ABSTRACT

Oxalis triangularis (A.St.-Hil) or commonly known as ‘Pokok Rama-rama’ in Malaysia is a beautiful ornamental plant which is propagated by bulbs. The plant grows to a height of 0.1 m - 0.2 m and is perfect for cultivating in pots or containers. Nowadays, with the emerging and advanced technologies, an efficient protocol has been established for a rapid multiplication of *Oxalis triangularis* in a large scale production under aseptic conditions. *In vitro* plant regeneration of *Oxalis triangularis* was successfully obtained in the present study via petiole and leaf as explants. The petiole explants cultured on MS medium supplemented with 0.5 mg/l α-Naphthaleneacetic acid (NAA) and 1.0 mg/l Kinetin (KIN) produced maximum number of adventitious shoots (12 shoots) while for leaf explants, the best treatment achieved on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l 6-Benzylaminopurine (BAP) which produced a maximum of 14 shoots within 8 weeks. Comparison between *in vivo* plants and *in vitro* was observed using a Scanning Electron Microscope. The morphological features for both petiole and leaf samples have no differences. Both contain same structures of stomata and trichomes. *In vitro* flowering which is very important in order to improve quality and shortened physiological process of flowering was observed when adventitious shoots explants cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP (90% *in vitro* flowering). In the synthetic seeds study, two different storage durations were tested (Day 7 and Day 30). The highest frequency of synthetic seeds production in *Oxalis triangularis* was recorded on Day 7 with 96.67% of conversion frequency. The number of shoots per encapsulated bead was 4.57 ± 0.72 with 100% roots emergence. Synthetic seeds were also sown on black soil and sterile black soil. However, both soil conditions gave negative results due to fungal

infection. For the biological activities study, anthocyanin from petiole and leaf explants could be extracted by acidified methanol and acidified acetone solvents, and subsequently by reading the absorbance of UV-visible spectrophotometer. The highest anthocyanin absorbance for *in vivo* and *in vitro* petiole explants were recorded from explants extracted in acidified acetone solvent at concentration of 5 ml with the reading of 1.093 and 0.968, respectively. Different results were obtained for leaf explants in *in vivo* and *in vitro*. *In vivo* leaf explants showed highest anthocyanin pigment when extracted with acidified acetone at solvent concentration of 20 ml with absorbance of 2.223, meanwhile, *in vitro* leaf explants in acidified acetone at solvent concentration of 50 ml with absorbance of 1.280. Extraction was further examined with the natural pigment for paint production. Extraction from *in vitro* leaf explants had more dense colours compared to *in vitro* petiole explants. Extraction of *in vitro* leaf explants was coated on glass slides and let to dry overnight before being subjected to weathering tests; heat and salt tests. Both weathering tests had shown similar results whereby, it caused degradation of anthocyanin pigment. In phytochemical screening of *Oxalis triangularis*, four different solvents were used to test the presence of terpenoids/steroids, tannins, saponins, reducing sugar and glycosides. Both *in vivo* and *in vitro* plant extract only revealed the presence of steroids and saponins. In the antioxidant activity, both explants (*in vivo* and *in vitro*) of *Oxalis triangularis* in methanolic extracts were evaluated using DPPH free radical scavenging activity. *In vitro* petiole in methanolic extracts showed the lowest reading of IC_{50} value (less than 20µg/ml), among all the extracts that indicated the higher of inhibition rate. *In vitro* plantlets obtained from leaf explants were acclimatized in the greenhouse. Three types of growing substrates (black soil, red soil and vermiculite) were tested to identify the best soil to grow *Oxalis triangularis* successfully. The present study showed that all the growing substrates (black soil, red soil

and vermiculite) tested could be used to grow *Oxalis triangularis* but the recommended soil would be black soil because it resulted in high survival rates and low fungal infections.

ABSTRAK

Oxalis triangularis (A.St.-Hil) atau biasa dikenali sebagai ‘Pokok Rama-rama’ di Malaysia merupakan tumbuhan hiasan yang cantik yang dipropagasi melalui umbi. Tumbuhan ini membesar sehingga ketinggian 0.1 - 0.2 m dan sesuai ditanam di dalam pasu atau bekas. Pada masa kini, dengan kemajuan dan kecanggihan teknologi, satu protokol yang berkesan telah berjaya dilaksanakan untuk penghasilan *Oxalis triangularis* dalam skala pengeluaran yang besar di dalam keadaan aseptik. Regenerasi tumbuhan *in vitro Oxalis triangularis* telah berjaya diperolehi dalam kajian ini melalui petiol dan daun sebagai eksplan. Eksplan petiol yang dikultur di atas medium Murashige dan Skoog (MS) ditambah dengan 0.5 mg/l asid α-Naphthaleneasetik (NAA) dan 1.0 mg/l Kinetin (KIN) menghasilkan bilangan pucuk berganda yang paling banyak (12 pucuk) manakala untuk eksplan daun, rawatan yang terbaik ialah di dalam medium MS ditambah dengan 1.0 mg/l NAA dan 1.5 mg/l 6-Benzilaminopurin (BAP) menghasilkan paling banyak 14 pucuk dalam masa 8 minggu. Perbandingan antara tumbuhan *in vivo* dan *in vitro* diperhatikan dengan menggunakan mikroskop elektron imbasan. Ciri-ciri morfologi untuk kedua-dua sampel petiol dan daun tidak menunjukkan perbezaan. Kedua-duanya mengandungi struktur stoma dan trikom yang sama. Pembungaan *in vitro* adalah sangat penting untuk meningkatkan kualiti dan memendekkan proses fisiologi pembungaan diperhatikan pada eksplan yang dikultur di atas medium MS ditambah dengan 0.5 mg/l NAA dan 0.5 mg/l BAP (90% pembungaan *in vitro*). Dalam kajian biji benih sintetik, dua tempoh jangka masa penyimpanan yang berlainan telah diuji (Hari ke-7 dan Hari ke-30). Frekuensi tertinggi penghasilan biji benih sintetik *Oxalis triangularis* dicatatkan pada hari ke-7 dengan frekuensi percambahan sebanyak 96.67%. Bilangan pucuk bagi satu kapsul biji benih adalah 4.57 ± 0.72 dengan

100% menunjukkan penghasilan akar. Biji benih sintetik turut dicambah di atas tanah hitam dan tanah hitam steril. Walau bagaimanapun, kedua-dua kondisi tanah memberi keputusan negatif akibat jangkitan kulat. Untuk kajian aktiviti biologi, antosianin daripada eksplan petiol dan daun boleh diekstrak dengan pelarut metanol berasid dan aseton berasid dan seterusnya keserapan dibaca melalui UV-spektrofotometer. Serapan antosianin yang tertinggi untuk eksplan petiol *in vivo* dan *in vitro*, kedua-duanya telah direkodkan daripada eksplan yang diekstrak dengan pelarut aseton berasid pada kepekatan 5 ml dengan bacaan masing-masing 1.093 dan 0.968. Berbeza dengan keputusan yang diperolehi untuk eksplan daun dalam *in vivo* dan *in vitro*. Eksplan daun *in vivo* menunjukkan pigmen antosianin yang tertinggi apabila diekstrak dengan pelarut aseton berasid pada kepekatan 20 ml dengan serapan 2.223, sementara itu, eksplan daun *in vivo* yang diekstrak dengan pelarut maseton berasid pada kepekatan 50 ml dengan serapan 1.280. Kajian pengekstrakan dilanjutkan dengan pigmen semulajadi untuk penghasilan cat. Ekstrak daripada eksplan daun *in vitro* mempunyai warna yang lebih pekat berbanding dengan eksplan petiol *in vitro*. Ekstrak daripada eksplan daun *in vitro* disalut pada slaid kaca dan dibiarkan kering semalaman sebelum ujian ketahanan terhadap cuaca dijalankan; ujian haba dan garam. Kedua-dua ujian ketahanan terhadap cuaca menunjukkan keputusan yang sama di mana ia menyebabkan degradasi pigmen antosianin. Dalam kajian penyaringan fitokimia *Oxalis triangularis*, empat jenis pelarut telah digunakan untuk menguji kehadiran terpenoid/steroid, tanin, saponin, gula penurun dan glikosida. Kedua-dua ekstrak tumbuhan *in vivo* dan *in vitro* hanya menunjukkan kehadiran steroid dan saponin. Bagi aktiviti antioksidan, kedua-dua eksplan (*in vivo* and *in vitro*) *Oxalis triangularis* di dalam ekstrak metanolik dinilai dengan menggunakan cerapan antioksidan penyahradikal bebas DPPH. Daun *in vitro* di dalam ekstrak metanolik menunjukkan bacaan nilai IC_{50} yang terendah

(kurang daripada 20µg/ml), berbanding ekstrak yang lain menunjukkan kadar perencatan yang rendah. Plantlet *in vitro* yang diperolehi dari eksplan daun diaklimatisasi ke rumah hijau. Tiga jenis substrat pertumbuhan (tanah hitam, tanah merah dan vermikulit) di uji untuk mengenal pasti tanah yang terbaik untuk pertumbuhan *Oxalis triangularis*. Dalam kajian ini, ke semua substrat (tanah hitam, tanah merah dan vermikulit) boleh digunakan untuk pertumbuhan *Oxalis triangularis* tetapi jenis tanah yang disarankan ialah tanah hitam kerana daripada kajian ini menunjukkan kadar kelangsungan hidup yang tinggi dan jangkitan kulat yang rendah.

ACKNOWLEDGEMENT

IN THE NAME OF ALLAH, MOST GRACIOUS, MOST MERCIFUL

I would like to thank my supervisor, Professor Dr. Rosna Mat Taha for her supervision. I would also like to acknowledge Postgraduate Research Fund (PPP), University of Malaya for the grant provided to support my Master's studies.

I also thank the members of Tissue Culture Laboratory and laboratory assistant, for their suggestions and moral support. Without their assistance, this experimental work might not have been accomplished. Their help is much indeed appreciated. It really brought us together to appreciate the true value of friendship and respect for each other.

Last but not least, special thanks to my beloved family, especially my parents for all the encouragement and financial assistance in completing my study successfully.

- ANIS IDAYU MAD YUSSOF-

- SGR090088-

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degree Celcius
µl	Microliter
µg/ml	Microgram/milliliter
%	Percentage
w/v	Weight/ volume
2iP	Isopentenyl adenine
ADS	Adenine sulphate
$AgNO_3$	Silver nitrate
BAP	6-Benzylaminopurine
$CaCl_2$	Calcium chloride
cm	Centimeter
$CoCl_2$	Cobalt chloride
DMRT	Duncan's Multiple Range Test
DPPH	1, 1-Diphenyl-2-picryl-hydrazyl
g	Gram
GA_3	Gibberelic acid
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
l	Liter
NAA	α-Naphthaleneacetic acid

NaOH	Natrium hydroxide
M	Molar
mg	Milligram
ml	Milliliter
MS	Murashige and Skoog
O_sO_4	Osmium tetroxide
pH	Negative logarithm of H_3O^+ ion concentration in mol/l
PGRs	Plant Growth Regulators
PVA	Polyvinyl alcohol
Rpm	Revolutions per minute
SPSS	Statistical Package for Social Science
SE	Standard Error
TDZ	Thidiazuron
UV	Ultraviolet

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION OF PLANT TISSUE CULTURE

The history of plant tissue culture begins in 1838-1839 when Schleiden (1838) and Schwann (1839) proposed that cell is the basic unit of organisms. They visualised that cell is capable of autonomy and therefore it should be possible for each cell if given an environment to regenerate into whole plant. Based on this premise, the Austrian botanist Gottlieb Haberlandt was the first who pointed out the possibilities of the culture of isolated tissues (Bonner, 1936). He suggested that the potentialities of individual cells via tissue culture and also suggested that the reciprocal influences of tissues on one another could be determined by this method. Since Haberlandt's original assertions method for tissue and cell culture had been realised, leading to significant discoveries in Biology and Medicine. His original idea presented in 1902 was called totipotency (Haberlandt, 1902).

Plants have a remarkable regenerative power, as evidenced by the relative ease with which they can be rooted, transplanted and grafted. Early attempts at growing plants *in vitro* from isolated parts were unsuccessful, because knowledge of plant nutrition and physiology was inadequate. With the discovery of essential plant hormones, some progress was made starting in the 1920s and 30s. A major advancement was made by Philip R. White in 1939 with his report of continuous culture of carrot and tobacco done completely *in vitro*. Further progress was made by Folke Skoog, who discovered new and important

properties of the hormone auxin. Skoog, along with Toshio Murashige, went on to develop the still widely used standard plant nutrient solution; Murashige and Skoog (MS) medium.

The work begun by Kenneth Vivian Thimann in the late 1950s, which demonstrated that kinetin broke the dormancy of lateral buds, allowing them to develop as if they were at the tips of plants, paved the way for rapid advancements. From then on, new and important results were announced almost every year. Today almost any plant can be grown under laboratory control from a wide range of starting tissues.

The first true plant tissue cultures were obtained by Gautheret (1934, 1935) from cambial tissue of *Acer pseudoplatanus*. He also obtained success with similar explants of *Ulmus campestre, Robina pseudoacacia,* and *Salix capraea* using agar-solidified medium of Knop's solution, glucose and cysteine hydrochloride. Later, the availability of Indole-3-acetic acid (IAA) and the addition of B vitamins allowed for the more or less simultaneous demonstrations by Gautheret (1939) and Nobécourt (1939a) with carrot root tissues and White (1939a) with tumor tissue of a *Nicotiana glauca* X *N. langsdorffii* hybrid, which did not require auxin, that tissues could be continuosly grown in culture and even made to differentiate roots and shoots (Nobécourt, 1939b; White, 1939).

During the 1990s, continued expansion in the application of *in vitro* technologies to an increasing number of plant species has been observed. Tissue culture techniques are being used with all types of plants, including cereals and grasses (Vasil and Vasil, 1994), legumes (Davey *et al.*, 1994), vegetable crops (Reynolds, 1994), potato (Jones, 1994) and other root and tuber crops (Krikorian, 1994a), oilseeds (Palmer and Keller, 1994),

temperate (Zimmerman and Swartz, 1994) and tropical fruits (Grosser, 1994), plantation crops (Krikorian, 1994b), forest trees (Harry and Thorpe, 1994) and of course, ornamentals (Debergh, 1994). Good progress is being made in extending cryopreservation technology for germplasm storage (Kartha and Engelmann, 1994). Progress is also being made in artificial seed technology (Redenbaugh, 1993).

Tissue culture may be defined as the aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions (Thorpe, 2007). Plant tissue culture, also referred to as *in vitro*, axenic, or sterile culture is an important tool in both basic and applied studies as well as in commercial application. Although Street (1977) has recommended a more restricted use of the term, plant tissue culture is generally used for the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions of *in vitro*. Plant tissue culture is a technique used to grow plants or plant tissues and organs and to propagate new plants starting from a single cell or a small sample of cells using highly filtered air in an enclosed under sterile conditions. It is often to produce clones of a plant. This technology is also used for conserving rare and endangered plants, difficult to propagate plants especially seedless plants species to create transgenic plants and cryopreservation purposes. The production of high quality planting material of crop plants and fruit trees propagated from vegetative parts has created new opportunities in global trading, benefited growers, farmers and nursery owners and also improved rural employment. However, to accomplish this technology, all this method requires some laboratory knowledge and skill as well as basic laboratory equipment to make it successful.

According to Ahloowalia *et al*., (2004), the cultured cells and tissue can take several pathways to produce a complete plant. Among these, the pathways that lead to the production of true-to-type plants in large numbers are the popular and preferred ones for commercial multiplication. The following terms have been used to describe various pathways of cells and tissue in culture (Bhojwani and Razdan, 1983; Pierik, 1989).

In regeneration and organogenesis pathway, groups of cells of the apical meristem in the shoot apex, axillary buds, root tips and floral buds are stimulated to differentiate and grow into shoots and ultimately into complete plants. In many cases, the axillary buds formed in the culture undergo repetitive proliferation and produce large number of tiny plants. The plants are then separated from each other and rooted either in the next stages of micropropagation or *in vivo* (in trays, small pots or beds in glasshouse or plastic tunnel under relatively high humidity). The explants cultured on relatively high amounts of auxin (example, 2,4-dichlorophenoxyacetic acid (2,4-D)) form an unorganised mass of cells, called callus. The callus can be further subcultured and multiplied. The callus shaken in a liquid medium produces cell suspension, which can be subcultured and multiplied into more liquid cultures. The cell suspensions form cell clumps, which eventually form calli and give rise to plants through organogenesis or somatic embryogenesis (Ammirato, 1983). In some cases, explants, example, leaf-discs and epidermal tissue can also generate plants by direct organogenesis and somatic embryogenesis without intervening callus formation, example, in orchardgrass, *Dactylis glomerata* L. (Hanning and Conger, 1986). In organogenesis the cultured plant cells and cell clumps (callus) and mature differentiated cells (microspores, ovules) and tissues (leaf discs, inter nodal segments) are induced to

differentiate into complete plants to form shoot buds and eventually shoots and rooted to form complete plants.

In somatic embryogenesis, this pathway, cells or callus cultures on solid media or in suspension cultures form embryo-like structures called somatic embryos which on germination produce complete plants. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis. Although, somatic embryogenesis has been demonstrated in a very large number of plants and trees, the use of somatic embryos in large-scale commercial production has been restricted to only a few plants such as carrot, date palm and a few forest trees. Somatic embryos are produced as adventitious structures directly on explants of zygotic embryos from callus and suspension cultures.

Somatic embryos and synthetic seeds (embryos encapsulated in artificial endosperm) hold potential for large scale clonal propagation of superior genotypes of heterogeneous plants (Redenbaugh *et al*., 1993; Mamiya and Sakamoto, 2001). They have also been used in commercial plant production and for the multiplication of parental genotypes in large scale hybrid seed production (Bajaj, 1995; Cyr, 2000). In many species, somatic embryos are morphologically similar to the zygotic embryos although some biochemical, physiological and anatomical differences have been documented. The synthetic auxin, 2,4-D is commonly used for embryo induction. In many angiosperms, example, carrot (Lee *et al*., 2001) and alfalfa (McKersie and Bowley, 1993) subculture of cells from 2,4-D containing medium to auxin-free medium is sufficient to induce somatic embryogenesis. The process can be enhanced with the application of osmotic stress, manipulation of medium nutrients and reducing humidity. Selection of embryogenic cell

lines has also been successfully used. For example, selection of unique morphotypes in grapevine cultures allows production of high quality embryos with predictable frequency (Jayasankar *et al*., 2002).

A major problem in large scale production of somatic embryos is culture synchronisation. This is achieved through selecting cells or pre-embryonic cell clusters of certain size and manipulation of light and temperature (McKersie and Bowley, 1993), temporary starvation (Lee *et al*., 2001) or by adding cell cycle synchronising chemicals to the medium (Dobrev *et al*., 2002). Cytokinins seem to play a key role in cell cycle synchronisation (Dobrev *et al*., 2002) and embryo induction, proliferation and differentiation (Schuller *et al*., 2000). Abscisic acid is crucial in all the stages of somatic development, maturation and hardening (Schuller *et al*., 2000; Nieves *et al*., 2001).

Embryo culture is similar to seed culture except the embryos are aseptic excision from the seeds before starting and its transfer to a suitable medium for development under optimum conditions. Meristem cultures begin with excised root or shoot tips. Meristems are the actively growing zones at the apexes of roots, shoots and lateral buds. In callus culture, the growth of undifferentiated tissue that normally would only appear on a wound is encouraged. Tremendous numbers of callus cells can be grown as a starting material for fully developed plants. These tumor-like cells must be induced to form from differentiated (already developed) tissues through the use of chemicals. Likewise, callus cells must be chemically treated to revert them to differentiated tissues.

Plants in nature are able to synthesise their own food material. In contrast, plants growing *in vitro* are heterotrophic; they cannot synthesise their own food material. Therefore one of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium where it requires all essential minerals. Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the culture media. Although the basic nutrient requirements of cultured plant tissues are similar to those of whole plants in practice nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species. Media compositions are thus formulated considering specific requirements of a particular culture system. For example, some tissues show better response on a solid medium while others prefer a liquid medium. Plant tissue and cell culture media are generally made up of some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organic supplements, solidifying agents or support systems and growth regulators.

The macronutrients provide the six major elements; nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) required for plant cell or tissue growth. The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species. Plant cells may grow on nitrates alone, but considerably better results are obtained when the medium contains both a nitrate and ammonium nitrogen source. pH medium tends to be alkaline when only nitrate is used. Addition of ammonium to nitrates can prevent the occurrence of alkaline. Certain species require ammonium or another source of reduced nitrogen for cell growth to occur.

The essential micronutrients for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). Chelated forms of iron and zinc are commonly used in preparing culture media. Iron may be the most critical of all the micronutrients. Iron sulphate, citrate and tartrate may be used in culture media, but these compounds are difficult to dissolve and frequently precipitate after media are prepared hence cannot be fully utilised by the tissues. Murashige and Skoog used an ethylene diaminetetraacetic acid (EDTA)-iron chelate to bypass this problem. EDTA is a chelate complex agent found to improve growth in culture medium because in this form more iron can be absorbed by plants.

