on explant response and different explant respond differently according to the amount and concentration of both these nutrients. Callus induction response of explant is linear to the concentration of both these hormones and response was rapid and quick by increasing its concentration but up to some limit beyond which no callus induction was observed. This may be due to the hyper accumulation of both hormones which decreases efficiency of explant for callus formation as in our results by doubling the concentration of both hormones than optimized concentrations no callus formation occurred.

NAA alone and along with BA did not induce callus in *Viola odorata* though reports are available for the seed derived callus on MS media supplemented with NAA and BA (Parkash *et al.* 1999). Our results also disagree with that of Baber and Kulhbushan (1999) work on *Viola tricolour* in which NAA plus 2, 4-D induced callus. This is because the synergistic effect of hormones varies from species to species and even among different parts of same species used as an explant. The previous data revealed that both NAA and BA induced callus with optimum rate in *Hybanthus floribundus* (Bidwell *et al.*, 2001) and *Hybanthus ennespermus* (Parkash *et al.*, 1999) both belong to Violaceae but not effective in the present study for callus induction. There are many factors due to which different plants and different parts of same plant respond differently to same hormone. This may be due to different explant used, different origin of explant, different phytochemistry and having different genetic make up. Recently, Zheng *et al.* (2009) reported that leaf explant from *in vitro* grown plants of *Lysimachia* were more responsive to callogenesis and organogenesis than those obtained from *in vivo* growth conditions. These factors also have effect on callus structure, colour, texture and growth rate as in the present study the explant derived from leaves have green colour, rapid growth rate and compact in texture as compared to callus derived from stem and petioles. Moreover

quality, health condition and age of explant may also have effect on its response to different hormones.

4.2. Shoot initiation

Best and optimum shooting occurred on shooting media by using green callus as compared to yellow or brown callus. The medium used for shooting was supplemented by GA_3 hormone which can promote growth and elongation of cells and it has been used earlier by Wang and Man (2006) in case of *Viola tricolour* for shoot initiation. TDZ and $AgNo_3$ used with GA_3 and NAA have role in promoting shoot formation. TDZ is one of the several substituted ureas that have been investigated recently for their cytokinin-like activity. TDZ is known to be more active than zeatin for stimulating the growth when added to a tissue culture medium at a low concentration (Sajid and Faheem, 2009). It has been considered to be more potent than most of the commonly used cytokinins (Huetteman and Preece, 1993).

$AgNo_3$ has proved to be a very potent inhibitor of ethylene action and is widely used in plant tissue culture. Silver ion mediated responses seem to be involved in polyamines; ethylene and calcium mediated pathways, and play a crucial role in regulating physiological process including morphogenesis (Kumar *et al.*, 1993). $AgNo_3$ is also known to promote multiple shoot formation in different plants. *In vitro* shoot formation was improved by incorporating $AgNo_3$ in the culture medium. Earlier, Ganesh and Sreenath (1996) reported *in vitro* sprouting of apical buds of *Coffea* under the influence of $AgNo_3$.

In the present study it was observed that shooting was poor on media devoid of both or one of these chemicals i.e $AgNo_3$ and TDZ. Furthur NAA was used along with $AgNo_3$ and TDZ for shoot initiation. It was found that the shoots developed on green calluses have grown rapidly and up to 4-5 cm in length after 5-6 weeks of incubation. Shooting of *Viola odorata* was easy and rapid as compared to rooting. This might be due to the high concentration of BA accumulated in the callus during callus formation which latter on has effect on shooting and rooting. It also explains that pre treatment of explant have

effect on post treatment response of explant i.e PGRs used earlier for callogenesis have effect on the activity and response of callus formed latter on for other PGRs. The same patron is also stated by (Wang and Man, 2006) in *Viola wttirockinii* in which rooting was easy as compared to shooting because media contain higher concentration of auxin as compared to cytokinin, which promote rooting. The number of shoots and branches formed on the callus also varied in our results among different calluses and among different media. It might be due to difference in efficiencies of calluses and media for shoot formation. The relative concentration of hormones, TDZ and $AgNo_3$ also have direct relation to regeneration and by increasing their concentration, shooting rate was also observed to be increased as in S1 and S2 media (Table 5).

However apart from this, shooting efficiency also depends on callus colour, callus age and the type of explant from which callus derived (orlikowska *et al.*, 1999). Green callus have higher organogenic potential than other types of calluses. The same results were also observed on some other plants (Maureen and pau, 1990; Vargas *et al.*, 2004). These observations made it possible to get high shooting rate by selecting green calluses in *Viola odorata* tissue culture.

4.3. Root initiation

Shoot failed to form roots on MS medium lacking auxin. Roots are under the process of development after 4 weeks of incubation from the bases of differentiated shoots in the presence of NAA and BA best on R2 medium (Table 3). Both these hormones BA and NAA act synergistically for rooting irrespective of our previous result where it failed to induce callus in C14 media. In these two hormones the concentration of NAA is more important for rooting and any variation its concentration greatly effect rooting. The same result was observed by Kumar *et al.* (1993) in *Clitoria ternate.*

4.4. Antioxidant activity

In the present study the free radical scavenging activity of methanolic extract of callus and wild plant of *Viola odorata* was determined. The callus has the ability to accumulate secondary metabolites similar to those found in the mother plant (Shilpa *et al.* 2010). Due

to these stored metabolites, plant and the callus extracts have antioxidant activity against free radicals. In the present study the activity of these secondary metabolites was determined by using DPPH free radical also used by Vukics *et al.* (2008) in case of *Viola tricolor*, an other species of Violaceae . The scavenging of stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Ebrahimzadeh *et al.*, 2008). DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore free radical scavengers (Dehpour *et al.*, 2009). It was found that the radical-scavenging activities of all the extracts increased with increasing concentration of secondary metabolites.

In the present study callus derived extract have greater antioxidant activity than wild plant derived extract because of greater accumulation of secondary metabolites. This variation in its activity demonstrates that different components are accumulated during different growth phases and in different environmental conditions. Because of difference in the amount of secondary metabolites, different parts of plant also have variation in their activities. Earlier it was suggested that the phenolic compounds contributed significantly to the antioxidant capacity of 112 wild grown Chinese herbs (Cai *et al.*, 2004).

In *Viola odorata* antioxidant activity is related to the amount of anthocyanins, one of the groups of flavonoids pigments. Anthocyanins occur in all tissues including leaves, stems, roots and flowers which are derivatives of anthcyanidin which include pendant sugars Anthoxanthins are their clear, white to yellow counterparts occurring in plants. Other bioactive compounds and secondary metabolites showing antioxidant activity are violin, flavonoids, alkaloids and phenolic compounds. The total phenol and flavonoids contents of leaf of *Viola odorata* were determined previously by Ebrahimzadeh *et al.* (2010). Previous data suggest that leaf of *Viola odorata* has higher antioxidant activity than other parts of plant, however antioxidant activity of callus is higher than other plant parts as revealed by the present study, suggesting that callus can be preserved and sub cultured for medicinal use related to antioxidants.

Conclusions

In conclusion an *in vitro* protocol was developed for rapid callus formation from leaf, stem and petiole of *Viola odorata* as well as organogenesis of the plant from leaf callus. The leaf based callus induction protocol is expected to be more useful for conservation of this endangered species by micropropegation and commercial cultivation. Moreover, antioxidant activity was also monitored for callus and wild plants of *Viola odorata*. It was also observed that callus have greater antioxidant activity than wild plants.

References

Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA (2004) Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem. 84: 551-562.

Amzallag GN, Lerner HR, Poljakoff-Mayber A (1990) Induction of increased salt tolerance in *Sorghum bicolor* by NaCl pretreatment. J. Experimental Bot. 41: 29-34.

Ahmad M, Sher H (1997) Traditional use of wild medicinal plants of swat, A technical report 2.1a, Environmental Rehabilitation project in Malakand Division (Chitral, Swat, Buner).

Ames SN, Shigenaga MK, Hagen TM (1993) Oxidants antioxidants and degenerative diseases of aging. PNAS. USA. 90: 7915-7922.

Anderson JA, Padhye SR (2004) Protein aggregation, radical scavenging capacity, and stability of hydrogen peroxide defense systems in heat-stressed vinca and sweet pea leaves. J. Am. Soc. Hortic. Sci. 129: 54-59.

Akazawa T (1972) Report of the investigation of the Kamitakatsu shell-midden site. University Museum, University of Tokyo, Bulletin 4.

Babber S, Kulbhushan S (1991) Study of anatomy of vitrified structure in *Viola tricolor* L. Ann. Biol. (Ludhiana) 7 (1): 93-95.