The preferred carbohydrate in plant cell culture media is sucrose, where sucrose is a main carbon source that needs to be added to the medium and is commonly used in cultured medium. Partial hydrolysis to produce glucose and fructose might happen when the medium being autoclaved. Glucose and fructose may be substituted in some cases, glucose being as effective as sucrose and fructose being somewhat less effective. Other carbohydrates that have been tested include lactose, galactose, rafinose, maltose and starch. Sucrose concentrations of culture media normally range between 2 and 3 percent. Use of autoclaved fructose can be detrimental to cell growth. Carbohydrates must be supplied to the culture medium because few plant cell lines that have been isolated are fully autotrophic, example, capable of supplying their own carbohydrate needs by carbon dioxide assimilation during photosynthesis.

Normal plants synthesise the vitamins required for their growth and development. Vitamins are required by plants in small quantities as catalysts in various metabolic processes. When plant cells and tissues are grown *in vitro*, some vitamins may become

limiting factors for cell growth. The vitamins most frequently used in cell and tissue culture media include thiamin (B1), nicotinic acid, pyridoxine (B6) and myo-inositol. Thiamin is the one vitamin that is basically required by all cells for growth. Myo-inositol is commonly included in many vitamin stock solutions. Although it is a carbohydrate and not a vitamin, it has been shown to stimulate growth in certain cell cultures. Its presence in the culture medium is not essential but in small quantities myo-inositol stimulates cell growth in most species.

Agar is the most commonly used gelling agent for preparing semi-solid and solid plant tissue culture media. Agar has several advantages over other gelling agents. First, when agar is mixed with water, it forms a gel that melts at approximately 60°C - 100°C and solidifies at approximately 45°C; thus, agar gels are stable at all feasible incubation temperatures. Additionally, agar gels do not react with media constituents and are not digested by plant enzymes. The firmness of an agar gel is controlled by the concentration and brand of agar used in the culture medium and the pH of the medium. Another gelling agent commonly used for commercial as well as research purposes is gelrite (used at the concentration of 0.2%), resulting in a clear gel which aids in detecting contamination. Alternative methods of support have included use of perforated cellophane, filter paper bridges, filter paper wicks, polyurethane foam, alginate, gelatine and polyester fleece. Whether explants grow best on agar or on other supporting agents varies from one species of plant to the next.

Plant growth regulators (PGRs) is a chemical that occurs naturally within plant tissues (endogenously), have a regulatory rather than a nutritional role in the growth and

development while plant growth regulator is a synthetic chemical with similar physiological activities to plant growth or compound having an ability to modify plant growth by some other means. Natural growth substances that are prepared synthetically or through fermentation process and have been added to plant tissue media are termed as plant growth regulators (exogenously). PGRs are the critical media components in determining the developmental pathway of the plant cells. The amount of PGRs in the culture medium is critical in controlling the growth and morphogenesis of plant tissues (Skoog and Miller, 1957). Four broad classes of growth regulators are important in plant tissue culture; an auxins, cytokinins, gibberellins, and abscisic acid. Skoog and Miller were the first to report that the ration of auxins to cytokinins determined the type and extent of organogenesis in plant cell cultures. Both an auxins and cytokinins are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species, and even cultivars in the type and amount of auxins and cytokinins required for induction of morphogenesis.

Auxins major functions is promoting both cell division and cell growth; promotion of stem elongation and growth; formation of adventitious roots; inhibition of leaf abscission; inducement of ethylene production and promotion of lateral bud dormancy. Site of production of hormone auxins can be found at apical meristem, seeds and other immature parts of plants like young leaves. Principal action of cytokinins is stimulation of cell division, but only in the presence of auxin; promotion of chloroplast development; delay of leaf aging; promotion of bud formation; flower development; embryo development; seed germination and inhibition of apical dominance. It can be found in root

apical meristems and immature fruits. For gibberellins, their function is to promote stem elongation; stimulation of enzyme production in germinating seeds; flowering and fruit development. This hormone can be found in roots and shoot tips, young leaves and embryo in seeds. Another major hormone is abscisic acid where their principal of actions are inhibition of bud growth; control of stomatal closure; some control of seed dormancy; inhibition of effects of other hormones and responses to water stress. It can be produced or found in fruits, seeds and almost all cells that contain plastids such as leaves, stems, and roots.

Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include those described by White, Murashige and Skoog, Gamborg *et al.,* Schenk and Hilderbrandt, Nitsch and Nitsch and Lloyd and McCown. Murashige and Skoog (MS) medium, Schenk and Hildebrand (SH) medium and Gamborg (B5) medium are all high in macronutrients, while the other media formulations contain considerably less of the macronutrients.

Plant tissue culture includes protoplast (cell without wall), cell, tissue and organ culture under sterile or aseptic conditions on a complete nutrient medium. The tissue culture technique is based on 'cell theory' and totipotency whereby each cell is believed to be able to develop into complete plant or individual plant. Totipotency implies that all the information necessary for growth and reproduction of the organism is contained in the cell. Although theoretically all plant cells are totipotent the meristematic cells are best able to express it. Micropropagation, the clonal production of complete plants is commonly used to

grow plants on a commercial scale. Tissue culture techniques also contribute significantly in the field of agriculture and agro-industry.

The surfaces of living plant materials are naturally contaminated from the environment with microorganisms, so surface sterilisation of starting material or also known as explant in chemical solutions is a critical preparation step. In many species explants of various organs vary in their rates of growth and regeneration, while some do not grow at all. The choice of explant material also determines if the plantlets developed via tissue culture are haploid or diploid. Some species can be started easily from a piece of cut explant; any part of plant, for example, leaf, stem and root. Many can be grown from seeds *in vitro*, allowing generation of plants that may otherwise be difficult to grow. Thus it is very important that an appropriate choice of explant be made prior to tissue culture. For instance, shoot tip culture can regenerate into virus and disease-free plants. In shoot tip culture, only small portions of shoot tips were used and this portion is protected by the leaf primordia, therefore free of any contaminants. Through protoplast fusion, protoplast from leaves of many species can be fused and as a result a hybrid plant is produced which has both parents' characters. Fusion of protoplast is usually done by using chemicals or small electric current. The fused protoplast can form new cell wall, divide and form callus which can result in plant regeneration. Usually only the superior traits/characteristics were chosen to be inherited to the offspring. Disease resistance or herbicide resistance plants or hybrids were usually chosen. Other modern technique, such as plant transformation, whereby selected gene is inserted into a plant is also possible using tissue culture as a basic technique.

1.1.1 Micropropagation

The process of plant micropropagation aims to produce clones (true copies of a plant in large numbers). Micropropagation is the practice of rapidly multiplying novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods using modern plant tissue culture procedure. It is the true-to-type propagation of a selected genotype using *in vitro* culture techniques to propagate plants using small fragments of the various part of plant slike growing tip, stem, leaves, flowers and sometimes even just individual cells of a parent plant. Micropropagation also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction or plants that do not reproduce rapidly by regular means.

Several stages are involved in micropropagation, each of which is influenced by an array of physical, nutritional and hormonal factors. There are five stages in micropropagation (de Fossard, 1976). The preparative stage (Stage 0); the initiation stage (Stage 1); the multiplication stage (Stage 2); the rooting stage (Stage 3); the transplanting stage (Stage 4), which are described in detail below.

At a Stage 0, *in vitro* introduction and establishment depends on the physiological and phytosanitary qualities of the plant, which are in part determined by the environmental conditions under which the plant is being grown (George and Debergh, 2008). The preparative stage, the plant material for *in vitro* culture is prepared; the aim is to obtain hygienic and physiologically better adapted starting materials. Clean stock materials that are free of viruses and fungi are important in the production of the healthiest plants.

Initially, this stage was introduced to overcome the huge problem of contamination. Raising stock plants in a greenhouse under more hygienic conditions can considerably reduce the risk of contamination, especially those related to fungal infestation. They may be prescreened for diseases, isolated and treated to reduce contamination (George, 1993; Holdgate and Zandvoort, 1997). However, it is more difficult to interpret the results with respect to bacterial contamination since most often we cannot distinguish between endogenous and exogenous bacteria. A healthy mother was selected before disinfecting it. A segment of the plant or commonly known as explant is covered in bacteria and fungus or mold spores. The explants are then brought to the production facility, surface sterilised and introduced into culture. They may at this stage be treated with antibiotics and fungicides (Kritzinger *et al.*, 1997) as well as antimicrobial formulations, such as parts per million (PPM) (Guri and Patel, 1998). The explants are then culture indexed for contamination by standard microbiological techniques, which are occasionally supplemented with tests based on molecular biology or other techniques (George, 1993; Leifert and Woodward, 1998; Leifert and Cassells, 2001).

For Stage 1, the initiation stage, the aim is to obtain reliable starting materials. Multiplication is not importan at this stage. The potential explants are cut from the plant. Then, it is placed in a sterile nutrient media under sterile conditions. Some plants are easily grown on simple media but others require more complicated media for successful growth. The plant tissue grows and differentiates into new tissues or organs depending on the medium. The culture medium is usually a variation of MS medium containing all essential elements, vitamins and hormones to stimulate the desired type of growth. For example, media containing cytokinins are used to induce multiple shoots from plant buds and it

happens in a vegetative form. The medium is made either solid medium by the addition of agar or liquid medium, without addition of agar.

Next is Stage 2, the multiplication stage. Following the successful introduction and growth of plant tissue, the establishment stage is followed by multiplication. The only function of this stage is to increase or maximise and maintain the stock; meristematic centres are induced and developed into buds and/or shoots. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds or thousands of plants. Depending on the type of tissue grown, multiplication can involve different methods and media. If the tissue is grown as small plants called plantlets, hormones are often added to promote the rapid regeneration of shoots from plant cells. To promote shoot multiplication, high concentrations of kinetin (KIN), benzylaminopurine (BAP) or N6-(2-isopentenyl)-adenine (2iP) are used along with an unstable form or low concentration of auxins. This combination promotes axillary or adventives shoot formation while inhibiting root growth. After a certain interval, the plantlets from multiple shoots can be cut to a single shoot and subculture into a new medium to multiply further.

In Stage 3, the rooting stage is to prepare the plantlet for transplanting from the artificial heterotrophic environment of the test tube to an autotrophic free-living existence in the greenhouse and to their ultimate location. Before transferring the plantlet into commercial soil, the plantlet must be produced well of roots. Roots can be induced by placing them in a medium containing roots induction medium. This time, hormones called auxins are used. For some species, this step can be skipped and the plants can be placed in hormone-free media, which is usually enough to get the plant to start producing roots. Root

induction takes place followed by root development *in vivo*. Until this stage, the plantlets have been grown in 'ideal' conditions, designed to encourage rapid growth. Due to lack of necessity, the plants are likely to be highly susceptible to disease and often do not have fully functional dermal coverings and will be inefficient in their use of water and energy. *In vitro* conditions is high in humidity and plants grown under these conditions do not form a working cuticle and stomata that keep the plants from drying out, thus when taken out of culture the plantlets need time to adjust to more natural environmental conditions. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to what would be considered a normal growth environment for the species in question. This is done by moving the plants to a location high in humidity.

Stage 4, the transplanting stage, is the final stage of plant micropropagation, whereby the plantlets are transfer to an evnvironment with greenhouse conditions for continued growth by conventional methods. Before being transferred to a greenhouse, most species require acclimatization procedure to increase survivability before potting in the greenhouse. This is done by transferring the plantlets from plant media to soil, then gradually increasing light, reducing humidity and allowing the plants to experience increased temperature variations. The plantlets are kept in the shade for weeks to prevent it from direct sunlight. Once hardened, thr acclimatized plants can be transferred to the greenhouse however more care is needed since the plants tend to be slightly less hardy in comparison to traditionally grown individuals.

In the process of micropropagation, a small piece of plant tissue (the explant) is removed from a healthy, well-maintained mother plant and sterilised in a different

concentration of dilute bleach solution to minimise contamination in the *in vitro* cultures. The source of tissue will vary by species but shoot tip, leaf, stem, lateral bud and flower tissues have been used successfully for various plants. Rhododendron, for example, will produce plantlets from flower petal tissue. In herbaceous plants such as potato, chrysanthemum, carnation, strawberry and African violet, the explant sources are meristems, apical and axillary buds, young seedlings, developing young leaves and petioles and unopened floral buds (Ahloowalia *et al.*, 2004). The sterilised explant is rinsed with sterile water before and after treatment with chemicals and placed in aseptically prepared containers on a specially formulated medium.

The explant may produce shoot proliferating cultures directly by enhanced lateral bud break or the tissue may undergo a certain period of unorganised growth (callus) prior to shoot differentiation. The pattern of growth of the cultures is principally determined by the PGRs content of the tissue culture medium (the auxins and cytokinins concentration). The surviving explants showing growth are maintained in the culture room and subculturing on fresh medium. Most cultures are established within 4 to 12 weeks depending on the species and in some instances, depending on the cultivar. A shoot proliferating culture is one which can be subdivided (subcultured) to produce divisions which will continue rapid multiplication in a large number without losing the genetic stability. Estimates of the rate of multiplication vary and are affected by many factors, but the production of thousands, and in some cases millions, of plants a year from a single explant has been demonstrated through tissue culture technology.

1.1.2 *In Vitro* Flowering

In vitro flowering has been reported in a number of plant species, example, *Dendrocalamus strictus* (Singh *et al.*, 2000), *Gentiana triflora* (Zhang and Leung, 2000, 2002), *Streptocarpus nobilis* (Floh and Handro, 2001), *Pharbitis nil* (Galoch *et al.*, 2002), *Ammi majus* (Pande *et al.*, 2002), *Hypericum brasiliense* (Abreu *et al.*, 2003), *Bambusa edulis* (Lin *et al*., 2003, 2004) and *Psygmorchis pusilla* (Vaz *et al.*, 2004). Important factors for *in vitro* flowering are carbohydrates, growth regulators, light and pH of the culture medium (Heylen and Vendrig, 1988).

Generally, cytokinin is a common requirement for *in vitro* flowering (Scorza, 1982). A number of studies reported the use of cytokinins for *in vitro* flowering in a number of species like *Citrus unshiu* (Garcia-Luis *et al.*, 1989), *Murraya paniculata* (Jumin and Ahmad, 1999), *Fortunella hindsii* (Jumin and Nito, 1996), *Gentiana triflora* (Zhang and Leung, 2000), *Pharbitis nil* (Galoch *et al*., 2002) and *Ammi majus* (Pande *et al*., 2002). An increase in cytokinin concentration has also been reported during *in vivo* flowering of *Xanthium strumarium* (Phillips and Cleland, 1972) where it was shown that cytokinin levels in excretory products from aphids feeding on flowering plants are higher when compared to those from aphids feeding on vegetative plants. Increased levels of cytokinins during flower induction have also been observed in some other species (Bernier, 1988). However, in the present study, 6-Benzylaminopurine (BAP) at any concentration did not show its effect on flowering. This means that all cytokinins do not show their effects on *in vitro* flowering. This observation that different cytokinins have different effects on *in vitro* flowering has also been made by Meeks-Wagner *et al.*, (1989) who showed that in

Nicotiana tabacum, kinetin promoted flower formation, whereas zeatin promoted shoot formation.

Sugar is a necessary carbon source for reliable induction and development of flowers. Addition of sugar to the medium is necessary for induction of floral stimulus. Sucrose is known to be the main carbon source of choice for *in vitro* flower culture studies (Rastogi and Sawhney, 1987). In the present investigation, the effect of different concentrations of sucrose on flower induction was studied by keeping all other parameters constant. Sucrose availability in aerial parts of the plant promotes flowering in *Arabidopsis thaliana* (Roldan *et al*., 1999). Sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* by moving between shoot and root (Havelange *et al.,* 2000).

1.1.3 Synthetic Seeds

Synthetic seeds can be defined as the artificial encapsulation of somatic embryo, shoot buds or aggregates of cell or any tissues which has the ability to form a plant in *in vitro* or *in vivo* condition. Synthetic seeds can be stored for a long time under appropriate condition. Synthetic seeds production and used technology is a rapidly growing branch of seed biotechnology. Toshio Murashige in 1970, first coined the term ‘synthetic seed’. Synthetic seed production requires a large scale production of viable plant material in laboratory condition using *in vitro* culture system. Synthetic seeds are functionally defined as somatic embryos engineered to be of use in commercial plant production (Gray, 1990b). The actual form of synthetic seed (examples, presence or absence of a synthetic seed coat, whether they are hydrated or dehydrated, quiescent or not, others) may vary depending on the specific crop application.

The first reference to the potential use of somatic embryos for propagation is generally credited to Murashige (1978) and efforts to engineer them into synthetic seeds have been ongoing ever since (examples, Gray *et al*., 1984, 1992; Kitto and Janick, 1985a,b,c; Redenbaugh *et al*., 1986, 1987a,b, 1988, 1991, 1993; Fujii *et al*., 1987a,b, 1989; Gray, 1987a, 1990b; Gray and Mortensen, 1987; Stuart *et al*., 1987; Carman, 1989; Janick *et al*., 1989; Kim and Janick, 1989a,b; McKersie *et al*., 1989; Redenbaugh, 1990; Senaratna, 1992; Attree and Fowke, 1993). However, basic developmental mechanisms that contribute to the desirability of seed, such as onset of quiescence and ability to withstand dehydration, are either missing in all but a few embryogenic systems or have been simply overlooked (Gray, 1990b).

Initially, synthetic seeds consisted of propagules produced only from alginate encapsulated somatic embryos (adventitious embryogenic structures from somatic tissue) originating from rapidly multiplying vegetative cell cultures (Standardi and Piccioni, 1998; Murashige, 1978). However, low production rates and quality, as well as the potential for somaclonal variation constrained production to only a few species (Standardi and Piccioni, 1998). As a result, the technology has expanded to include propagules such as shoots, cell aggregates, nodes and any other totipotent (potential to develop into any cell or tissue type or whole organism) tissue (Standardi and Piccioni, 1998). The use of *in vitro*-derived axillary buds and shoot tips provide inexpensive, easily obtained propagules for storage (Danso and Ford-Lloyd, 2003). Nodal segments of eggplant (Huda *et al*., 2007) and potato (Sarkar and Naik, 1998) both relatives of tomato, have been successfully used to produce synthetic seeds.

Synthetic seeds technology has many advantages and applications. Crop, hybrid plants, genetically modified plants can be easily propagated using this technique. Synthetic seeds can also be applied for endangered species to maintain their existence. The technology provides a possible method for germplasm storage, transport and utilisation of clonal plant populations (Ozden-Tokatli *et al*., 2008; Naik and Chand, 2006; Singh *et al*., 2006; Danso and Ford-Lloyd, 2003). Another advantage is ease of handling while in storage, to transport and has potential for long term storage without losing viability. Other than that, it serves as a channel for new plant lines produced through biotechnological advances to be delivered directly to the greenhouse or field. While great strides have been taken in the development of this technology, its potential has yet to be fully recognised and an analog to natural seed has yet to be realised (Kumar *et al*., 2005).

1.1.4 Pigment Extraction

Anthocyanins are a group of reddish blue, water-soluble pigments common in many flowers, fruits and vegetables and they can be included in the category of natural additives (Francis *et al.,* 1989). Anthocyanins (in Greek '*anthos'* means flower, and '*kyanos'* means blue) are the more important plant pigments visible to the human eye and becoming increasingly important not only as food colourants but also as antioxidants. Anthocyanins are involved in attraction of insects and animals for pollination and seed dispersal purposes as they constitute the chemical basis of flower colour in angiosperms (Strack and Wray, 1994; Harborne and Williams, 1995).

The interest of the food industry in natural colorant replacing synthetic dyes has increased significantly over the decades, mainly due to safety issues (Garcia-Falcon *et al.*,

2007). Although anthocyanins are less stable in various environmental conditions, they include varieties of colour such as orange, red, maroon and blue which makes it an attractive alternative as colouring agents in food industries (Markakis, 1982; Francis, 1989). The intensity and stability of the anthocyanin pigments is dependent on various factors including structure and concentration of the pigments, pH, temperature, light intensity, quality and presence of other pigments together, metal ions, enzymes, oxygen, ascorbic acid, sugar and sugar metabolites, sulfur oxide and others (Mazza and Minitiati, 1993; Francis, 1989). Anthocyanins have four different structures, which are in equilibrium and include flavylium cation, quinoidal base, carbinol pseudobase and chalcon. The relative amounts of these structures in equilibrium are varied and depend on the pH and anthocyanin structure (Mazza and Minitiati, 1993).

The most significant function of anthocyanins is their ability to impart colour to the plants or plant products in which they occur. Anthocyanins and 3-deoxyanthocyanidins however have roles in flowering plants other than as attractants. They can act as antioxidants, phytoalexins or as antibacterial agents (Kong *et al*., 2003). Anthocyanins may be important factors along with other flavonoids in the resistance of plants to insect attack (Harborne, 1988). For example, cyanidin 3-glucoside was shown to protect cotton leaves against the tobacco budworm (Hedin *et al*., 1983).

1.1.5 Phytochemical and Antioxidant Activity

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. They are non-essential nutrients, meaning that they are not required by the human body for sustaining life. They can have complementary and overlapping

mechanisms of action in the body, including antioxidant effects, modulation of detoxification enzymes, stimulation of the immune system, modulation of hormone metabolism, and antibacterial and antiviral effect. It is well-known that plants produce these chemicals to protect them but recent researches demonstrate that they can also protect humans against diseases.