Ballard HE, Sytsma KJ, Kowal RR (1999) Shrinking the violets: Phylogenetic relationships of infrageneric groups in *Viola* (Violaceae) based on internal transcribed spacer DNA sequences. System. Bot. 23: 439-458.

Bandoniene D, Murkovic M, Pfannhause W, Venskutonis PR, Gruzdiene D (2002) Detection and activity evaluation of radical scavenging compounds by using DPPH free radical and online HPLC-DPPH methods. Eur. Food Res. Technol. 214: 143-147.

Banyal S, Vinay KR (1983) Reversal of osmotic stress effects by gibberellic acid in Brassica campestris .Recovery of hypocotyl growth, protein and RNA levels in the presence of GA. Scand. Soci. plant Physiol. 59 (1): 111-114.

Bhaskaran S, Smith RH (1990) Regeneration in cereal tissue culture: A review. Crop Sci. 30: 1328-1336.

Bibi S, Ghulam D, Farukh H, Perveen S (2006) Elemental composition of *Viola odorata* Linn. Pak. J. Plant Sci. 12 (2): 141-143.

Bidwell SD, Pederick JW, Sommer K, Woodrow IE (2001) Micropropagation of the Nickel hyperaccumalator, *Hybanthus floribundus* (family-Violaceae), Plant Cell, Tiss. and Org. Cult. 67(1): 89-92.

Bokesch HR, Pannell LK, Cochran PK, Sowder RC, McKee TC, Boyd MR (2001) A novel anti-HIV macrocyclic peptide from Palicourea condensate. J. Nat. Prod. 64: 249-250.

Bondet V, Brand-Williams W, Berset C (1997) Kinetics and mechanisms of antioxidant activityusing the DPPH free radical method. Lebensm. Wiss. Technol. 30: 609-615.

Cai YZ, Luo Q, Sun M, Corke H (2004) Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associate with anticancer. Life Sci. 74: 2157-2184.

Canter PH, Thomas H, Ernst E (2005) Bringing medicinal plants in cultivation: opportunities and challenges for biotechnology. Trends in Biotechnol 23: 180-185.

Chang C, Chen C, Tsai Y, Chang (2000) A tissue culture protocol for a rare plant *Lilium speciosum* Var. gloroiosoides Baker . Bot. Bull. Acad. Sin. 41: 139-142.

Craig WJ, (1999) Health-promoting properties of common herbs, Am. J. Clin. Nutr. 3: 491-499.

Craik DJ (2001) Plant cyclotides: circular, knotted peptide toxins. Toxicon. 39: 1809-1813.

Dehpour AA, Ebrahimzadeh MA, Nabavi SF, Nabavi SM (2009) Antioxidant activity of methanol extract of *Ferula assafoetida* and its essential oil composition, Grasasy Aceites. 60 (4): 405-412.

Dinç M, Yıldırımlı S (2002) A new species of *Viola* (Violaceae) from Turkey. Bot. J. Linn. Soci. 138: 483-487.

Dinç M, Bagci Y, Yıldırımlı S (2003) A new species of *Viola* L. (Violaceae) from South Anatolia. Bot. J. Linn. Soc. 41: 477-482.

Di Matteo V, Esposito E (2003) Biochemical and therapeutic effects of antioxidants in the treatment of Alzeimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, Current Drug Target CNS and Neurological Disorder: 95-107.

Drozdowicz YM, Jones RL (1992) Regulation of acid release from barley aleurone by gibberellic acid and abscisic acid. In MK Walker-Simmons, JL Reid, eds, Preharvest Sprouting in Cereals, American association of cereal chemists, St. Paul, MN: 254-261.

Duh PD (1998) Antioxidant activity of burdock (*Arctium lappa* Linne): Its scavenging effect on free radical and active oxygen. JAOCS. 75: 455-465.

Ebrahimzadeh MA, Nabavi SF, Nabavi SM, Eslami B (2010) Antihypoxic and antioxidant activity of *Hibiscus esculentus* seeds. Grasas Aceites. 61 (1): 30-36.

Ebrahimzadeh MA, Pourmorad F, Bekhradnia AR (2008) Iron chelating activity screening, phenol and flavonoid content of some medicinal plants from Iran. Afr. J. Biotech. 7: 3188-3192.