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Free radical damage may lead to cancer. The oxidation induced by reactive oxygen species (ROS) can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases (Liao and Yin, 2000). Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS (Halliwell *et al.,* 1995; Sies, 1993), continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng *et al.,* 1997). Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated (Soares *et al.,* 1997).

Antioxidant compounds in food play an important role as a health protecting factor. Antioxidants interact with and stabilise free radicals and may prevent some of the damage free radicals might otherwise cause. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Examples of antioxidants include beta-carotene, lycopene, vitamins C, E, and A and other substances.

1.1.6 Acclimatization

In vitro micropropagation plants require an acclimatization period during the transition from sterile culture container to field or greenhouse conditions. The acclimatization process usually consists of placement in high humidity conditions (under mist or humidifiers) with a gradual decrease of humidity and increase in light intensity over time (Lee *et al.*, 1985). Although some plants have no problem during hardening off, significant losses are often incurred in some species (Wetzstein and Sommer, 1983). High mortality and increased crop time needed for acclimatization economically limit the use of *in vitro* techniques for propagation purposes in some species, particularly woody plants. Acclimatization is the physiological adaptation of a plant to changes in climate or environment, such as light, temperature or altitude.

1.2 GENERAL INTRODUCTION OF *OXALIS*

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Oxalidales
Family	: Oxalidaceae
Genus	: *Oxalis*
Species	: *Oxalis triangularis*
Binomial name	: *Oxalis triangularis*

Oxalis belongs to the family Oxalidaceae and is a large genus with over 800 species widely spread in the tropical but also in temperate regions of both the New and Old World with the exception of Australasia. They are mainly grown as ornamental plants, although the tubers and leaves of some species are used as food and some are considered weeds. Many species come from Southern Africa and Southern America. The family in Southern Africa is represented by two genera: *Oxalis* and *Biophytum*. *Oxalis* comprises about 700 species worldwide with centres of diversity in both Southern Africa and Southern America. About 270 species occur in Southern Africa. The members are widespread but are mainly confined to Namibia and the Western and Eastern Cape in Southern Africa. The genus needs assessment regarding rare and endangered species.

Members of the genus *Oxalis*, with their delicate, brightly coloured flowers, clover-like leaves and corolla lobes twisted in the bud, are mainly native to Southern Africa and

Southern America. Many people know them for their sour-tasting stalks. The name *Oxalis* is derived from the Greek word '*oxys*', meaning sharp, in reference to the sour acid juice of the plants. Oxalate poisoning of livestock may occur where pastures are infested with *Oxalis* because of the accumulation of oxalate in some species. A few Southern American species of *Oxalis* are stem succulents and some Southern African species produce small to medium-sized tubers. Some of these tubers are edible and cultivated for food, and leaves can also be eaten. However, excessive consumption of leaves containing oxalate may lead to loss of bone density. Several species have somewhat fleshy leaves.

Many of the species are known as wood-sorrels (in American English typically written 'woodsorrels' or 'wood sorrels') as they have an acidic taste reminiscent of the unrelated Sorrel (*Rumex acetosa*) proper. Some species are called yellow-sorrels or pink-sorrels after the color of their flowers instead. Other species are colloquially known as false shamrocks and some are rather misleadingly called "sourgrasses". For the genus as a whole, the term 'Oxalises' is also used.

Most of the cultivated kinds are tropical herbs used as window plants. The leaves are usually clover-like and respond to darkness with 'sleep' movements by folding back their leaflets. Several species grow wild in North America, including the white wood sorrel (*Oxalis acetosella*) widely distributed in the North Temperate Zone and one of the plants identified as the shamrock. This and, to a lesser extent, other species have long been used for salads and greens because of their pleasantly acidic taste; these species contain oxalic acid. *Oxalis tuberosa* has a starchy tuber that has been valued in the high Andes for centuries. Although species of *Oxalis* are called sorrels, the genus is unrelated to the true

sorrel or dock (genus *Rumex*) of the buckwheat family. *Oxalis* is classified in the division Magnoliophyta, class Magnoliopsida, order Geramales and family Oxalidaceae.

Oxalis, established by Linnaeus in 1753, is characterised by stemless (acaulescent) or stemmed (caulescent) herbs or subshrubs with corms. This genus is the only one of the dicotyledonous genera to produce corms (tuberous, bulb-like rootstocks). The species are perennials and low-growing, reaching heights varying from 0.05 m to 0.3 m.

The leaves, borne in basal rosettes, are also characteristic of the genus. The leaves of Oxalidaceae are alternate with no stipules (small leafy growths at the leaf nodes), simple or compound and usually trifoliolate (having three leaflets) with leaflets varying from more or less heart-shaped (broadly obovate and cuneate at the base) to narrowly obovate and predominantly stalked (petiolate). Petioles are long and slender. Occasionally the leaves are sessile (without a stalk) or subsessile. The leaflets are often flushed with purple below and maybe hairy or have calli (thickenings). In some species, the leaflets display sleep movements, drooping or folding at night or in cold weather. Some species exhibit rapid changes in leaf angle in response to temporarily high light intensity to decrease photo-inhibition.

Flowers are solitary or in umbel-like clusters on long, erect peduncles (stalks). They are radially symmetric (actinomorphic) with five free sepals (calyx) and a tubular corolla with five lobes (petals), which are sometimes joined at the base and sometimes separate. In some species the tube is long and slender, whereas in others it is rather short or either narrowly or broadly funnel-shaped. Similar to the leaves, the flowers close at night and in

dull weather. The lobes of the corolla are prominently twisted in the bud, revealing delicate, glossy, brightly coloured petals as the corolla opens. White, rose-pink, pink, red, shades of purple and yellow are colours observed in the genus. *Oxalis* is also characterised by obovate (inversely ovate), broadly obovate or suborbicular (almost with a ± circular outline), clawed corolla lobes.

Ten stamens, in two series, five long and five short, united at the base, and often golden yellow, are characteristic of *Oxalis*. Pollen grains produced in the anthers display different types. These pollen types proved to be significant in distinguishing different sections and species in the genus. The ovary is superior, 5-locular with five free styles. The fruit of *Oxalis* is a 5-lobed capsule, much longer than broad, with nearly parallel sides.

The seedpod forms inside the flower (a superior ovary). The ovary develops into a 5-chambered fruit which is usually a capsule but may be a berry, each containing several seeds. Some species have explosively dehiscent fruits as a very effective mechanism for seed dispersal. In cold conditions, the European species *Oxalis acetosella* (Wood Sorrel) may be cleistogamous (can produce viable seeds without opening). The seeds of some species have a fleshy aril at the base. The seeds, one too many, are explosively ejected. To disperse the seeds, the inner cells of the aril turn inside out suddenly and separate from the seed coat, and the seeds are catapulted from the plant. Because of this, some members of the Oxalidaceae are invasive weeds and may disperse themselves through the greenhouse with volunteer seedlings coming up in every pot. A non-succulent species *Oxalis corniculata* (creeping wood sorrel) with small yellow flowers will be familiar to many cactus collectors and is difficult to eradicate.

Members of the genus are propagated by their corms and seed. There are species to suit any situation; good or bad soil, full sunshine or complete shade or semi-shade, and they grow well provided they get enough water. The plants spread rapidly and can be used as seasonal ground covers, but containers keep growth in check and prevent plants from becoming weedy. Many of the species are dormant in the dry season. The genus is not known to be subjected to pests or diseases in the garden, but the only reason for this may be that it has not yet been significantly cultivated (Du Plessis and Duncan, 1989).

Some Southern African species of *Oxalis* are grown as ornamentals, for example, *Oxalis flava, Oxalis lanata* and *Oxalis purpurea*. However, the horticultural potential of these delicate plants still needs to be explored.

1.2.1 Medicinal Uses of *Oxalis*

Van Wyk *et al*., (2002) reported that the *Oxalis* species, for example, *Oxalis pes-carpae* and other plants containing soluble oxalic acid which are not really poisonous but they can lead to human and animal fatalities if excessive amounts are consumed. In Southern Africa (as in many other parts of the world), outbreaks of oxalate poisoning have been reported, resulting from pastures infested with *Oxalis* or *Rumex* species. Under field conditions, sheep are mostly affected but cattle and horses may suffer from degenerative conditions of the bones after prolonged exposure to low levels of oxalates. *Oxalis pes-caprae* occurs mainly in the Western Cape and has become a troublesome weed in many parts of the world. However, the sour leaves of *Oxalis pes-caprae* are an essential ingredient of waterblommetjie stew and other stews (Van Wyk and Gericke, 2000).

Tuberous woodsorrels provide food for certain small herbivores, such as the Montezuma Quail (*Cyrtonyx montezumae*), though the oxalic acid content probably makes the plants toxic to many mammals. The foliage is eaten by some Lepidoptera, such as the Dark Grass Blue (*Zizeeria lysimon*) and Polyommatini Pale Grass Blue (*Pseudozizeeria maha*) which feeds on Creeping Woodsorrel and others.

The edible tubers of the Oca (*Oxalis tuberosa*), somewhat similar to a small potato, have long been cultivated for food in Colombia and elsewhere in the Northern Andes Mountains of South America. The leaves of Scurvy-grass Sorrel (*Oxalis enneaphylla*) were eaten by sailors travelling around Patagonia as a source of vitamin C to avoid scurvy.

In the past, it was a practice to extract crystals of calcium oxalate for use in treating diseases and as a salt called *sal acetosella* or "sorrel salt" (also known as "salt of lemon"). Growing Oca tuber root caps are covered in fluorescent slush rich in harmaline and harmine and apparently suppress pests; this phenomenon has been studied to some extent at the Colorado State University. Creeping Wood-sorrel and perhaps other species are apparently hyperaccumulators of copper. The Ming Dynasty text *Precious Secrets of the Realm of the King of Xin* from 1421 describes how *Oxalis corniculata* can be used to locate copper deposits as well as for geobotanical prospecting. Thus, it ought to have some potential for phytoremediation of contaminated soils.

1.2.2 *Oxalis triangularis*

Figure 1.1: Intact plant of *Oxalis triangularis.*

Oxalis triangularis (A.St.-Hil) or commonly known as 'Pokok Rama-rama' in Malaysia is one of the prettiest ornamental varieties. The leaves are large and purple-darker toward the edges, more vibrant in the centers. Each of the three segments is the shape of a triangle. The plant is thus called *Oxalis triangularis*, as an obvious reference to the three-sided leaves that are borne in clusters of three and held at tip of slender succulent petioles that can arch downwards due to the sheer weight of the leaves. The velvety heart-shaped trifoliate leaves will fold down from sunset till the morning light (response to the darkness). The pale pink flowers are also larger than those of the medicinal types and they are held higher above the foliage.

The leaves contain oxalic acid, which gives them their sharp flavor. Any remedies made out of *Oxalis triangularis*, should be taken in moderation. The leaves should not be

eaten in large amounts since oxalic acid can bind up the body's supply of calcium leading to nutritional deficiency. Oxalic acid can aggravate conditions such as arthritis, gout and kidney stones.

Oxalis triangularis is a low growing clump-forming herbaceous plant that grows to a height of 0.1 - 0.2 m with the spread of 0.1 - 0.3 m. the plant propagated by division of vegetative offsets from matured clumps or by rhizomes (bulbs). The plant grows to a height of less than one foot making it a perfect container plant, ground cover, or under planting for taller plants. It is easily grown in a sandy soil in a warm dry position in the sun or light shade (Chittendon, 1956; Huxley, 1992). It also grows well in a dry shady corner (Phillips and Rix, 1998).

The best way of cultivating *Oxalis triangularis* is in pots or containers. A spot in bright shade or part sun is best, preferably with morning sun and afternoon shade. *Oxalis triangularis* can tolerate shade, but produces best foliage colour and flowering in bright indirect light. The plant prefers acid, neutral and alkaline soils and regular watering moderately in active growing.

1.3 RESEARCH OBJECTIVES

1) To identify the optimum medium for *in vitro* regeneration of *Oxalis triangularis* and to establish an efficient regeneration system of this species for production on a large scale basis through tissue culture method. Hence, to compare the ultrastructure changes of this species grown *in vivo* and *in vitro* using Scanning Electron Microscopy.

2) To induce *in vitro* flowering of *Oxalis triangularis* by manipulation of various combinations and concentrations of plant growth regulators.

3) To investigate the production of synthetic seeds from *in vitro* microshoots for mass propagation.

4) To analyse and compare extraction of anthocyanins from this species grown *in vivo* and *in vitro* and to screen phytochemical constituents presence in *Oxalis triangularis*.

5) To evaluate the suitability of *Oxalis triangularis* plantlets on different substrates for acclimatization process.

CHAPTER 2

IN VITRO PLANT REGENERATION OF *Oxalis triangularis*

2.1 EXPERIMENTAL AIMS

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of intact plants. The Murashige and Skoog (MS) salts compositions are the most widely used especially in plant regeneration procedures. The culture medium consisted of MS supplemented with vitamins and different types and concentrations of plant growth regulators (PGRs). PGRs were used to induce adventitious shoots, so that comparison between MS without hormone and MS supplemented with PGRs can be observed. *In vitro* plant regeneration was also conducted to identify which part of explant from mother plant is more effective to germinate into complete plantlets through tissue culture technique. The studies were also carried out using scanning electron microscopy (SEM). Petiole and leaf from intact plants (*in vivo*) and explants grown *in vitro* were viewed using scanning electron microscope. The aim of this study is to observe the morphological differences between both samples obtained from *in vivo* and *in vitro*.

2.2 MATERIALS AND METHODS

2.2.1 Source of Explants

Six-month-old *Oxalis triangularis* which were grown from bulbs in pots with commercial soil in the greenhouse of the botanical garden of Institute Biological of Sciences, University of Malaya, Kuala Lumpur were used. Petiole and leaf explants of *Oxalis triangularis* were used as starting materials for regeneration studies. 30 replicates of samples were used.

2.2.2 Sterilization of Explants

All explants used must be surface sterilised before they can be cultured on MS medium. These shoots were removed and slightly washed with Dettol to clean any dirt found in the shoots before being placed under running tap water for 15 minutes. Subsequently, these shoots were washed in descending concentrations of diluted commercial bleach (chlorox) containing a few drops of Tween 20 followed by three rinses (for each concentration) in distilled water. A few drops of Tween 20 were only used in the first diluted commercial bleach only. Rinses with chlorox as shown below:-

a) Rinse with 70% of chlorox and a few drops of Tween 20 (3 times) followed by distilled water (3 times).
b) Rinse with 50% of chlorox (3 times) followed by distilled water (3 times).
c) Rinse with 30% of chlorox (3 times) followed by distilled water (3 times).
d) Rinse with 10% of chlorox (3 times) followed by distilled water (3 times).

For the last step of surface sterilized these shoots were transferred to the laminar flow chamber to prevent air contamination and maintained the aseptic conditions. They were then immersed in 70% ethanol for 1 minute followed, by three rinses in sterile distilled water. These shoots were dried for about 30 minutes before being cut into smaller pieces (≈ 1 cm).

2.2.3 Preparation of Culture Media

One of the most important factors governing the growth and morphogenesis of plant tissue culture is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. The Murashige and Skoog (MS) salts compositions are the most widely used especially in plant regeneration procedures. The culture medium consisted of MS supplemented with vitamins and different types and concentration of PGRs. Here is the basic of media preparation used in most of the experiments in this study, the difference is only the addition of PGRs which may vary for each objective of the study.

Conical flask was added with 700 ml distilled water. Magnetic stirrer was placed in the flask and 4.4 g of MS including vitamin was added. Next, 30 g of sucrose was added and the pH of the medium was adjusted to 5.8 using 1.0 N Hydrochloric acid (HCl) or 1.0 N Sodium hydroxide (NaOH). Gelrite weighed 36 g was added as a solidifying agent to provide surface and support for *in vitro* growing explants before autoclaving to sterilize the medium. Lastly, the volume of the medium then was filled up with distilled water to 1 litre using a graduated cylinder. Mixture was stirred to dissolve completely. For the medium with PGRs, hormone(s) was added after the pH was adjusted to 5.8. The autoclaving

condition was usually 121°C (15 psi) for 20 minutes. After autoclaved, MS medium were dispensed into the sterile containers. The medium was allowed to cool prior to use. Below are the combinations and concentrations of hormones which were used for plant regeneration study.

Table 2.1: List of combinations and concentrations of hormones for plant regeneration of *Oxalis triangularis*.

No.	Combinations and concentrations of hormones
1	MS basal (without hormone)
2	MS + 0.5 mg/l NAA + 0.5 mg/l BAP
3	MS + 0.5 mg/l NAA + 1.0 mg/l BAP
4	MS + 0.5 mg/l NAA + 1.5 mg/l BAP
5	MS + 0.5 mg/l NAA + 2.0 mg/l BAP
6	MS + 1.0 mg/l NAA + 0.5 mg/l BAP
7	MS + 1.0 mg/l NAA + 1.0 mg/l BAP
8	MS + 1.0 mg/l NAA + 1.5 mg/l BAP
9	MS + 1.0 mg/l NAA + 2.0 mg/l BAP
10	MS + 1.5 mg/l NAA + 0.5 mg/l BAP
11	MS + 1.5 mg/l NAA + 1.0 mg/l BAP
12	MS + 1.5 mg/l NAA + 1.5 mg/l BAP
13	MS + 1.5 mg/l NAA + 2.0 mg/l BAP
14	MS + 2.0 mg/l NAA + 0.5 mg/l BAP
15	MS + 2.0 mg/l NAA + 1.0 mg/l BAP

'**Table 2.1**, continued'

No.	Combinations and concentrations of hormones
16	MS + 2.0 mg/l NAA + 1.5 mg/l BAP
17	MS + 2.0 mg/l NAA + 2.0 mg/l BAP
18	MS + 1.0 mg/l NAA + 1.5 mg/l KIN
19	MS + 1.0 mg/l IBA + 1.5 mg/l KIN
20	MS + 1.0 mg/l IBA + 1.5 mg/l BAP
21	MS + 1.0 mg/l IAA + 1.5 mg/l TDZ
22	MS + 1.0 mg/l 2,4-D + 1.5 mg/l 2iP
23	MS + 0.5 mg/l NAA + 1.0 mg/l KIN
24	MS + 0.5 mg/l IBA + 1.0 mg/l KIN
25	MS + 0.5 mg/l IBA + 1.0 mg/l BAP
26	MS + 0.5 mg/l IAA + 1.0 mg/l TDZ
27	MS + 0.5 mg/l 2,4-D + 1.0 mg/l 2iP

2.2.4 Culture Conditions

All the tissue culture equipment such as a scalpel and forceps must be in aseptic conditions to prevent any contamination. These culture apparatus were autoclaved for 20 minutes. UV light and hot bead steriliser was turned on for at least 30 minutes prior to use. All laminar flow surfaces were wiped with 70% ethanol before and after use to ensure the sterility inside the chamber.

2.2.5 Subculture

All the aseptic cultures were maintained in the culture room (16 hours light and 8 hours dark photoperiod and the temperature was maintained at 25 ± 1°C). The plantlets obtained were subcultured on the same medium at 2 months interval to maintain supply of fresh nutrient medium.

2.2.6 Induction of Plant Regeneration *In Vitro*

Explants were cut using a scalpel into a small size (≈ 1 cm), both for petiole and leaf explants. Size of explants must be uniform. Explants were then cultured on the MS medium supplemented with PGRs as mention in Table 2.1. The *in vitro* adventitious shoots of *Oxalis triangularis* were observed for 8 weeks.

2.2.6.1 Experimental Outline

Surface sterilised of intact plants (shoots).

↓

Excision of explants into smaller sizes (≈ 1 cm).

↓

Cultured onto MS medium supplemented with PGRs.

↓

Maintained in the culture room.

↓

Regenerated plantlets were subcultured on the same medium at 2 months interval.

2.2.7 Microscopic studies (Scanning Electron Microscopy, SEM)

Petiole and leaf samples were utilised to observe the differences between *in vivo* and *in vitro* grown plants. Both explants taken were of similar developmental stages. Observations and comparisons were made on the differences of stomata and trichomes on both petiole and leaf samples.

2.2.7.1 Preparation for SEM examination

Both *in vitro* and *in vivo* petiole and leaf explants of *Oxalis triangularis* were fixed in 4% glutaraldehyde in buffer, minimum of 4 hours and washed in buffer. After that, both explants were fixed in 1% osmium tetroxide (O_sO_4) overnight, then, washed with distilled water and dehydrated in ascending series of ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100%). Followed by dehydration in mixture of ethanol and acetone with the ratio of:-

Ethanol : Acetone

a) 3 : 1

b) 1 : 1

c) 1 : 3

Samples were then dehydrated in pure acetone before being dried in carbon dioxide using critical point drying (CPD). After finishing with CPD, samples were mounted on stubs with carbon adhesive cement or double-sided adhesive tape and coated with gold prior viewing by using Scanning Electron Microscope.

2.2.7.2 Experimental Outline

(Using 2 fixatives)

Fixed in 4% glutaraldehyde in buffer minimum 4 hours.