Gams H (1925) *Viola* L. In: Hegi G, ed. Illustrierte flora von mittel-Europa, München. J. F. Lehmanns Verlag (5): 586-657.

Ganesh SD, Sreenath HL (1996) Silver nitrate enhanced shoot development in cultured apical shoot buds of *Coffea arabica* Cv Cauvery (S4347). J. Plantation Crop. 24: 577-580.

Gazi MR, Kanda K, Yasuda M, Kato F (2004) Optimization of cultural conditions and some properties of radical scavenging substances from sporobolomyces salmonicolor. Pak. J. Biol. Sci. (7): 1365-1370.

Gerber M, Boutron MC, Hercberg S, Riboli E, Scalbert A, Siess MH (2002) Food and cancer. State of the art about the protective effect of fruits and vegetables, Bull. Cancer 89: 293-312.

Gran L (1973) Isolation of oxytocic peptides from *Oldenlandia affininis* by solvent extraction of tetraphenylborate complexes and chromatography on Sephadex LH-20. Lloydia. 36: 207-208.

Gustafson KR, Sowder II RC, Henderson LE, Parsons IC, Kashman Y, Cardellina II, JH, McMahon JB, Buckheit Jr RW, Pannell LK, Boyd MR (1994) Circulins A and B: novel HI inhibitory macrocyclic peptides from the tropical tree Chassalia parvifolia. J. Am. Chem. Soc. 116: 9337-9338.

Halliwell B (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence. Lancet. 344 (8924): 72-724.

Hallock YF, Sowde RC, Pannell LK, Hughes CB, Johnson DG, Gulakowski R, Cardellina JH, Boyd MR (2000) Cycloviolins A-D, anti-HIV macrocyclic peptides from *Leonia cymosa*. J. Org. Chem. 65: 124-128.

Hamabata A, Garcia MM, Romero T, Bernal LI (1988) kinetics of the acidification capacity of aleurone layer and its effects upon solubilization of resewe substances from starchy endosperm of wheat. Plant physiol. 186: 643-644.

Hansel R, Keller R, Rimpler H, Schneider G (1993) Hagers Handbuch der Pharmazeutischen Praxis. Berlin, Springer-Verlag. 1141-53.

Henry M, Malécot V, Marcussen T, Munzinger J, Yockteng R (2005) Origin of the parma Violet cultivars (Violaceae). Poster P1279. Presented at the XVII International Botanical Congress, Vienna. 17-23.

Heywood VH (1993) Flowering Plants of the World. University Press, London.

Hildebrandt A C and A J Riker (1946) Some common sugars, polysaccharides, and organic acids as carbon sources for plant tissue cultures. Amer. J. Bot. 33:229-230.

Hildebrandat C and A J Riker (1949) The influence of various carbon compounds on the growth of marigold, Paris-daisy, periwinkle, sunflower and tobacco tissue in vitro. Amer. J. Bot. 36: 74-85.

Honda H, Liu CZ, Kobayashi T (2001) Large-scale plant micro propagation. Adv. Biochem Engr/Biotech. 72: 158-182.

Huetteman A, Preece EJ (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell, Tiss, Org, Cult. 33: 105-119.

Hussain K, Majeed A, Nawaz K, Nisar MF, Khan F, Afghan S, Ali K (2010) Comparative study for salt stress among seed, root stock and direct regenerated violet (*Viola odorata* L.) seedlings in relation to growth, ion contents and enzyme activities Afric. J. Biotech. 9 (14): 2108-2117.

Ishii K (1998) Tissue culture and genetic engineering of forest trees with particular reference to the tropics .Plant biotech. Cambridge University Press , London. 103.

Iwashima M, Mori J, Ting X, Matsunaga T, Hayashi K, Shinoda D, Saito H, Sankawa U, Hayashi T (2005) Antioxidant and antiviral activities of plastoquinones from the brown alga *Sargassum micracanthum*, and a new chromene derivative converted from the plastoquinones. Biol. Pharm. Bull. 28: 374-377.

Jackson D, Bergeron K (2005) Alternative nature online herbal. Edited by K. Bergeron http:// altnature.com/gallery/violet.htm.

Jain SM, Brar DS, Ahloowalia BS (1998) Somaclonal variation and induced mutations in crop improvement. Kluwer Academic Publishers, UK.

Jaime A, da Silva T (2003) Anthemideae: advances in tissue culture, genetics and transgenic biotechnology Afri. J. Biotech. 2 (12):547-556.