↓

Washed in buffer twice (10 minutes each).

↓

Fixed in 1% OsO_4 (Aqueous or in buffer) 1 hour.

↓

Washed in distilled water twice (10 minutes each)

(wash and store in buffer if not continuing processing).

↓

(Dehydration)

Dehydrated in ascending series of ethanol;

30%, 50%, 70%, 80%, 90% and 95% (15 minutes each).

↓

Dehydrated in 100% ethanol twice (15 minutes each).

↓

Dehydrated in ethanol : acetone mixture

ethanol : acetone

3 : 1 (15 minutes)

1 : 1 (15 mininutes)

1 : 3 (15 minutes)

↓

Pure acetone, 3 times (20 minutes each).

↓

Critical point drying (CPD) process 1 hour.

↓

Mounted on stubs with carbon adhesive cement or double-sided adhesive tape.

↓

Coat with gold/carbon in coater.

↓

View by using Scanning Electron Microsope.

2.2.8 Data Analysis

Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with different letters in the same column differ significantly at p = 0.05.

2.3 RESULTS

2.3.1 Induction of Plant Regeneration *In Vitro*

In this study to raise stock culture, explants for induction of plant regeneration were collected from healthy intact plants which were grown from bulbs in pots with commercial soil in the greenhouse. Subsequently, petiole and leaf explants were taken from four-month-old sterile mother plant to induce multiple shoots plantlets. Both explants, petiole and leaf were able to produce multiple shoots in solid medium, while root explants did not have any response after 8 weeks being cultured and even after being subcultured into fresh medium. Each shoot produced one set of leaves which consist of three leaves (trifoliate leaves).

Most explants started to emerge roots within 2 weeks and started to germinate within 3 weeks from culture initiation. Initially, 3 or 4 shoots developed, later up to 14 shoots were formed on petiole and leaf explants in 8 weeks. Shoots started to produce leaves after 6 weeks of culture. Figure 2.1 showed roots formation of *Oxalis triangularis* after 8 weeks from leaf explant on MS supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP. Figure 2.2 showed (a) Petiole explants of *Oxalis triangularis* cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP. (b) Leaf explants of *Oxalis triangularis* cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP showing roots formation. Figure 2.3 showed petiole and leaf explants started to produce roots within 2 weeks of culture on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP.

Based on Table 2.2 and Table 2.3, all combinations of hormones could induce *in vitro* plant regeneration from petiole and leaf explants by producing adventitious shoots with different percentages. Figure 2.4 showed formation of adventitious shoots of *Oxalis triangularis* after 4 weeks being cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP from leaf explant. The highest frequency (100%) formation of maximum number of adventitious shoots for leaf explants was observed in MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP, while for petiole explants in MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l KIN. Maximum number of shoots proliferation occurred on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP from leaf explants. Figure 2.5 showed adventitious shoots of *Oxalis triangularis* after 8 weeks on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP from leaf explant. Compared to explants that were cultured on MS without hormone, which served as a control, addition of PGRs showed promising results in inducing complete *in vitro* plant regeneration.

Table 2.2: Effect of different concentrations and combinations of hormones on petiole explants of *Oxalis triangularis* cultured on MS medium at 25 ± 1°C under 16 hours light and 8 hours dark photoperiod.

MS + Hormones		**Shoot formation (%)**	**No. of shoots per explant Mean ± SE**	**Root formation (%)**	**Observations**
NAA (mg/l)	**BAP (mg/l)**				
0	0	100.00 ± 0.00 a	3.83 ± 0.33 a	100.00 ± 0.00 a	All explants started to emerge roots within 2 weeks of cultured and adventitious shoots within 3 weeks of cultured. No formation of callus was observed.
0.5	0.5	100.00 ± 0.00 a	9.90 ± 0.48 fgh	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	11.03 ± 0.47 hi	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	4.43 ± 0.56 ab	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	4.53 ± 0.52 ab	100.00 ± 0.00 a	
1.0	0.5	100.00 ± 0.00 a	5.27 ± 0.53 abc	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	9.57 ± 3.78 fgh	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	7.90 ± 0.56 def	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	5.03 ± 0.70 abc	100.00 ± 0.00 a	
1.5	0.5	100.00 ± 0.00 a	6.20 ± 1.07 bcd	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	10.13 ± 0.54 ghi	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	6.23 ± 0.91 bcd	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	8.60 ± 0.74 efg	100.00 ± 0.00 a	

'**Table 2.2**, continued'

MS + Hormones		Shoot formation (%)	No. of shoots per explant Mean ± SE	Root formation (%)	Observations
NAA (mg/l)	**BAP (mg/l)**				
2.0	0.5	100.00 ± 0.00 a	6.83 ± 0.59 cde	100.00 ± 0.00 a	All explants started to produce roots within 2 weeks of culture and adventitious shoots within 3 weeks of cultured. No formation of callus was observed.
	1.0	100.00 ± 0.00 a	8.97 ± 0.70 fgh	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	5.07 ± 0.72 abc	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	6.37 ± 0.74 bcd	100.00 ± 0.00 a	
0.5 NAA + 1.0 KIN		100.00 ± 0.00 a	12.07 ± 0.64 i	100.00 ± 0.00 a	
0.5 IBA + 1.0 KIN		100.00 ± 0.00 a	9.17 ± 0.86 fgh	100.00 ± 0.00 a	
0.5 IBA + 1.0 BAP		100.00 ± 0.00 a	5.73 ± 0.73 abc	100.00 ± 0.00 a	
0.5 IAA + 1.0 TDZ		100.00 ± 0.00 a	8.30 ± 0.62 defg	100.00 ± 0.00 a	
0.5 2,4-D + 1.0 2iP		100.00 ± 0.00 a	11.03 ± 0.87 hi	100.00 ± 0.00 a	

Each value represents the mean ± SE of 30 replicates.
The mean with different letters in the same column differ significantly at 0.05 level.

Table 2.3: Effect of different concentrations and combinations of hormones on leaf explants of *Oxalis triangularis* cultured on MS medium at 25 ± 1 °C under 16 hours light and 8 hours dark photoperiods.

MS + Hormones		Shoot formation (%)	No. of shoots per explant Mean ± SE	Root formation (%)	Observations
NAA (mg/l)	BAP (mg/l)				
0	0	100.00 ± 0.00 a	3.07 ± 0.30 a	100.00 ± 0.00 a	All explants started to produce roots within 2 weeks of culture and adventitious shoots within 3 weeks of cultured. No formation of callus was observed.
0.5	0.5	100.00 ± 0.00 a	12.63 ± 0.71 h	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	7.70 ± 0.76 def	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	10.53 ± 0.81 g	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	9.37 ± 0.54 fg	100.00 ± 0.00 a	
1.0	0.5	100.00 ± 0.00 a	8.70 ± 0.66 efg	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	7.67 ± 0.63 def	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	14.20 ± 0.84 h	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	8.57 ± 0.66 efg	100.00 ± 0.00 a	
1.5	0.5	100.00 ± 0.00 a	10.17 ± 0.65 g	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	5.77 ± 0.89 bcd	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	6.67 ± 0.73 cde	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	8.53 ± 0.70 efg	100.00 ± 0.00 a	
2.0	0.5	100.00 ± 0.00 a	5.77 ± 0.66 bcd	100.00 ± 0.00 a	
	1.0	100.00 ± 0.00 a	7.03 ± 0.69 cde	100.00 ± 0.00 a	
	1.5	100.00 ± 0.00 a	4.10 ± 0.68 ab	100.00 ± 0.00 a	
	2.0	100.00 ± 0.00 a	5.03 ± 0.59 abc	100.00 ± 0.00 a	

'**Table 2.3**, continued'

MS + Hormones	Shoot formation (%)	No. of shoots per explant Mean ± SE	Root formation (%)	Observations
1.0 NAA + 1.5 KIN	100.00 ± 0.00 a	7.87 ± 0.70 def	100.00 ± 0.00 a	All explants started to produce roots within 2 weeks of culture and adventitious shoots within 3 weeks of cultured. No formation of callus was observed.
1.0 IBA + 1.5 KIN	100.00 ± 0.00 a	5.77 ± 0.91 bcd	100.00 ± 0.00 a	
1.0 IBA + 1.5 BAP	100.00 ± 0.00 a	6.93 ± 1.26 cde	100.00 ± 0.00 a	
1.0 IAA + 1.5 TDZ	100.00 ± 0.00 a	3.63 ± 0.56 ab	100.00 ± 0.00 a	
1.0 2,4-D + 1.5 2iP	100.00 ± 0.00 a	3.37 ± 0.43 a	100.00 ± 0.00 a	

Each value represents the mean ± SE of 30 replicates.
The mean with different letters in the same column differ significantly at 0.05 level.

Figure 2.1: Roots formation of *Oxalis triangularis* after 8 weeks from leaf explant on MS supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP.

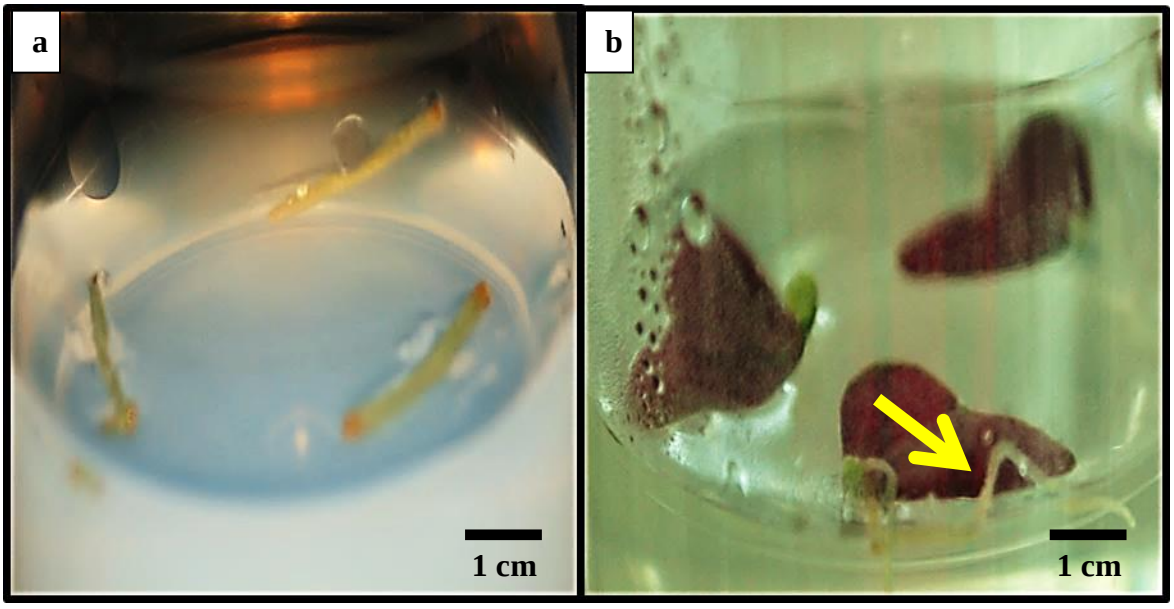


Figure 2.2:a) Petiole explants of *Oxalis triangularis* cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP.

b) Leaf explants of *Oxalis triangularis* cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP showing roots formation.

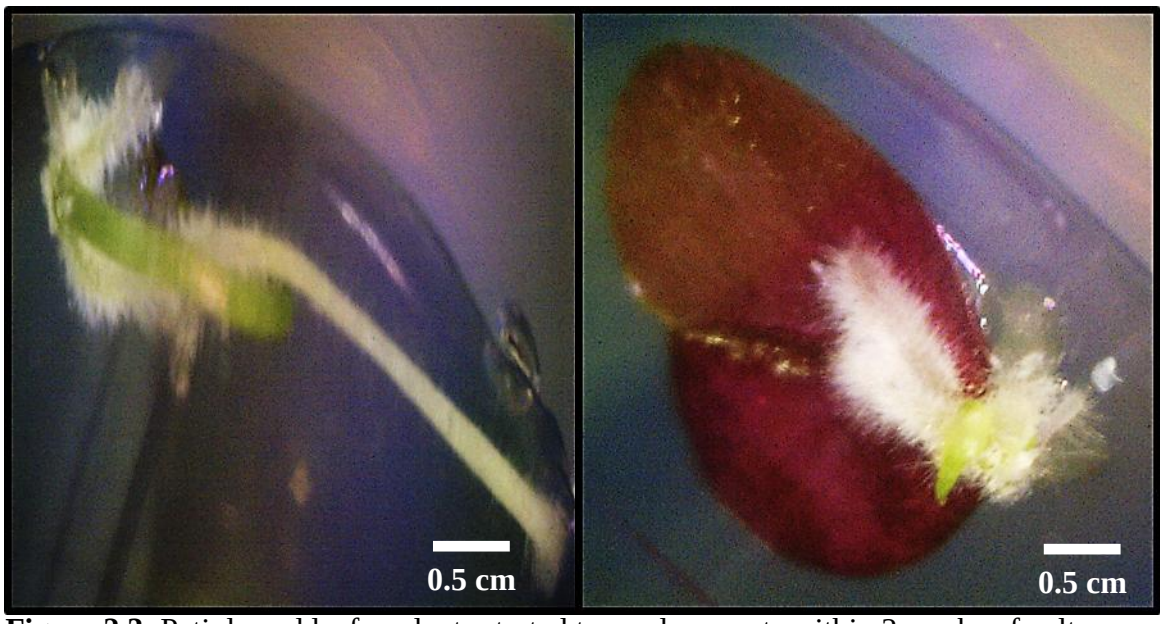


Figure 2.3: Petiole and leaf explants started to produce roots within 2 weeks of culture on on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP.

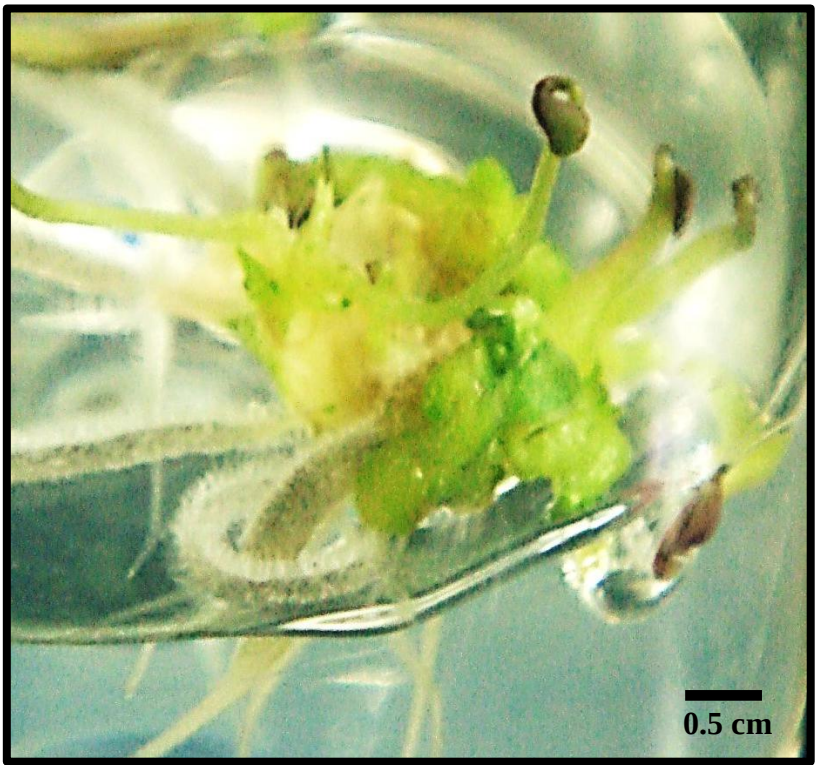


Figure 2.4: Formation of adventitious shoots of *Oxalis triangularis* after 4 weeks being cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP from leaf explant.

Figure 2.5: Adventitious shoots of *Oxalis triangularis* after 8 weeks on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP from leaf explant.

2.3.2 Microscopic Studies (Scanning Electron Microscopy, SEM)

Structures comparisons between petiole samples of *in vivo* grown plants and explants grown *in vitro* of *Oxalis triangularis* were shown in Figure 2.7(a) until Figure 2.7(h). While structures comparisons between leaf samples of *in vivo* grown plants and explants grown *in vitro* of *Oxalis triangularis* were shown in Figure 2.8(a) until Figure 2.8(h). Both, *in vivo* and *in vitro* petiole grown plants of *Oxalis triangularis* contained two types of trichomes which are short trichome and long trichome (Figure 2.6 (e and f) and Figure 2.7 (b and c)).

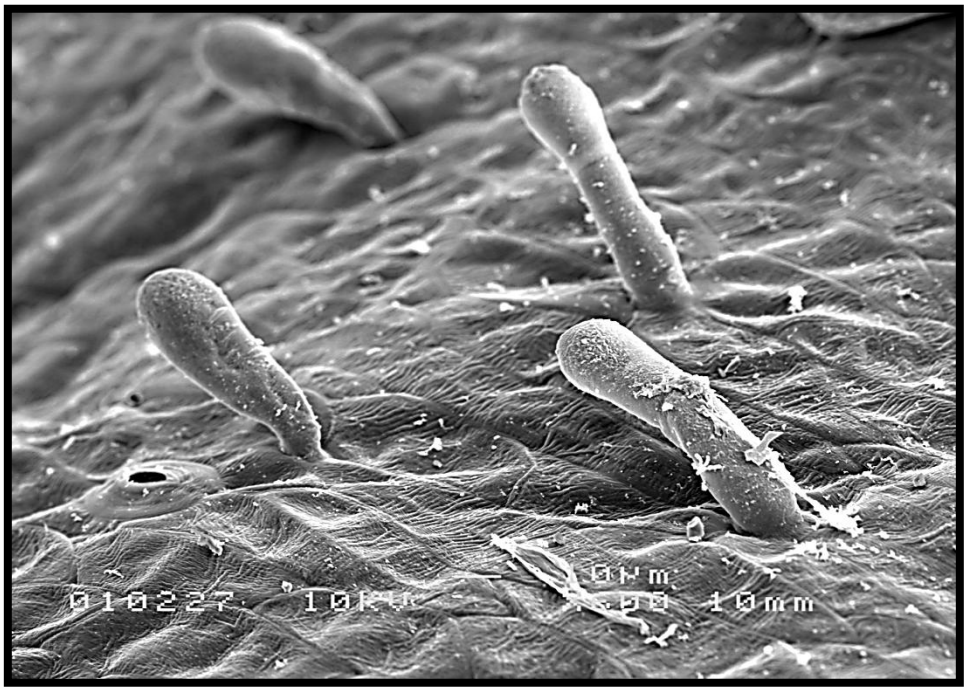


Figure 2.6(a): SEM micrograph showing *in vitro* petiole explant of *Oxalis triangularis* at 600x magnification. Short trichomes were seen on the petiole.

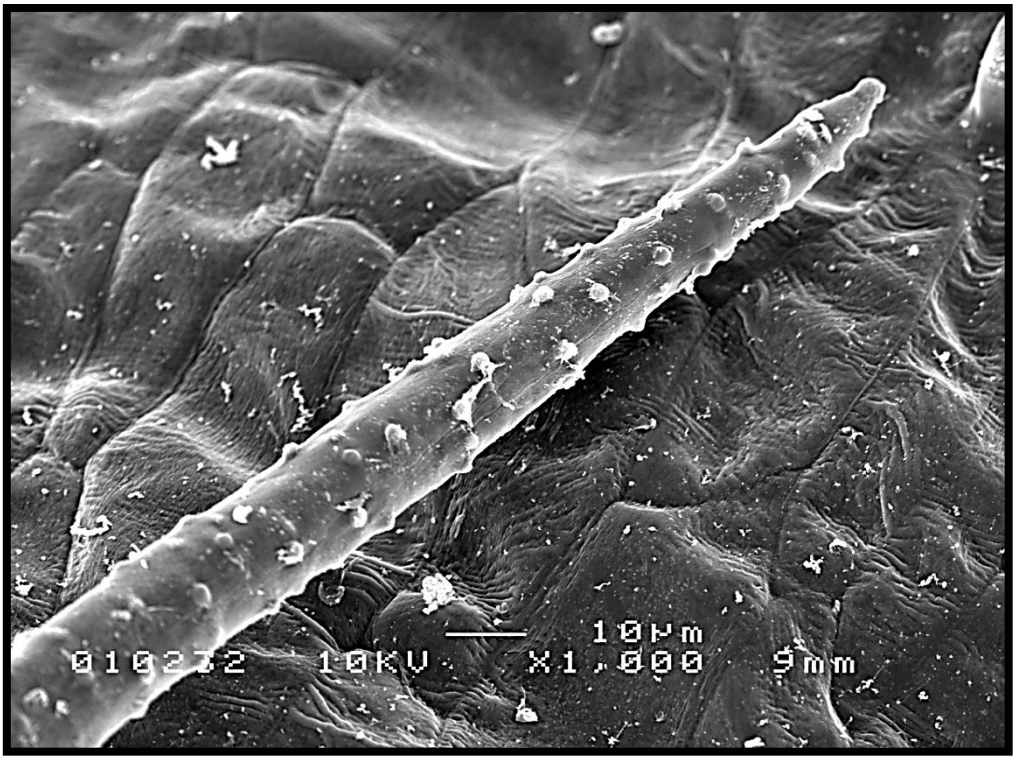


Figure 2.6(b): SEM micrograph showing *in vitro* petiole explant of *Oxalis triangularis* at 1000x magnification. Long trichome was seen on the petiole.

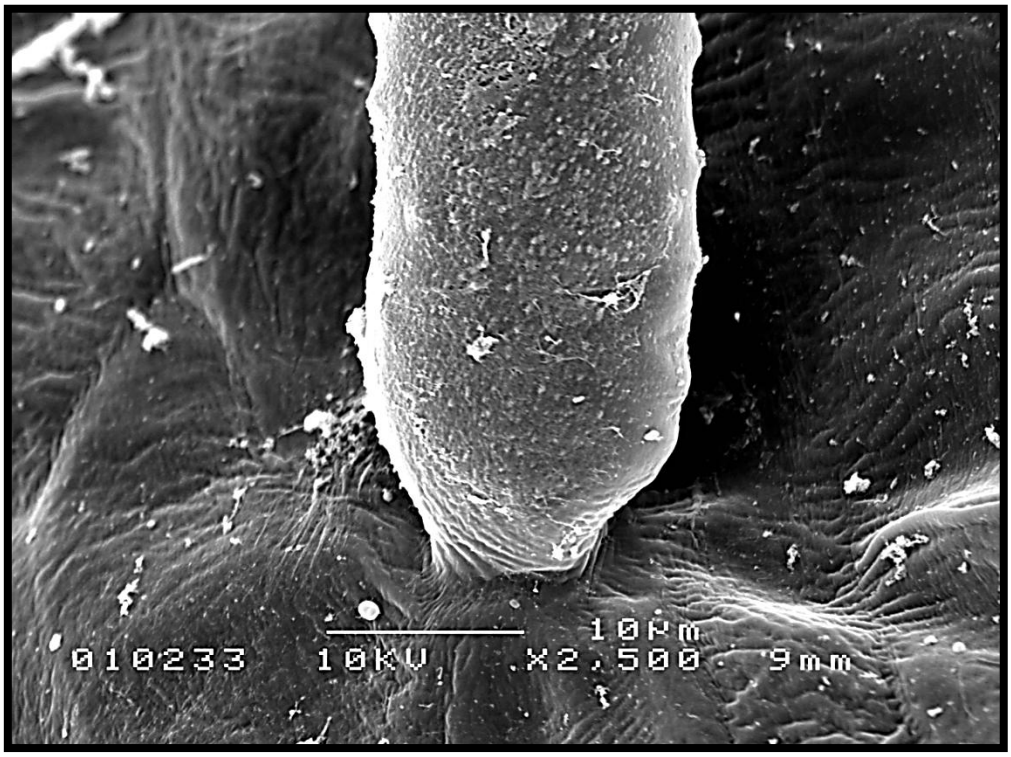


Figure 2.6(c): SEM micrograph showing *in vitro* petiole explant of *Oxalis triangularis* at 2500x magnification. Attachment of trichome with the surface of cell was seen on the petiole.

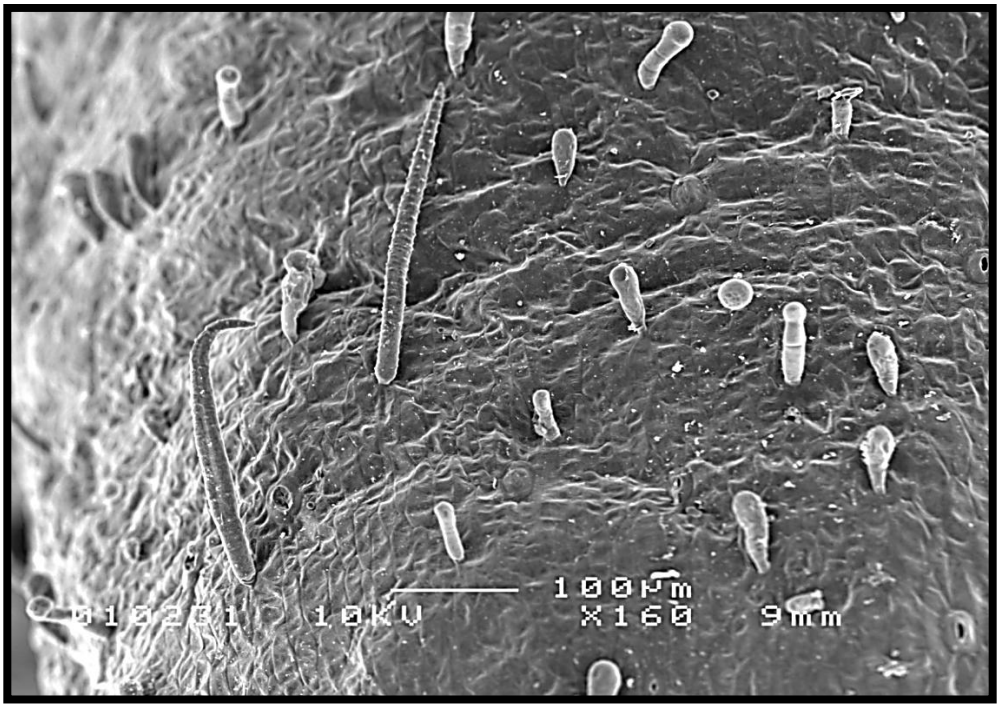


Figure 2.6(d): SEM micrograph showing *in vitro* petiole explant of *Oxalis triangularis* at 160x magnification. Distributions of both long and short trichomes were seen on petiole.

Figure 2.6(e): SEM micrograph showing petiole of *in vivo* plant of *Oxalis triangularis* at 1000x magnification. Short trichome was seen on the petiole.

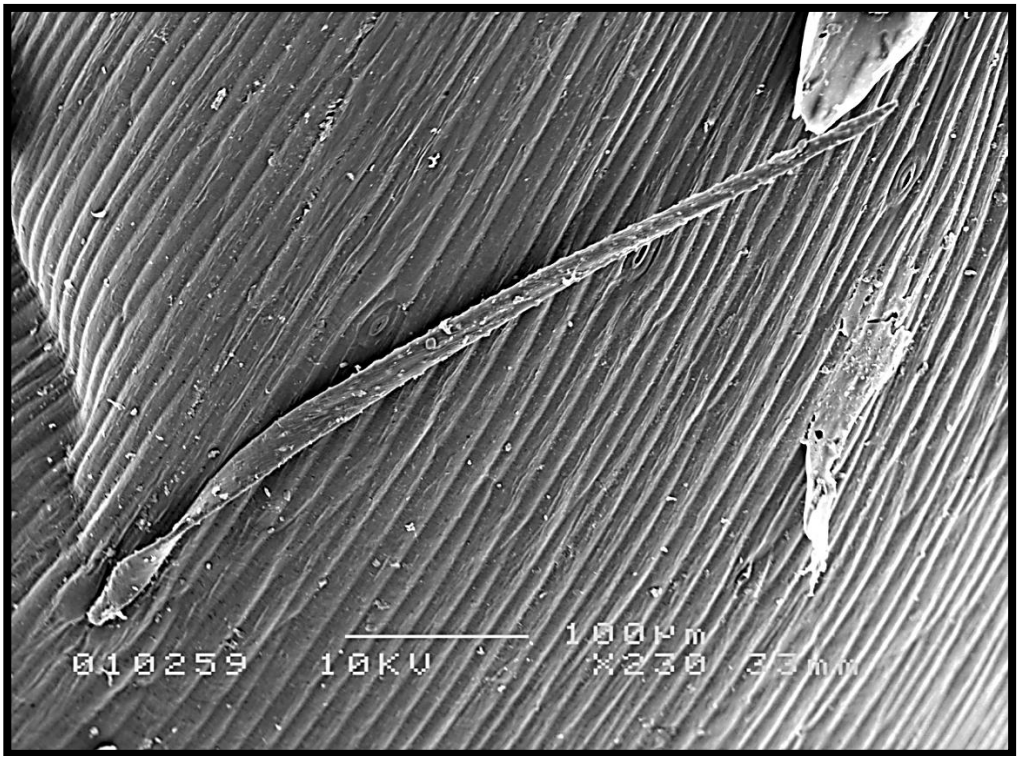


Figure 2.6(f): SEM micrograph showing petiole of *in vivo* plant of *Oxalis triangularis* at 230x magnification. Long trichome was seen on the petiole.

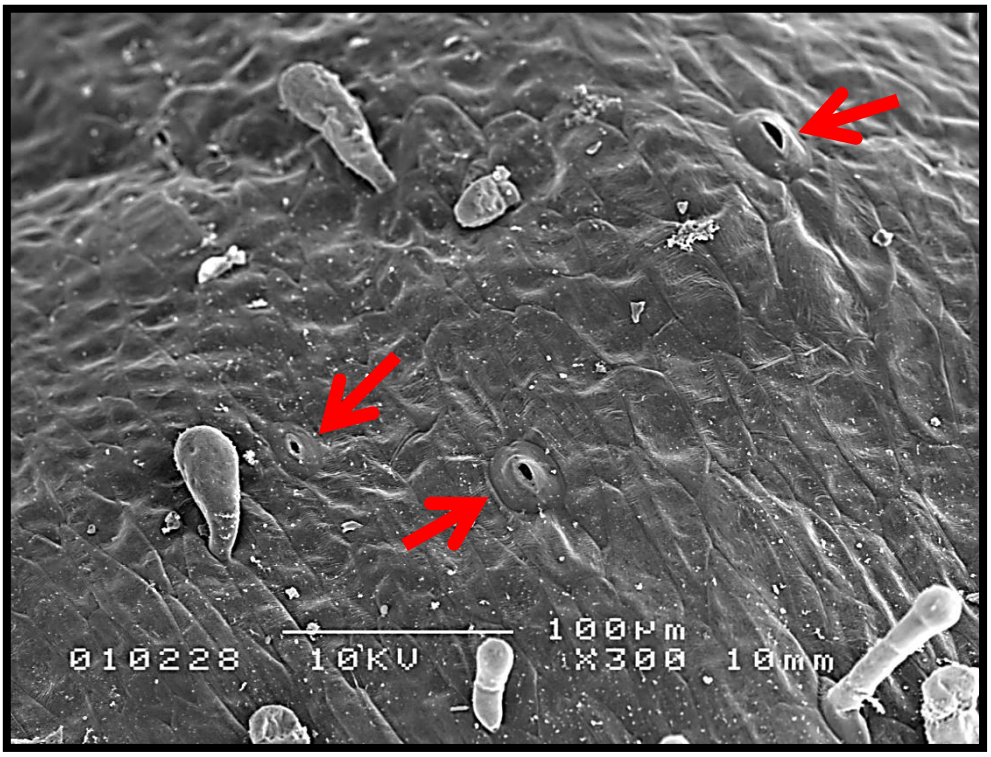


Figure 2.6(g): SEM micrograph showing *in vitro* petiole explant of *Oxalis triangularis* at 300x magnification. Stomata were seen on the petiole.

Figure 2.6(h): SEM micrograph showing *in vitro* petiole explant of *Oxalis triangularis* at 1000x magnification. Stoma was clearly seen on the petiole.

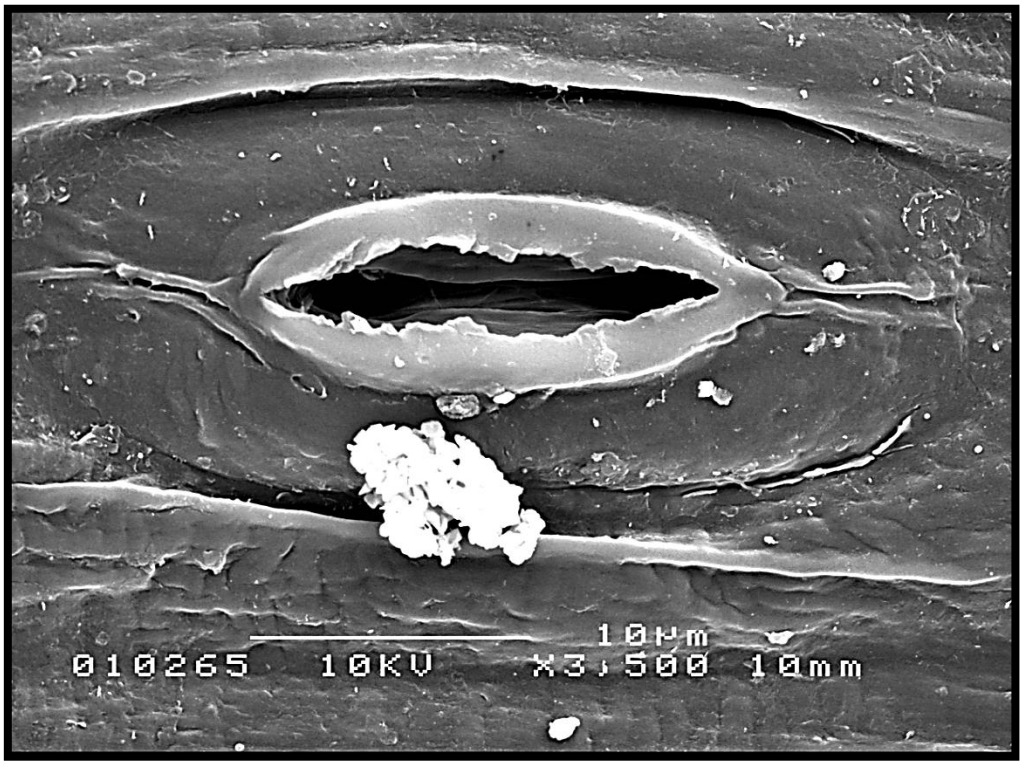


Figure 2.6(i): SEM micrograph showing petiole of *in vivo* plant of *Oxalis triangularis* at 3500x magnification. Stoma was clearly seen on the petiole.

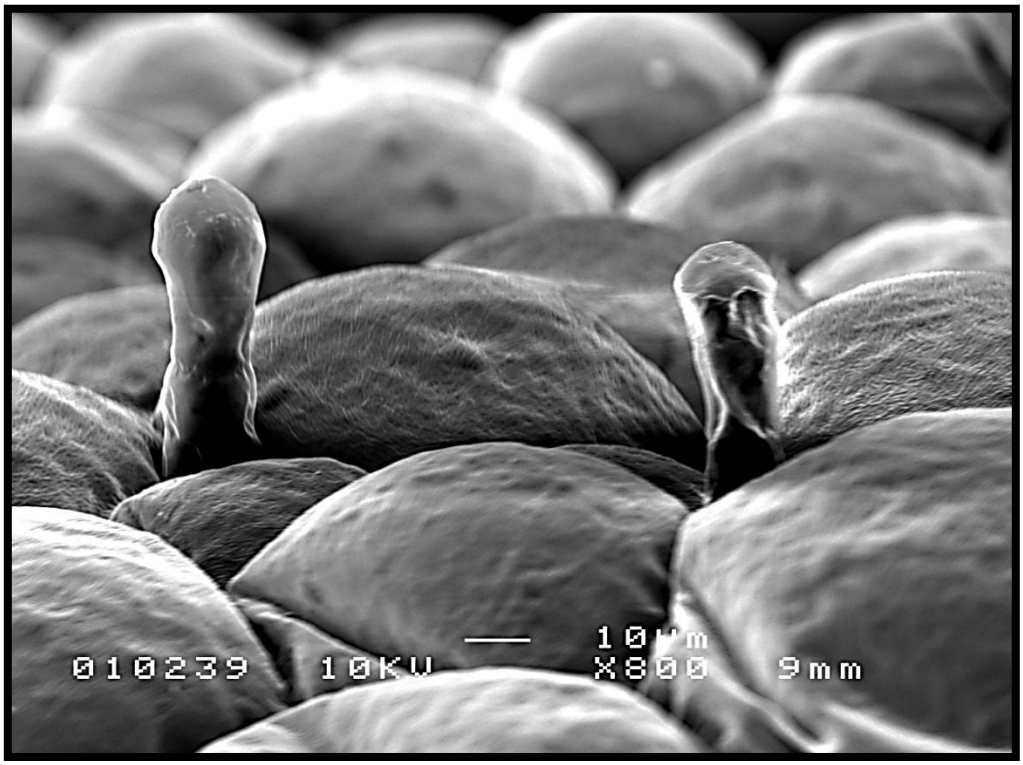


Figure 2.7 (a): SEM micrograph showing *in vitro* leaf explant of *Oxalis triangularis* at 800x magnification. Short trichomes were seen on the leaf.

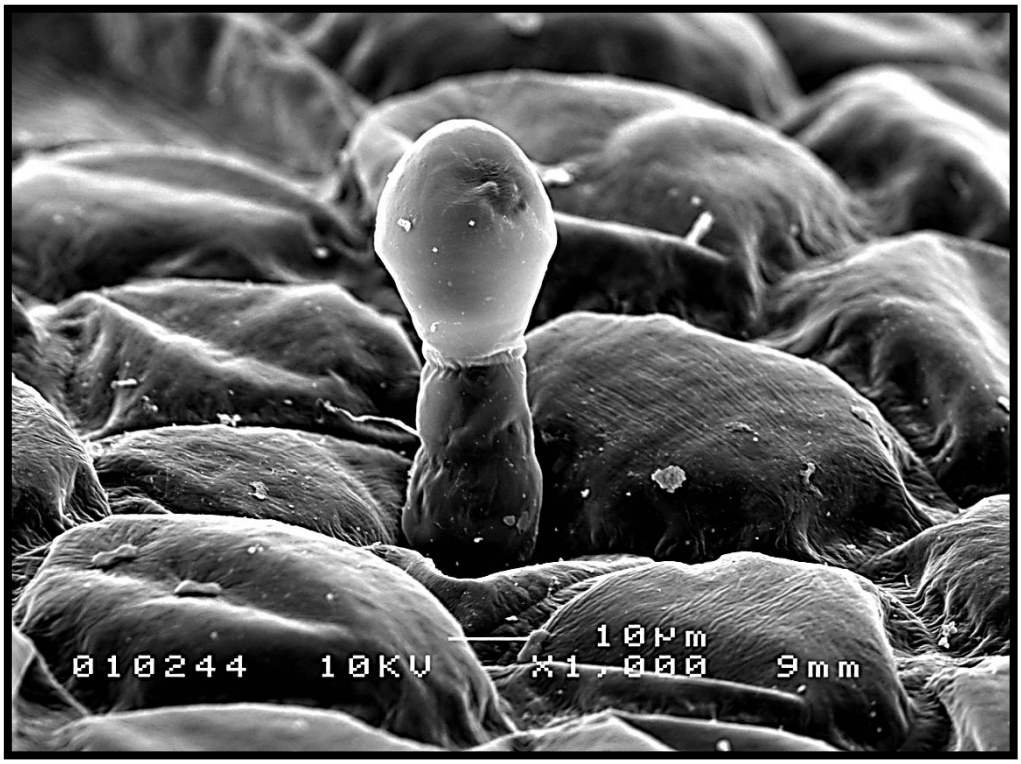


Figure 2.7(b): SEM micrograph showing *in vitro* leaf explant of *Oxalis triangularis* at 1000x magnification. Short trichome was found on the leaf.

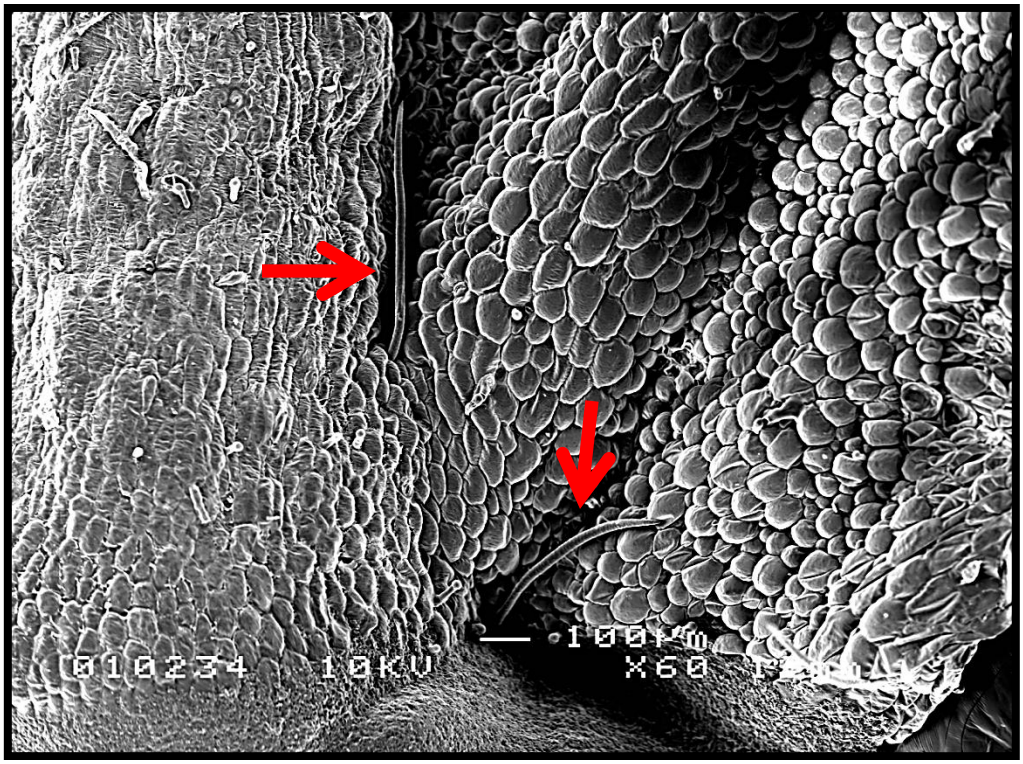


Figure 2.7(c): SEM micrograph showing *in vitro* leaf explant of *Oxalis triangularis* at 60x magnification. Long trichomes were seen on the leaf.