Jennings C, West J, Waine C, Craik DJ, Anderson M (2001) Biosynthesis and insecticidal properties of plant cyclotides: the cyclic knotted proteins from *Oldenlandia affinis*. Proc. Natl. Acad. Sci. USA. 98: 10614-10619.

Kathi K (1991) The illustrated herb encyclopedia (a complete culinary, cosmetic, medicinal and ornamental guide to herbs). Mallard Press, New York. 207.

Khalaf NA, Ashok KS, Atif A, Zaha E, Husni F (2008) Antioxidant activity of some common plants, Turk. J. Bio. 51-55.

Khan IA (1996) Cytomorphological studies on saffron (*Crocus sativus* L.) Ind. J. Agri. Res. 30 (1): 48-52.

Kirtikar K R, Basu BD (1918) Indian medicinal plants part 1, Indian press Allahabad, India.

Koleva II, Beek TA, Linssen JPH, DeGroo A, Evstatieva LN (2001) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13-817.

Kögl F, Erxleben H, and Haagen-Smit A (1934) uber die Isolierung der Auxine a und b aus pflanz-lichen Materialen. Zeitschr. Physiol. Chem. 225: 215-229.

Kulisic T, Radonic A, Katalinic V, Milos M (2004) Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85: 633-640.

Kumar KP, Soniya EV, Lawrence B and Nair GM (1993) Micropropagation of *Clitoria ternata* L. (Papilionaceae) through callus regeneration and shoot tip multiplication. J. Spices and Aroma. Crop. 2: 41-46.

Lamaison JL, Petitjean FC, Carnat A (1991) The violet flower: a comparison between *Viola lutea, Viola calerata* and *Viola odorata*. Plants Medicinal-*et*- Phytothera 25: 79-88.

Lambinon J, Langhe JE, Delvosalle L, Duvigneaud J (1992) New flora of Belgium, the Grand Duch Luxembourg, northern France and neighboring desr_gions (Pt_ridophytes and Spermatophytes). Heritage of the National Botanic Garden of Belgium. 1092.

Larkin PJ, Scowcroft SC (1981) Somaclonal variation - a novel source of variability from cell culture for plant improvement. Theor. Appl. Genet. 60: 197-214.

Liao Z, Chen M, Tan F, Sun X, Tang K (2004) Micropropegation of endangered Chinese Aloe. Plant Cell, Tiss. Org. cult. 76: 83-86.

Lindholm P, Go¨ ransson U, Johansson S, Claeson P, Gullbo J, Larsson R, Bohlin L, Backlund A (2002) Cyclotides-a novel type of cytotoxic agents. Mol. Cancer Therap. 1: 365-369.

Marcussen T, Borgen L (2000) Allozymic variation and relationships within *Viola* subsection *Viola* (Violaceae). Plant Syst. Evol. 223: 29-57.

Marcussen T, Borgen L, Nordal I (2001) *Viola hirta* (Violaceae) and its relatives in Norway. Nor. J. Bot. 21: 5-17.

Marcussen T, Borgen L, Nordal I (2005) New distributional and molecular information call into question the systematic position of the West Asian *Viola sintenisii* (Violaceae). Bot.l J. Linn. Soci. 147: 91-98.

Masuda T, Yonemori S, Oyama Y, Takeda Y, Tanaka T, Andoh T, Shinohara A, Nakata (1999) M Evaluation of the antioxidant of environmental plants: activity of the leaf extracts from seashore plants. J. Agric. Food Chem. 47: 1749-1754.

Matteo VD, Esposito E (2003) Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, Curr. Drug Targets. 2: 95-107.

Maureen MMF, Pau HM (1990) Comparison of 2, 4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. Plant Cell Tiss. Org. Cult. 20 (3): 157-163.

Meratan AA, Ghaffari SM, Niknam V (2009) In vitro organogenesis and antioxidant enzymes activity in *Acanthophyllum sordidum*. Physiol. Plant. 53: 5-10.

Milkuk J (1999) Propagation of endangered species by tissue culture. Acta Universitalis Palackianeae Olomucensis Facultas Rerum Naruralium Biologia. 37: 27-33.

Miller CO, Skoog F, Okumura FS, von Saltza M, Strong FW (1956) Isolation, structure and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Soc. 78: 1375-1380.

Miller CO, Skoog F, Von Saltza MH, Strong FM (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. 77: 1392.

Miller NJ, Rice-Evans CA, Davies MJ, Gopinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. 84 (4): 407-412.

Molyneux P (2004) The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26: 211-219.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Novotna I (1984) Inheritance of productivity in pansy (*Viola wittrockiana* Gams) and its use in breeding. Sborni'k Uvtiz Zahradnictvi 11: 315-321.

Naylor SL, Sakaguchi AY, Barker D, White R, Shows TB (1984) Proc. natn. Acad. Sci. U.S.A. 81: 2447–2451.

Orlikowska T, Nowak E, Marasek A, Kuchrska D (1999) Effect of growth regulators and incubation period on *in vitro* regeneration of adventitious shoots from gerbera petioles. Plant Cell Tiss. Org. Cult. 59: 95-102.

Parmessur YS, Alijanabi S, Saumatally S, Dookun SA (2000) Sugarcane yellow virus and sugarcane yellow phytoplasma elimination by tissue culture . Plant Pathl. 51: 561- 569.

Parkash E, Sha PS, Vallikhan P, Sairam R, Rao KR (1999) Regeneration of plants from seed-derived callus of *Hybanthus enneaspermus* (L) Muell, a rare ethnbotanical herb, J. Plant Cell rep. (18): 873-878.

Pavlov A, Kovatcheva P, Georgiev V, Koleva I, Ilieva M (2002) Biosynthesis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures. *Z. Naturforsch.*(57): 640-644.

Pham-Huy LA, He H and Pham-Huyc C (2008) free radicals, antioxidants in disease and health. Int. J. Bio. Sci. 4 (2): 89- 96.

Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53: 4290-4303.

Qaiser M, Omer (1985) In: Nasir E. & S. I. Ali (eds.).Flora of Pakistan,"Violaceae" no. 166.

Radice S, Caso OH (1997) Somatic embryogenesis and organogenesis in cultured cotyledons of *Silybum marianum* (L) Gaertn. Biocell. 21: 59-64.

Ramachandra RS, Ravishankar GA (2002): Plant cell cultures: chemical factories of secondary metabolites. Biotech. Adv. 20: 101-153.

Ravishankar GA, Rao SR (2000): Biotechnological production of phytopharmaceuticals. J. Biochem. Mol. Biol. Biophys. 4: 73-102.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans CA (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Boil. medi. 26 (9-10): 1231-1237.

Rupinder K, Ramesh K (1998) Effect of nitrogen, phosphorus and plant spacing on seed yield in pansy (*Viola tricolor*) cv 'Swiss Giant'. J. Ornamental Hort. (New Series) 11: 21-25.

Sabatay T (1941). Ann. Chim. Annual. Chim. Appl. 3:23.

Said HM (1969) Pharmacopoeia of Eastern medicine. The time press Karachi 544.

Sajid ZA, Faheem A (2009) Effect of Thidiazuron(TDZ) on *in vitro* micropropegation of *Solanum tuberosum* L. CVS. Desiree and cardinal Pak. J. Bot. 41(4): 1811-1815.

Sato T, Kwon O, Miyake H, Tangiguchi T, Maeda E 1995 Regeneration of plantlets from petiole callus of wild *Viola* (*Viola patrinii* DC.). Plant Cell Rep. 14: 768-772.

Scho pke, T, HasanAgha MI, Kraft R, Otto A, Hiller K (1993) Ha molytisch active Komponenten aus Viola tricolor L. und *Viola arvensis*. Murray. Sci. Pharm. 61: 145-153.

Sharma TR (2000) Molecular detection and identification of plant pathogens. Symposium on Biotechnology of Plant Protection, BHU, Banaras (UP), Feb. 25-27.

Shilpa K, Selvakkumar C, Senthil AK, Lakshmi BS (2010) *In vitro* root culture of *Ocimum sanctum* L. and evaluation of its free radical scavenging activity. Plant Cell Tiss. Org. Cult. 9: 9661-4.

Sinjorgo K M C, De Vries M A, Heistek J C, VanZeijl M J, VanderVeen S W and Douma A C (1993) The effect of external pH on the gibberellic acid response of barley aleurone. J. Plant Physiol. 142: 506–509.

Skirvin RM, M Norton, KD McPheeters (1993) Somaclonal variation: has it proved useful for plant improvement. Acta Hort. 336: 333-340.