Figure 2.7(d): SEM micrograph showing leaf of *in vivo* plant of *Oxalis triangularis* at 800x magnification. Short trichome was clearly seen on the leaf.

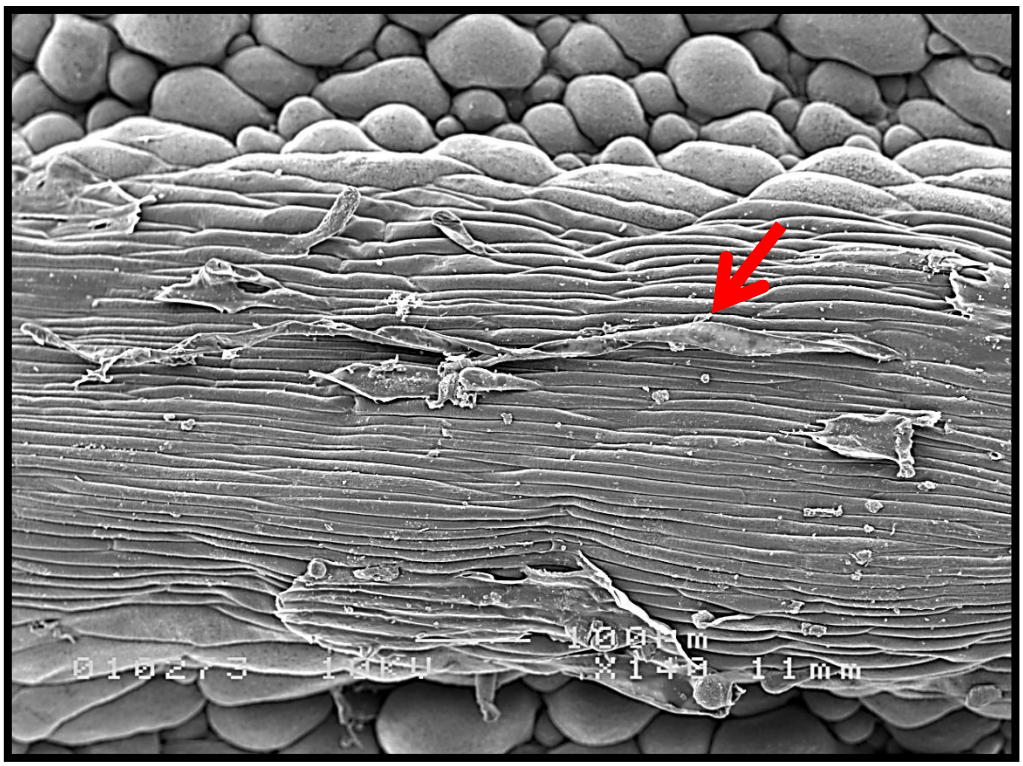


Figure 2.7(e): SEM micrograph showing leaf of *in vivo* plant of *Oxalis triangularis* at 140x magnification. Long trichome was seen on the leaf.

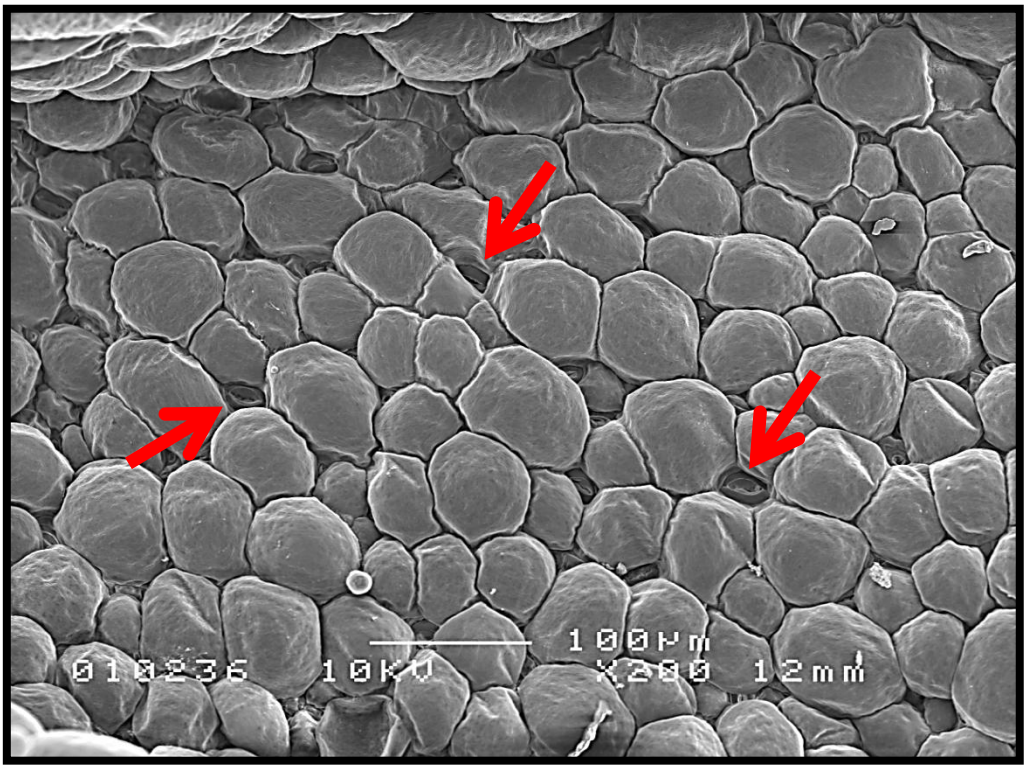


Figure 2.7 (f): SEM micrograph showing *in vitro* leaf explant of *Oxalis triangularis* at 200x magnification. Stomata were seen on the leaf.

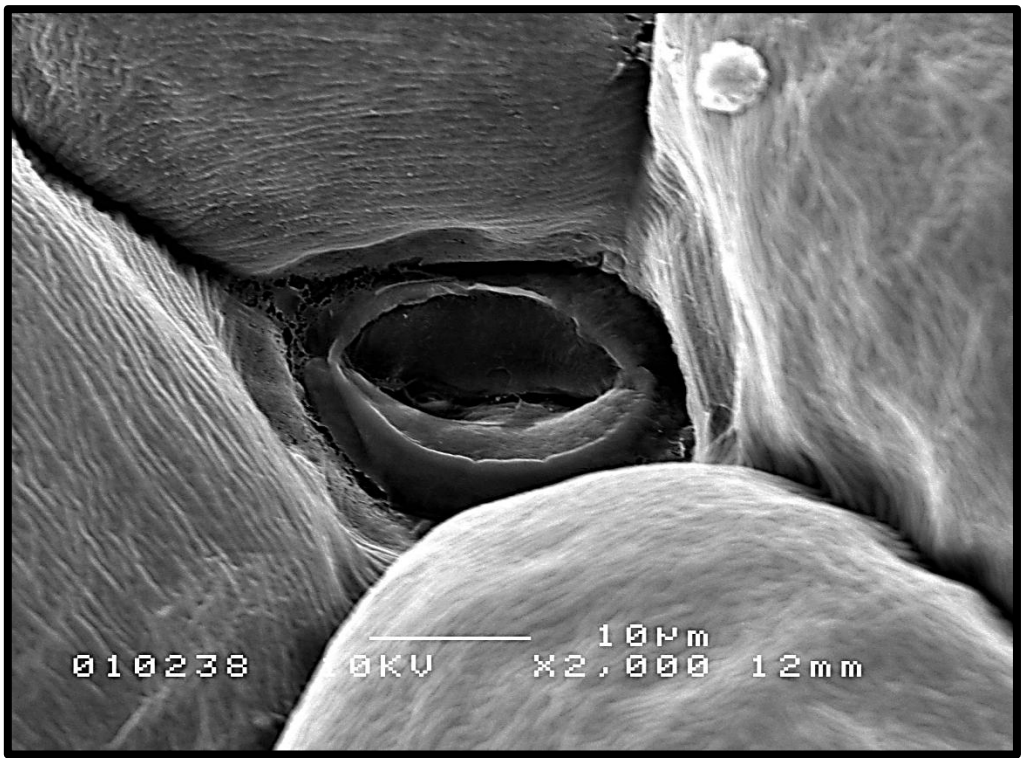


Figure 2.7(g): SEM micrograph showing *in vitro* leaf explant of *Oxalis triangularis* at 2000x magnification. Stoma was seen on the leaf.

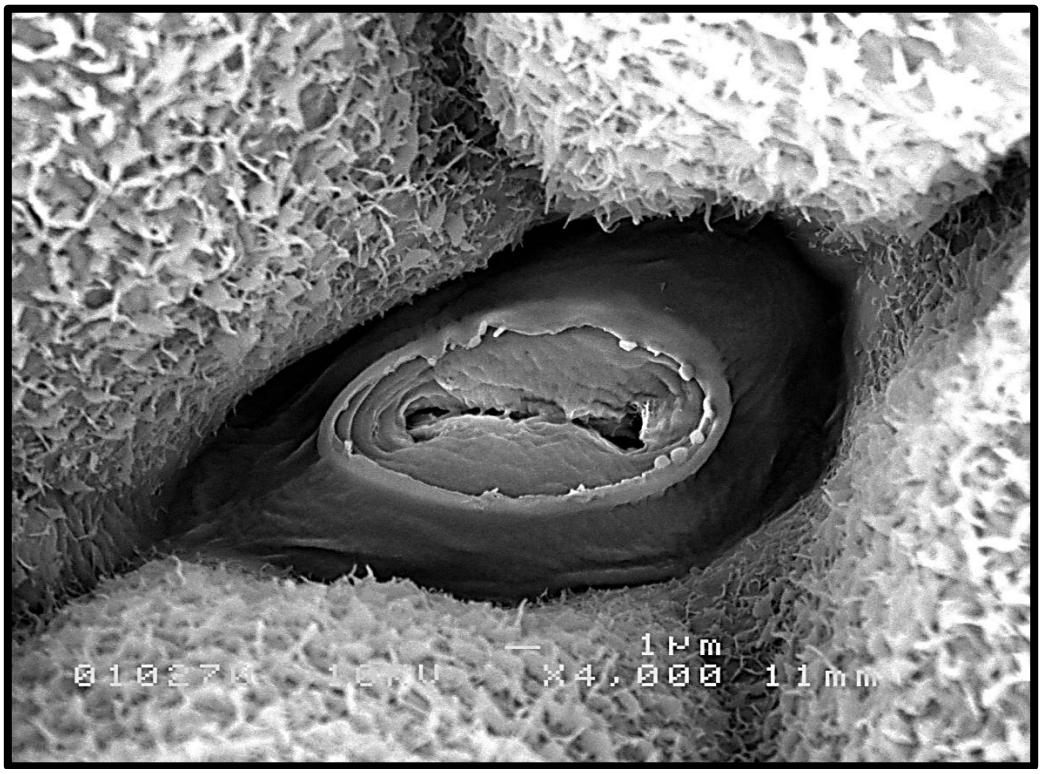


Figure 2.7(h): SEM micrograph showing leaf of *in vivo* plant of *Oxalis triangularis* at 4000x magnification. Stoma was seen on the leaf.

2.4 SUMMARY OF RESULTS

1) An efficient protocol has been established for plant regeneration of *Oxalis triangularis* through *in vitro* culture technique under aseptic conditions.

2) *Oxalis triangularis* can be germinated on MS basal medium and high frequency of adventitious shoots could be induced with addition of PGRs.

3) Adventitious shoots were best observed from petiole and leaf explants on MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l KIN with 12 shoots per explant and 1.0 mg/l NAA and 1.5 mg/l BAP with 14 shoots per explant, respectively.

4) Observations of petiole and leaf samples from *in vivo* and explants grown *in vitro* of *Oxalis triangularis* were made using scanning electron microscope. There were no morphological differences between *in vivo* and *in vitro* petiole and leaf samples.

CHARTER 3

IN VITRO FLOWERING OF *Oxalis triangularis*

3.1 EXPERIMENTAL AIMS

Flowering plants also known as Angiospermae are the most diverse plants on earth and flower is a part of plants which makes the plant very beautiful to the human eyes. Flower is also the reproductive structure for flowering plants apart from being merely ornamental plants. *Oxalis triangularis* is one of the prettiest varieties in the *Oxalis* family; their leaves are dark purple and have pale purple flower. The aim of this study was to induce *in vitro* flowering via tissue culture technique to shorten the time consuming for flowering from traditional plant breeding methods. Different types of plant growth regulators (PGRs) and concentrations were tested to accomplish these objectives.

3.2 MATERIALS AND METHODS

3.2.1 Source of Explants

Leaf explants were obtained from optimum *in vitro* regeneration of *Oxalis triangularis* which were obtained in CHAPTER 2: *IN VITRO* PLANT REGENERATION OF *Oxalis triangularis* (Table 2.3). 20 replicates of samples were used.

3.2.2 Preparation of Culture Media

Preparation of media composition for *in vitro* flowering was the same as the procedure for media composition of plant regeneration of *Oxalis triangularis* (2.2.3 Preparation of Culture Media). However the addition of plant growth regulators (PGRs) is varied in order to achieve the objective of the research approach. Below is the list of MS supplemented with PGRs used in this study:-

Table 3.1: List of MS medium supplemented with plant growth regulators to induce *in vitro* flowering of *Oxalis triangularis*.

No.	**Lists of MS Medium** **supplemented with plant growth regulators**
1	MS without hormone (control)
2	MS + 0.5 mg/l NAA + 0.5 mg/l BAP
3	MS + 0.5 mg/l NAA + 1.0 mg/l BAP
4	MS + 0.5 mg/l NAA + 1.5 mg/l BAP
5	MS + 0.5 mg/l NAA + 2.0 mg/l BAP

'**Table 3.1**, continued'

No.	Lists of MS Medium supplemented with plant growth regulators
6	MS + 1.0 mg/l NAA + 0.5 mg/l BAP
7	MS + 1.0 mg/l NAA + 1.0 mg/l BAP
8	MS + 1.0 mg/l NAA + 1.5 mg/l BAP
9	MS + 1.5 mg/l NAA + 1.5 mg/l BAP
10	MS + 0.5 mg/l KIN + 0.1 mg/l IAA
11	MS + 1.0 mg/l KIN + 0.1 mg/l IAA
12	MS + 1.0 GA_3 mg/l + 0.5 mg/l BAP
13	MS + 6.8 mg/l $AgNO_3$
14	MS + 7.14 mg/l CoCl2
15	MS + 0.25 mg/l ADS + 1.5 mg/l BAP + 0.5 mg/l IAA
16	MS + 8.25 mg/l NH_4NO_3 + 1.0 mg/l IBA
17	MS + 8.25 mg/l NH_4NO_3 1.0 mg/l BAP

3.2.3 Culture Conditions

All the tissue culture equipment such as a scalpel and forceps must be in aseptic conditions to prevent any contamination. These culture apparatus were autoclaved for 20 minutes. UV light and hot bead steriliser was turned on for at least 30 minutes prior to use. All laminar flow surfaces were wiped with 70% ethanol before and after use to ensure the sterility inside the chamber.

3.2.4 Subculture

All the aseptic cultures were maintained in the culture room (16 hours light and 8 hours dark photoperiod and the temperature was maintained at 25 ± 1°C). The growth plantlets were subcultured on the same medium at 2 months interval to maintain supply of fresh nutrient medium.

3.2.5 Induction of *In Vitro* Flowering

Leaf explants from *in vitro* adventitious shoots of *Oxalis triangularis* were cut ≈ 1 cm in the laminar flow chamber using a sterile scalpel and cultured onto MS basal medium. Two-month-old young adventitious shoots of *Oxalis traingularis* were subcultured on MS medium supplemented with PGRs as stated in Table 3.1 to promote *in vitro* flowering. Response of PGRs to induce *in vitro* flowering were recorded.

3.2.5.1 Experimental Outline

Adventitious shoots were taken from optimum

in vitro regeneration of *Oxalis triangularis* (Table 2.3).

↓

Leaf explants were cut into small pieces (≈ 1cm) in the laminar flow chamber.

↓

Cultured on MS basal medium.

↓

Plantlets were subcultured on MS medium supplemented with PGRs.

↓

Maintained in the culture room.

3.2.6 Data Analysis

Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with different letters in the same column differ significantly at p = 0.05.

3.3 RESULTS

For *in vitro* flowering study, two-month-old *Oxalis triangularis* plantlets were subcultured on MS medium supplemented with PGRs to induce *in vitro* flowering (Table 3.1). After 8 weeks of treatment periods, initiations of flower buds (Figure 3.1) were observed and the results were shown in Table 3.2. MS without hormone was used as control.

As can be seen in Table 3.2, combinations of hormones NAA and BAP showed greater response in inducing *in vitro* flowering. MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP resulted in the highest frequency of *in vitro* flowering (90.00 ± 0.07 %) and the least frequency of *in vitro* flowering was observed on MS medium supplemented with 0.5 mg/l NAA and 2.0 mg/l BAP (10.00 ± 0.07 %). Other than combinations of NAA and BAP hormones including control treatment (MS without hormone), showed no response of *in vitro* flowering. In this experiment, up to 7 flowers per plantlet (Figure 3.2) were developed within 8 weeks of culture on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP (2.20 ± 0.43), followed by MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP (1.15 ± 0.45), where maximum of 6 flowers per plantlet was obtained.

Table 3.2: Effect of various combinations and concentrations of hormones on *in vitro* flowering of *Oxalis triangularis*.

Treatments (mg/l)	**Flowering response**	**Flowering (%)**	**No. of flowers per plantlet (Mean ± SE)**
MS without hormone (control)	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 0.5 NAA + 0.5 BAP	Flowering	90.00 ± 0.07 e	2.20 ± 0.43 c
MS + 0.5 NAA + 1.0 BAP	Flowering	25.00 ± 0.10 bcd	0.90 ± 0.43 ab
MS + 0.5 NAA + 1.5 BAP	Flowering	25.00 ± 0.10 bcd	0.95 ± 0.49 ab
MS + 0.5 NAA + 2.0 BAP	Flowering	10.00 ± 0.07 ab	0.10 ± 0.07 a
MS + 1.0 NAA + 0.5 BAP	Flowering	15.00 ± 0.08 abc	0.15 ± 0.11 a
MS + 1.0 NAA + 1.0 BAP	Flowering	40.00 ± 0.11 d	1.15 ± 0.45 b
MS + 1.0 NAA + 1.5 BAP	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 1.5 NAA + 1.5 BAP	Flowering	30.00 ± 0.11 cd	1.45 ± 0.66 bc
MS + 0.5 KIN + 0.1 IAA	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 1.0 KIN + 0.1 IAA	No response	0.00 ± 0.00 a	0.00 ± 0.00 a

‘**Table 3.2**, continued’

Treatments (mg/l)	**Flowering response**	**Flowering (%)**	**No. of flowers per plantlet (Mean ± SE)**
MS + 1.0 GA_3 + 0.5 BAP	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 6.8 $AgNO_3$	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 7.14 $CoCl_2$	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 0.25 ADS + 1.5 BAP + 0.5 IAA	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 8.25 NH_4NO_3 + 1.0 IBA	No response	0.00 ± 0.00 a	0.00 ± 0.00 a
MS + 8.25 NH_4NO_3 + 1.0 BAP	No response	0.00 ± 0.00 a	0.00 ± 0.00 a

Each value represents the mean ± SE of 20 replicates.
The mean with different letters in the same column differ significantly at 0.05 level.

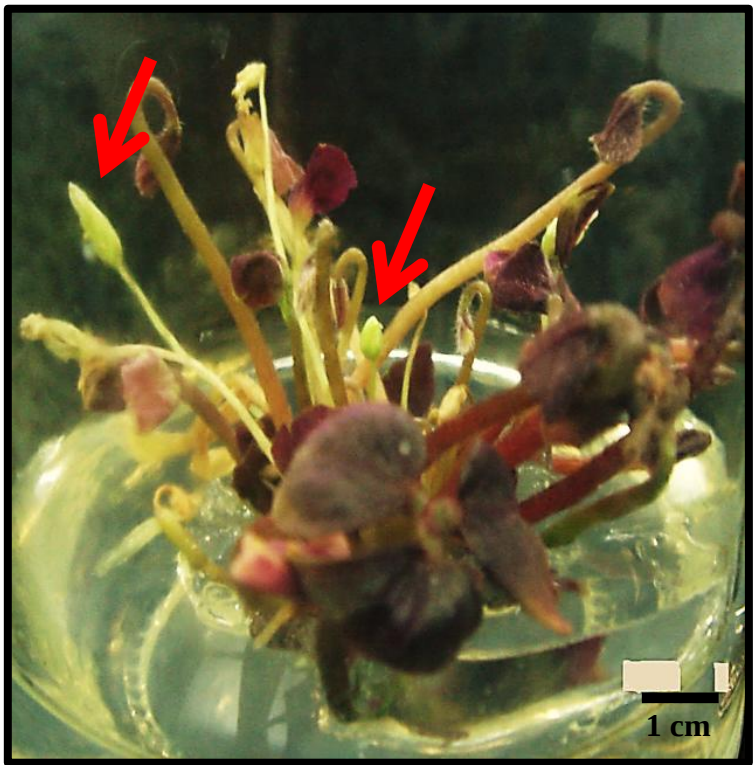


Figure 3.1: Flower buds of *in vitro* flowering of *Oxalis triangularis* were observed after 8 weeks multiple shoots being cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5mg/l BAP.