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue cultured in vitro. Symp. Soc. Exp. Biol. 11: 118-131.

Snyman SJ, Watt MP, Huckett FC, Botha BI (2000) Direct somatic embryogenesis for rapid, cost-effective production of transgenic sugarcane (*Saccharum* spp. hybrids). Pro. South Africa. Sugar Technol. Assoc. 74: 186-187.

Svangard E, Goransson U, Hocaoglu Z, Gullbo J, Larsson R *et al* (2004) J. Nat. Prod. 67: 144-147.

Tam JP, Lu YA, Yang JL, Chiu KW (1999) An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. Proc. Natl. Acad. Sci. USA. 96: 8913-8918.

Tepe B, Daferera D, Sokmen A, Sokmen M, Polissiou M (2005) Food Chem. 90: 333.

Thimann K V (1934) Studies on the growth hormone of plants. VI. The distribution of the growth substance in plant tissues. J. Gen. Physiol. 18: 23- 34.

Thorpe TA (1990) The current status of plant tissue culture, in plant tissue culture: applications and limitations, Elsevier, Amsterdam. 1-33.

Thomas M, Liv B, Inger N (2005) New distributional and molecular information call into question the systematic position of the West Asian *Viola sintenisii* (Violaceae). Bot. j. Linnean Soci. (147): 91-98.

Trabi M, Craik DJ (2002) Circular proteins-no end in sight. Trends Biochem. Sci. 27: 132-138.

Valentine DH, Merxmüller H, Schmidt A (1968) *Viola* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Cambridge: Cambridge University Press. (2): 270-282.

Vargas TE, Mejı´as A, Oropeza M, de Garcı´a E (2004) Plant regeneration of *Anthurium andreanum* cv Rubrun. Electron. J. Biotechnol. 7 (3): 23-25.

Vasil K I and Hildebrandt A C (1966) Growth and chlorophyll formation in plant callus tissue grown in vitro. Planta 68: 69-82.

Vasil K I (2008) A history of plant biotechnology: from the Cell Theory of Schleiden and Schwann to biotech. Crops: 1424.

Vanisree M, Tsay HS (2004): Plant Cell Cultures, an alternative and efficient source for the production of biologically important secondary metabolites. International J. Appl. Sci. Engin. 2: 29-48.

Vinutha B, Prashanth D, Salma K (2007) J. Ethnopharmacol. 109-59.

Vukics V, Ringer T, Kery A, Bonn GK, Guttman A (2008) Analysis of heartsease (*Viola tricolor* L.) flavonoid glycosides by micro-liquid chromatography coupled to multistage mass spectrometry. J. Chroma. A. 1206: 11-20.

Wang J, Man ZB (2006) Plant regeneration of pansy (*Viola wittrockiana*) Caidie via petiole-derived callus, scientia Horticult. 111: 266-270.

Went F W (1928) Wuchsstoff und Wachstum. Rec. Trav. Bot. Neerl. 25: 1-116.

Wijowska M, Kuta E, Przywara L (1999) In vitro culture of unfertilized ovules of *Viola odorata* L. Acta Biol Cracov Ser. Bot. 41.

Witkowska-Banaszczak E, Byka W, Matlawska I, Goslinska O, Muszynski Z (2005) Fitoterapia 76: 458-461.

Williams JRS, Hoult JB, Harborne J, Greenham, Eagles J (1995) A biologically active lipophilic flavonoid from Tanacetum parthenium, Phytochemistry 38: 267-270.

Yen GC and Chen HY (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agri. Food Chem. 43(1): 27-32.

Yen GC, Duh PD (1994) Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. J. Agric. Food Chem. 42: 629-632.

Yordabov ND, Christova AG (1997) Quantitative spectrophotometric and EPR-determination of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH). Fresen. J. Anal. Chem. 358: 610-613.

Yuzepchuk SV (1949) *Viola* L. In: Shishkin BK, ed. Flora of the U.S.S.R. Jerusalem: Israel program for scientific translations (15): 263-360.

Zaidi MA, Crow Jr, SA (2005) Biologically active traditional medicinal herbs from Bolochistan, Pak. J. Ethnopharmacol. 96: 331-334.

Zheng W, Xu XD, Dai H, Chen LQ (2009) Direct regeneration of plants derived from in vitro cultured shoot tips and leaves of three Lysimachia species. Sci. Hort. 122: 138-141.