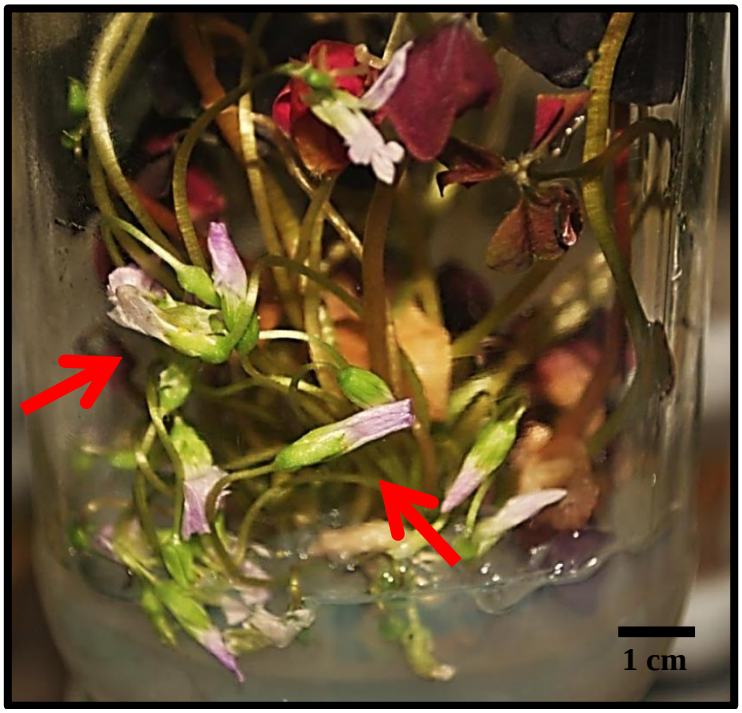


Figure 3.2: *In vitro* flowering of *Oxalis triangularis* after 9 weeks multiple shoots were cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5mg/l BAP.

3.4 SUMMARY OF RESULTS

1) Successful *in vitro* flowering of *Oxalis triangularis* could be obtained with the addition of plant growth regulators in MS medium.

2) MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP gave 90.00 ± 0.07 % of *in vitro* flowering and was the best hormone combinations to induce *in vitro* flowering compared to the other combinations of hormones.

3) *In vitro* flowering gave promising results whereby up to 7 flowers per plantlet were developed within 8 weeks of adventitious shoots being cultured on MS medium supplemented with PGRs such as on MS medium supplemented with 0.5 mg/l NAA and 0.5mg/l BAP.

CHAPTER 4

SYNTHETIC SEEDS OF *Oxalis triangularis*

4.1 EXPERIMENTAL AIMS

Oxalis triangularis does not produce seeds naturally and are cultivated via division from bulbs. One of the advantages of synthetic seeds production is especially useful for plants which do not produce viable seeds. In this study, production of synthetic seeds was investigated and its viability when stored at two different storage periods. Artificial seeds of *Oxalis triangularis* also being tested by directly sowing the synthetic seeds on various different sowing substrates to examine the capability of synthetic seeds of this species for agro-industry purposes.

4.2 MATERIALS AND METHODS

4.2.1 Source of Explants

Optimum *in vitro* regeneration of *Oxalis triangularis* which were obtained in CHAPTER 2: *IN VITRO* PLANT REGENERATION OF *Oxalis triangularis* (Table 2.2) was selected to continue the study for production of synthetic seeds. Leaf explants were taken from *in vitro* adventitious shoots and cultured onto MS basal media.

4.2.2 Preparation of Culture Media

4.2.2.1 Preparation of MS Stock Solution

MS stock solution for macronutrients without calcium, micronutrients, vitamins and irons were prepared separately in the conical flasks. To prepare 1 litre of stock solution for macronutrients without calcium, a conical flask was put on a stirrer plate. 700 ml distilled water was added in the conical flask. A magnetic stirrer was placed in the flask and stirred, while adding chemical compound (Table 4.1) into the conical flask. The volume of the stock solution then was filled up with distilled water to 1 litre using a graduated cylinder and stored at 4°C prior to use. The same procedure was repeated to prepare stock solution for micronutrients, vitamins and irons and for each stock solution prepared, chemical compound were added as stated in Table 4.1. All the MS stock solution was stored at 4°C to maintain the freshness of the medium.

Table 4.1: List of components of MS medium to prepare stock solution.

Component of MS medium (stock solution)	Concentration (g/l)
A) Macronutrients without calcium	
- Ammonium nitrate (NH_4NO_3)	16.5
- Potassium nitrate (KNO_3)	19.0
- Magnesium sulphate ($MgSO_4.7H_2O$)	3.7
- Potassium dihydrogen orthophosphate (KH_2PO_4)	1.7
B) Micronutrients	
- Manganese sulphate ($MnSO_4.4H_2O$)	1.56
- Zinc sulphate ($ZnSO_4.7H_2O$)	0.86
- Potassium iodide (KI)	0.083
- Cupric sulphate ($CuSO_4.5H_2O$)	0.0025
- Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.025
- Cobaltus chloride ($CoCl_2.6H_2O$)	0.0025
- Boric acid (H3BO3)	0.062
C) Vitamins	
- Nicotinic acid	0.05
- Thiamine HCl	0.5
- Pyridoxine HCl	0.5
D) Irons	
- Sodium EDTA Ferric salt (Na_2 EDTA)	3.73
- Ferrous sulphate (Fe $SO_4.7H_2O$)	2.78

4.2.2.2 Preparation of Sodium Alginate Solution

Conical flask was added with 70 ml of distilled water. Magnetic stirrer was placed to stir the medium. Subsequently, 10 ml of macronutrients without calcium, 1 ml micronutrients, 1 ml iron and 1 ml vitamin were added, followed by 3 g of sucrose and 0.1 g myo-inositol into the solution. While stirring the mixture, the heater was turned on at low temperature before 3 g of alginate powder was added to completely dissolve the alginate powder. After the solution has mixed well, the heater was turned off and the medium cooled at room temperature before the pH was adjusted at 5.8. Next, 1.0 mg/l of NAA and 1.5 mg/l of BAP were added to the solution. Lastly, distilled water was top-upped to increase the volume to 100 ml and was autoclaved at 121°C (15 psi) for 20 minutes.

4.2.2.3 Preparation of Calcium chloride Dehydrate Solution

Calcium chloride dehydrate solution ($CaCl_2.2H_2O$) act as complexing agent to allow the hardening of encapsulated bead of synthetic seeds. Conical flask was added with 400 ml of distilled water and 14.7 g of $CaCl_2.2H_2O$. Magnetic stirrer was placed into the flask to stir the mixture. Lastly, distilled water was top-upped to increase the volume to 500 ml. Complexing agent was autoclaved at 121°C (15 psi) for 20 minutes.

4.2.3 Culture Conditions

All the tissue culture equipment such as a scalpel and forceps must be in aseptic conditions to prevent any contamination. These culture apparatus were autoclaved for 20 minutes. UV light and hot bead steriliser was turned on for at least 30 minutes prior to use. All laminar flow surfaces were wiped with 70% ethanol before and after use to ensure the sterility inside the chamber.

4.2.4 Subculture

All the aseptic cultures of synthetic seeds were maintained in the culture room at 16 hours light and 8 hours dark photoperiod and the temperature was maintained at 25 ± 1°C. The plantlets obtained were subcultured on the same medium at 2 months interval to maintain supply of fresh nutrient medium.

4.2.5 Encapsulation of Microshoots

Under aseptic conditions, five-week-old microshoots were submerged in a sodium alginate solution and subsequently sucked through a micropipette into a modified 5 ml pipette. They were then, dropped one by one in the $CaCl_2.2H_2O$ solution. The microshoots were allowed to get encapsulated (hardened) by allowing them to remain in $CaCl_2.2H_2O$ solution for 30 minutes. It will form a bead like structure in the sodium alginate solution, then these beads were taken out and transferred into the sterile distilled water to wash out the excess $CaCl_2.2H_2O$ solution around the beads for 10 minutes. All beads were then dried out on the sterile tissue paper before being cultured on MS basal medium.

4.2.6 Growth of Encapsulated Beads at Different Storage Period

Encapsulated beads were stored in the dark condition at low temperature (4°C ± 1°C) before being cultured after storage for 7 days and 30 days on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP to check the viability of synthetic seeds. Encapsulated beads were also cultured on MS basal medium as a control right after multiple shoots had been encapsulated (Day 0). 30 replicates of samples were used. The optimum survival percentage of the encapsulated beads was evaluated.

4.2.6.1 Experimental Outline

Gelling and complexing agent were prepared.

↓

Autoclaved for 20 minutes.

↓

Optimum regeneration from the plant regeneration study was taken as a source of explant.

↓

Five-week-old microshoots of *Oxalis triangularis* were encapsulated with sodium alginate.

↓

Harden in $CaCl_2$ solution to form a bead like structure.

↓

Beads were washed with sterile distilled water.

↓

Cultured on MS basal medium.

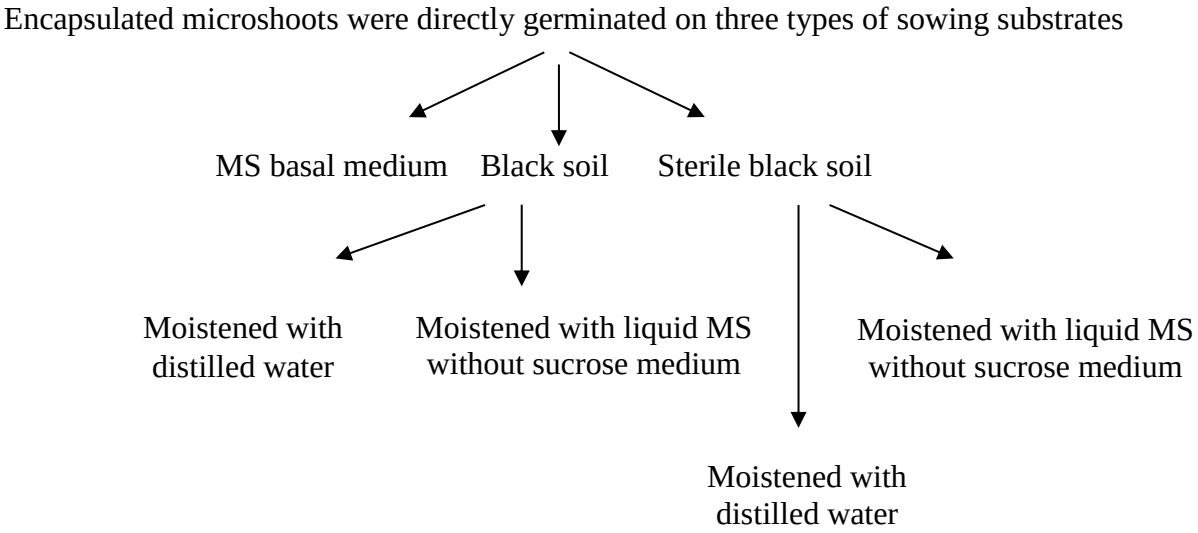
↓

Maintained in the culture room.

4.2.7 Germination of Synthetic Seeds on Various Sowing Substrates

Synthetic seeds were also directly sown on three different types of sowing substrates. MS basal medium, black soil and sterile black soil were used in this experiment. Black soil and sterile black soil are divided into two conditions; moistened with distilled water and moistened with liquid MS without sucrose medium. The most suitable sowing substrate was identified. 20 replicates of samples were used in this study.

4.2.7.1 Experimental Outline



4.2.8 Data Analysis

Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with different letters in the same column differ significantly at p = 0.05.

4.3 RESULTS

4.3.1 Growth of Encapsulated Beads at Different Storage Period

In the synthetic seeds study, five-week-old microshoots of *Oxalis triangularis* were used as explants. Effects of storage period (7 and 30 days) were studied to check the viability of synthetic seeds of *Oxalis triangularis* at low temperature (4°C) in the dark. The present study showed that conversion percentage of synthetic seeds of *Oxalis triangularis* was increased up to 96.67% on Day 7 and then dropped slowly on Day 30 (90.00%). From Table 4.2, conversion frequency of encapsulated propagules on Day 7 had increased with a value of 96.67% in comparison to Day 0 (60%) which served as control. Conversion frequency is a term referring to the percentage of synthetic seeds germination. However, conversion frequency of encapsulated beads started to drop on Day 30 with only 90% of synthetic seeds germination of *Oxalis triangularis* was recorded. It also expressed the highest percentage of survival of encapsulated beads on Day 7 followed by Day 30 and Day 0 with 86.21%, 77.78% and 66.67%, respectively.

Shoots emerged from the encapsulated beads of *Oxalis triangularis* on all the responded synthetic seeds cultured on MS basal medium. After 4 weeks of observation, highest number of shoots per encapsulated bead was achieved on the maximum conversion frequency of storage days on Day 7 with 4.57 ± 0.72 shoots, while on Day 30 recorded 3.97 ± 0.65 shoots and the lowest number of shoots was obtained on Day 0 (2.10 ± 0.57 shoots). However, all the responded synthetic seeds of *Oxalis triangularis* converted to plantlets formed well developed roots which emerged through the sodium alginate beads (100% shoots emerging).

4.3.2 Germination of Synthetic Seeds on Various Sowing Substrates

Comparison of germination of complete plantlets from synthetic seeds was also done on three types of different substrates which are MS basal medium, black soil and sterile black soil. Only synthetic seeds of *Oxalis triangularis* cultured on MS basal medium showed successful germination into complete plantlets (Table 4.3). For the soil samples, both types of soils were moistened with distilled water and liquid MS without sucrose. Encapsulated beads were sown directly after encapsulation of microshoots. In black soil moistened with distilled water, the encapsulated beads started to show negative response with shrinking beads after 7 days of being sown and eventually contaminated with fungal infection around the beads. In sterile black soil which was moistened with distilled water, encapsulated beads eventually shrunk without showing any positive response to germinate the beads into complete plantlets. Meanwhile, for both black soil and sterile black soil which were moistened with liquid MS without sucrose medium, all encapsulated beads were contaminated with fungal infection after 7 days of observation.

Table 4.2: Germination of synthetic seeds of *Oxalis triangularis* after being stored at different durations.

Storage durations (Days)	Conversion frequency (%)	Percentage of survival (%)	No. of shoots (Mean ± SE)	Root formation (%)
0 (control)	60.00	66.67	2.10 ± 0.57 a	100.00 ± 0.00 a
7	96.67	86.21	4.57 ± 0.72 b	100.00 ± 0.00 a
30	90.00	77.78	3.97 ± 0.65 b	100.00 ± 0.00 a

Each value represents the mean ± SE of 30 replicates.
The mean with different letters in the same column differ significantly at 0.05 level.

Table 4.3: Synthetic seeds germination of *Oxalis triangularis* in different sowing substrates.

Observation of synthetic seeds of *Oxalis triangularis*				
Medium/ substrate				
MS basal medium	**Black soil**		**Sterile black soil**	
	Moistened with distilled water	**Moistened with liquid MS without sucrose medium**	**Moistened with distilled water**	**Moistened with liquid MS without sucrose medium**
Encapsulated beads can be regenerated into complete plantlets when cultured on MS basal medium.	At first, after a few days the encapsulated beads started to shrink and eventually contaminated.	All encapsulated beads were contaminated with fungal infection.	Encapsulated beads did not give any response and ultimately shrinking.	All encapsulated beads were contaminated with fungal infection.

Each medium was represented by 20 replicates.

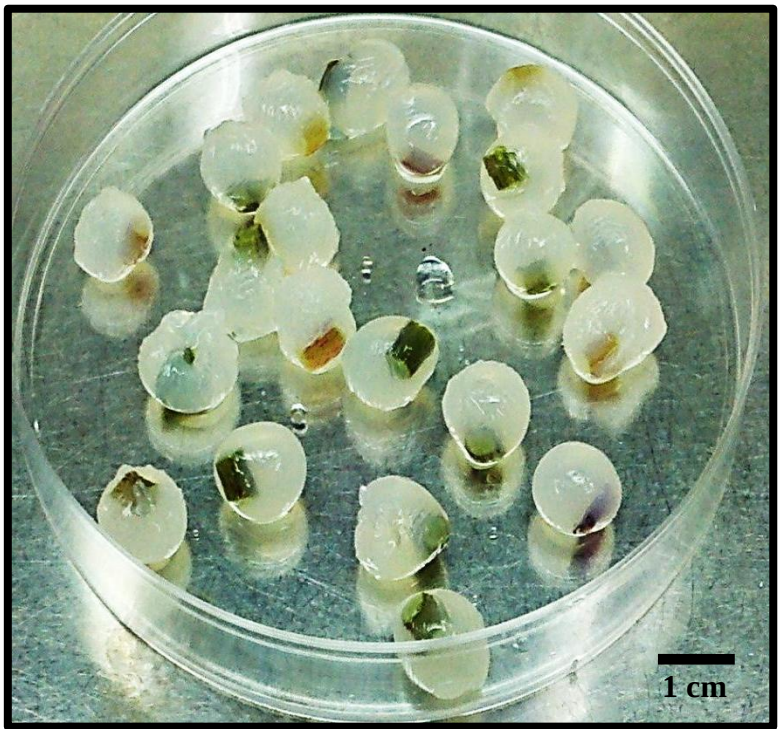


Figure 4.1: Encapsulated beads of *Oxalis triangularis* before being stored at low temperature (4°C ± 1°C).

Figure 4.2: Shoots emerged from the encapsulated microshoots of *Oxalis triangularis* after 7 days being cultured on MS basal medium.

Figure 4.3: Microshoots of *Oxalis triangularis* from synthetic seeds after 4 weeks being cultured on MS basal medium.

4.4 SUMMARY OF RESULTS

1) The best storage period for synthetic seeds of *Oxalis triangularis* was obtained on Day 7 with 96.67% frequency of conversion. The number of shoots per encapsulated microshoots was 4.57 ± 0.72 with 100 % roots emergence.

2) Synthetic seeds of *Oxalis triangularis* were successfully germinated when cultured under *in vitro* conditions compared with *in vivo* conditions.

3) Synthetic seeds serve as an alternative way to micropropagate *Oxalis triangularis* on a large scale since *Oxalis triangularis* did not produce seeds and propagated through division of bulb scales.

4) Synthetic seeds also have potential as an alternative method to preserve *Oxalis triangularis* for long term storage without losing viability.

CHAPTER 5

PIGMENT EXTRACTION OF *Oxalis triangularis*

5.1 EXPERIMENTAL AIMS

Anthocyanin are the flavonoid compounds that produce plant colours ranging from orange and red to various shades of blue and purple. Anthocyanin occurs in all higher plants, mostly in flowers, fruits and also in leaves. They have long been the subject of investigation by botanists and plant physiologists because of their roles as pollination attractants and phytoprotective agents. They have also been very useful in taxonomic studies. In this chapter, study was carried out to determine suitable solvents used for anthocyanin extraction because the choice of an extraction method is a great importance in the analysis of anthocyanin. Study was continued with the extraction from *in vitro* leaf explants to determine the potential of natural pigment for paint production.

5.2 MATERIALS AND METHODS

5.2.1 Source of Explants

Petiole and leaf explants were used to determine concentrations of anthocyanin found in *in vivo* and *in vitro* samples for both growth conditions. Both samples conditions were taken at the same developmental stages. *In vitro* explants were obtained from optimum regeneration of *Oxalis triangularis* which were obtained in CHAPTER 2: *IN VITRO* PLANT REGENERATION OF *Oxalis triangularis* (Table 2.2 and Table 2.3).

5.2.2 Anthocyanin Extraction

In this study, acidified acetone (acetone + 0.15% hydrocloric acid, HCl) and acidified methanol (methanol + 0.15% HCl) solvents were used to extract the anthocyanin from plant materials (petiole and leaf) for both grown *in vivo* and *in vitro*. Both petiole and leaf grown *in vivo* and *in vitro* of *Oxalis triangularis* were dried in the oven at 40°C for 20 minutes. 2 g of petiole from *in vivo* and *in vitro* grown plants were homogenized with different concentrations of acidified methanol using mortar and pestle under low light conditions, separately. The extracts were then centrifuged at 5000 rpm for 5 minutes. After centrifuge, only supernatant were pipetted into cuvette. The procedure was repeated with the solvent acidified acetone and leaf samples *in vivo* and *in vitro* for both solvents. The extracts were analysed for the absorption spectra wavelength range between 520 nm to 545 nm to determine concentrations of anthocyanin.

5.2.2.1 Experimental Outline

Plant materials (petiole and leaf) grown *in vivo* and *in vitro* were dried in the oven at 40°C for 20 minutes.

↓

Samples were homogenised using mortar and pestle with an acidified methanol and acidified acetone at different concentrations.

↓

Extracts was centrifuged at 5000 rpm for 5 minutes.

↓

Supernatant was pippeted into cuvette.

↓

Anthocyanin pigment was analysed by UV-visible spectrophotometer.

5.2.3 Natural Pigment for Paint Production

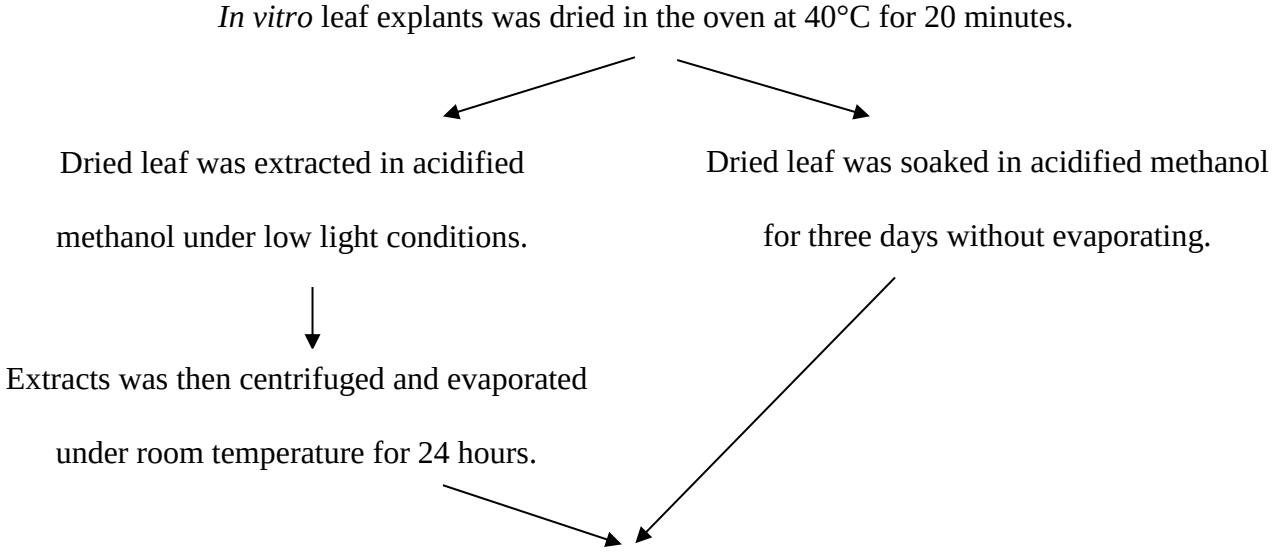
5.2.3.1 Method for Colour Extraction

In vitro leaf explants was dried in the oven at 40°C for 20 minutes. There are two methods for colour extraction. The first method consists of drying process, subsequently the dried leaf were extracted in acidified methanol under low light conditions with a ratio of dried leaf to methanolic acid is 1:5. The next step, the extracts was centrifuged to separate supernatant and pellet. Purple colour extracts of *in vitro* leaf explants was then evaporated for 24 hours at room temperature. In the second method, after the drying process, *in vitro* leaf explants were soaked in acidified methanol for three days without evaporating.

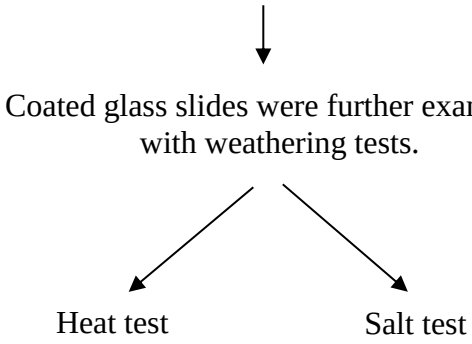
5.2.3.2 Coating Process

The resultant purple colour extracted from *in vitro* leaf explants were mixed with polyvinyl alcohol (PVA) with a ratio of extracts to PVA is 1:1. Mixture was stirred and coated onto glass slides and left to dry for another 24 hours. Comparison between two methods for colour extraction was observed and only the best method was chosen before coated glass slide was subjected to the weathering tests (heat and salt tests). For the salt test, coated glass slides were soaked in three different concentrations of Natrium chloride (NaOH); 0.5% w/v, 1.0% w/v and 1.5% w/v. The absorbance was measured every 15 minutes under Scanning Electron Microscope. While for the heat test, coated glass slide were expose under room temperature and in oven (40°C). Then, the absorbance was measured from Day 1 until Day 30 under Scanning Electron Microscope.

5.2.3.3 Experimental Outline



Both *in vitro* extracts were mixed with polyvinyl alcohol and coated on glass slides. Left to dry for another 24 hours.



5.2.4 Data Analysis

The absorbance of anthocyanin extraction was measured by scanning through a UV-visible spectrophotometer. Natural pigment for colour extraction study, absorbances of anthocyanin from weathering tests was measured using spectrophotometer.

5.3 RESULTS

5.3.1 Anthocyanin Extraction

Petiole and leaf of *Oxalis triangularis* obtained from *in vivo* and *in vitro* were used as samples to detect the presence of anthocyanin pigment at different types and concentrations of solvents. Two types of solvents were used which are acidified acetone and acidified methanol. The pigment extraction was determined by the absorption peaks at wavelength of 536 nm by UV-visible spectrophotometer analysis. Figure 5.1 until Figure 5.12 are the UV-visible spectroscopy reading of anthocyanin extraction using acidified acetone and acidified methanol from petiole explants grown *in vivo* and *in vitro* of *Oxalis triangularis*. While, Figure 5.13 until Figure 5.16 are the UV-visible spectroscopy reading of anthocyanin extraction using acidified acetone and acidified methanol from leaf explants grown *in vivo* and *in vitro* of *Oxalis triangularis*

Absorbance reading for petiole extracts of *Oxalis triangularis* decreased when the concentrations of extracts increased (Table 5.1). The highest anthocyanin absorbance for petiole grown *in vivo* and *in vitro* were recorded on samples extracted in acidified acetone, extract concentration of 400mg/ml with absorbance 1.093 and 0.968, respectively. In contrast with extraction in acidified acetone, *in vivo* and *in vitro* petiole extracts shown lower absorbance readings when extracted in acidified methanol.

Different observation were made in leaf samples of *Oxalis triangularis* for both solvents extraction. Absorbance reading of UV-visible spectrophotometer showed no obvious difference for each concentrations of extract tested (Table 5.2). Both *in vivo* leaf

extracts in acidified acetone and acidified methanol recorded high anthocyanin pigment at extract concentrations of 100mg/ml. However, *in vitro* leaf extracts gave different results for anthocyanin detection. In acidified acetone, high anthocyanin recorded at concentrations of extract of 40mg/ml with absorbance value of 1.280 while in acidified methanol the value recorded at concentrations of extract of 66.667 mg/ml was 0.551.

Table 5.1: Absorbance reading at the wavelength of 536 nm for petiole extracts of *Oxalis triangularis.*

Concentrations of extract (mg/ml)	**Absorbance**			
	Acidified acetone		**Acidified methanol**	
	In vivo	***In vitro***	***In vivo***	***In vitro***
400	1.093	0.968	0.484	0.532
200	0.141	0.144	0.209	0.339
160	0.227	0.268	0.221	0.170

Table 5.2: Absorbance reading at the wavelength of 536 nm for leaf extracts of *Oxalis triangularis.*

Concentrations of extract (mg/ml)	**Absorbance**			
	Acidified acetone		**Acidified methanol**	
	In vivo	***In vitro***	***In vivo***	***In vitro***
100	2.223	0.829	2.101	0.381
80	2.173	0.572	2.045	0.466
66.667	1.536	0.809	1.834	0.551
50	0.797	0.726	1.596	0.491
40	0.656	1.280	1.516	0.431

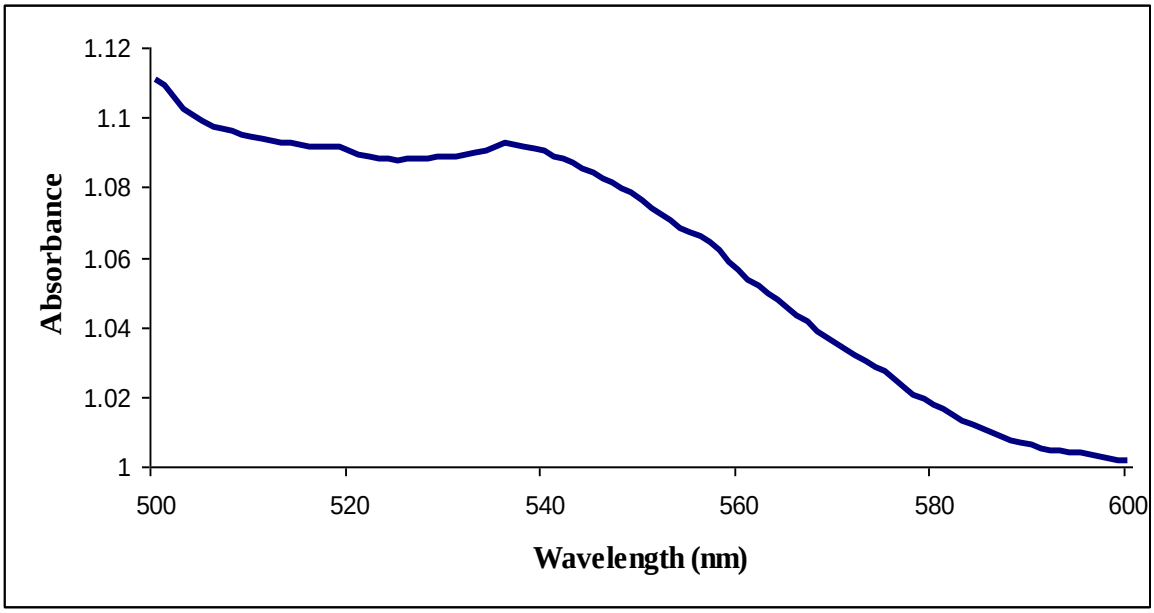


Figure 5.1: UV-visible spectroscopy reading of anthocyanin from *in vivo* petiole at the ratio of extracts to acidified acetone is 1:5.

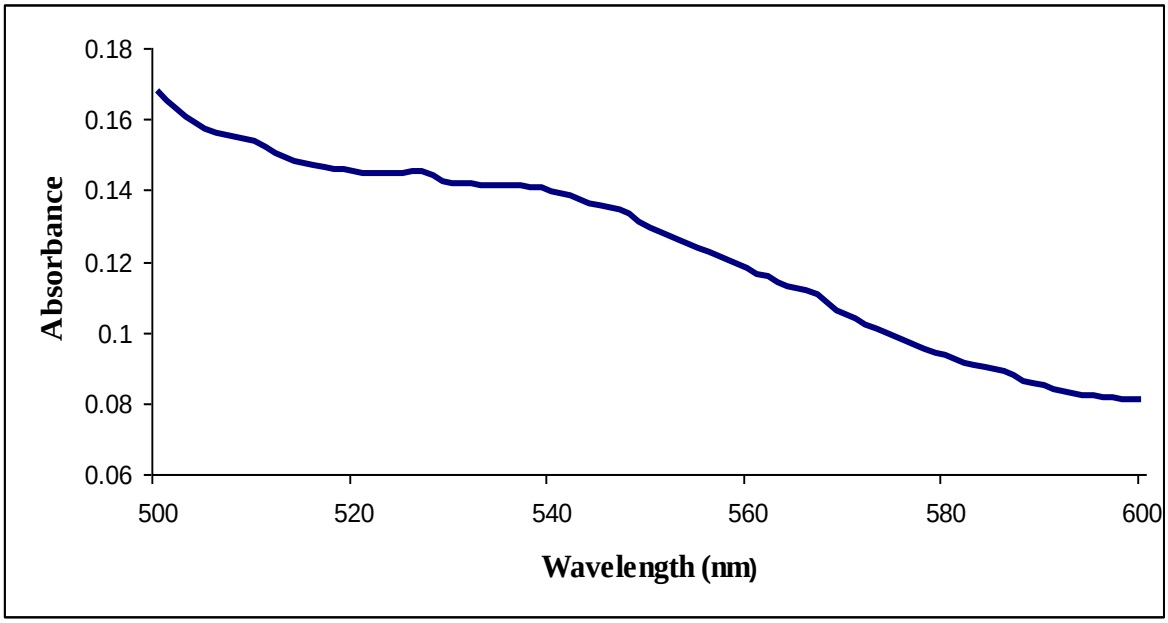


Figure 5.2: UV-visible spectroscopy reading of anthocyanin from *in vivo* petiole at the ratio of extracts to acidified acetone is 1:10.

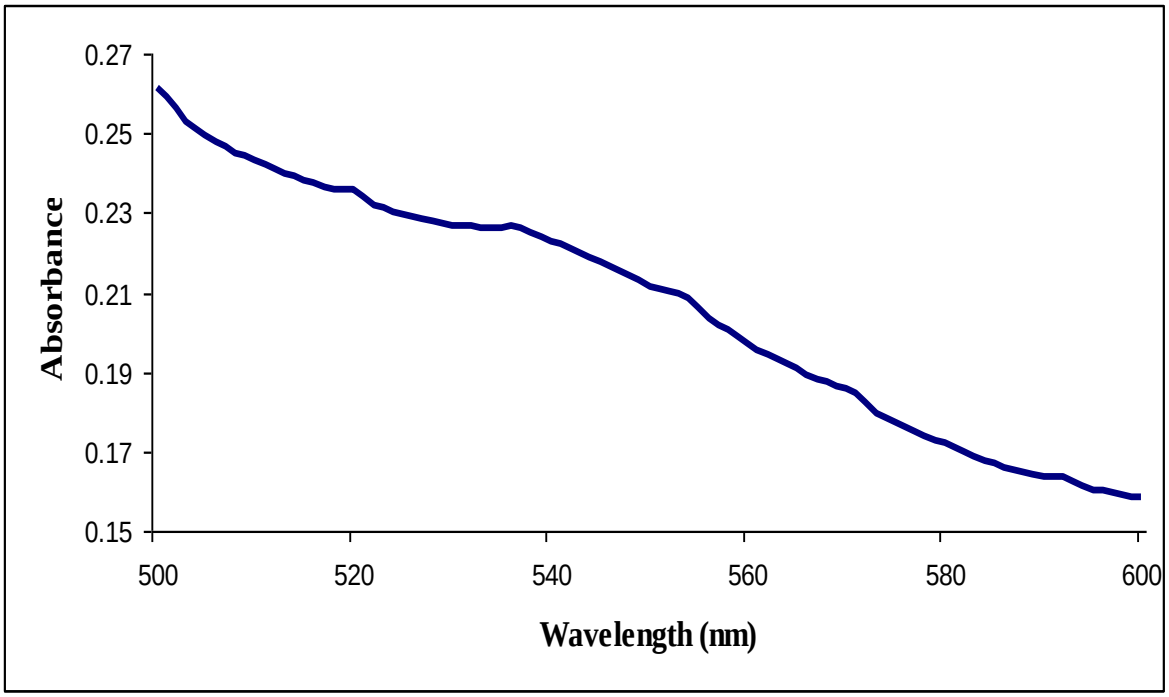


Figure 5.3: UV-visible spectroscopy reading of anthocyanin from *in vivo* petiole at the ratio of extracts to acidified acetone is 1:12.5.

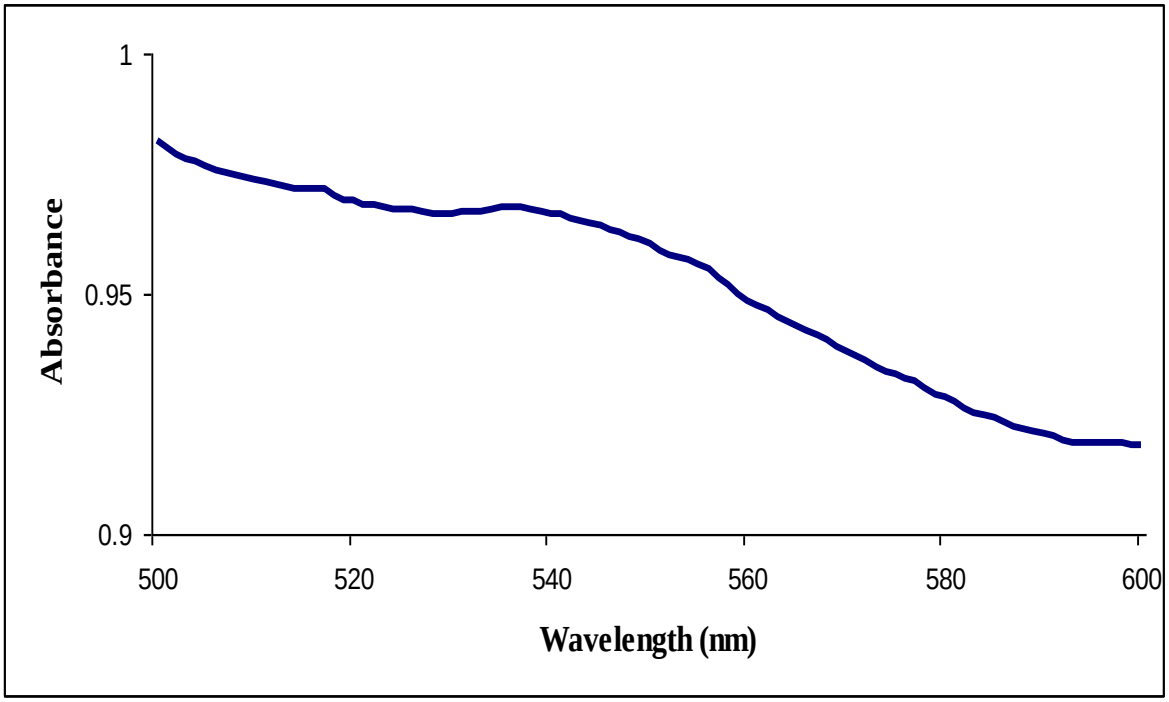


Figure 5.4: UV-visible spectroscopy reading of anthocyanin from *in vitro* petiole at the ratio of extracts to acidified acetone is 1:5.

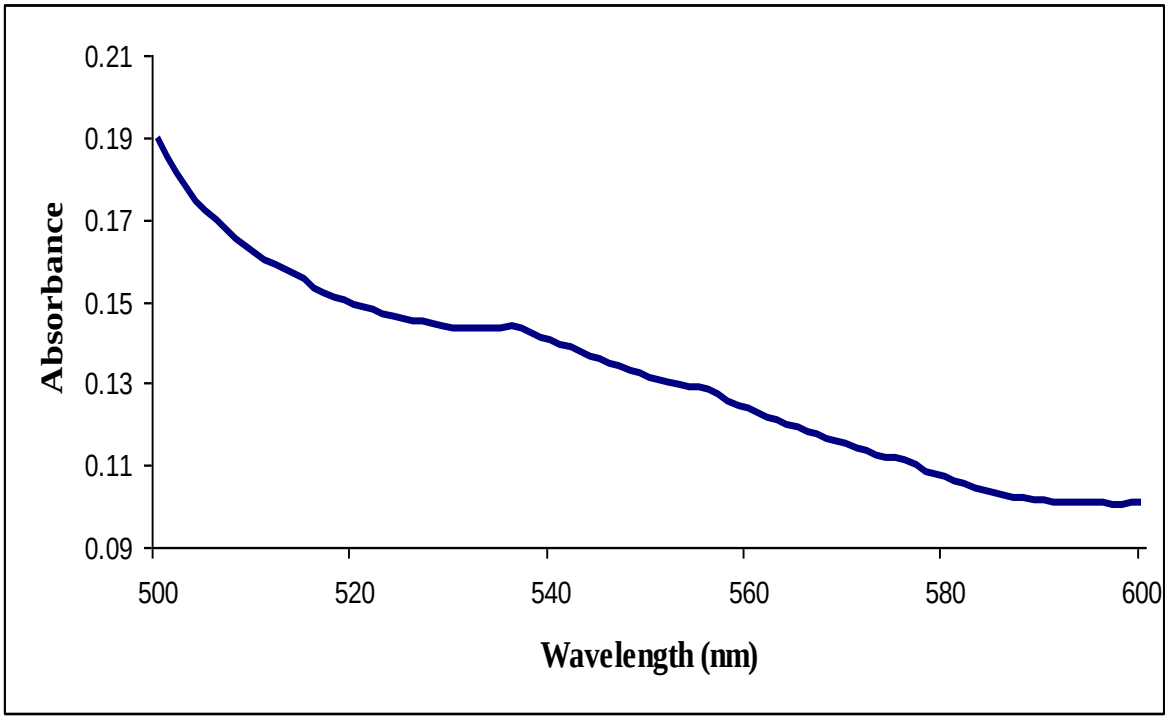


Figure 5.5: UV-visible spectroscopy reading of anthocyanin from *in vitro* petiole at the ratio of extracts to acidified acetone is 1:10.

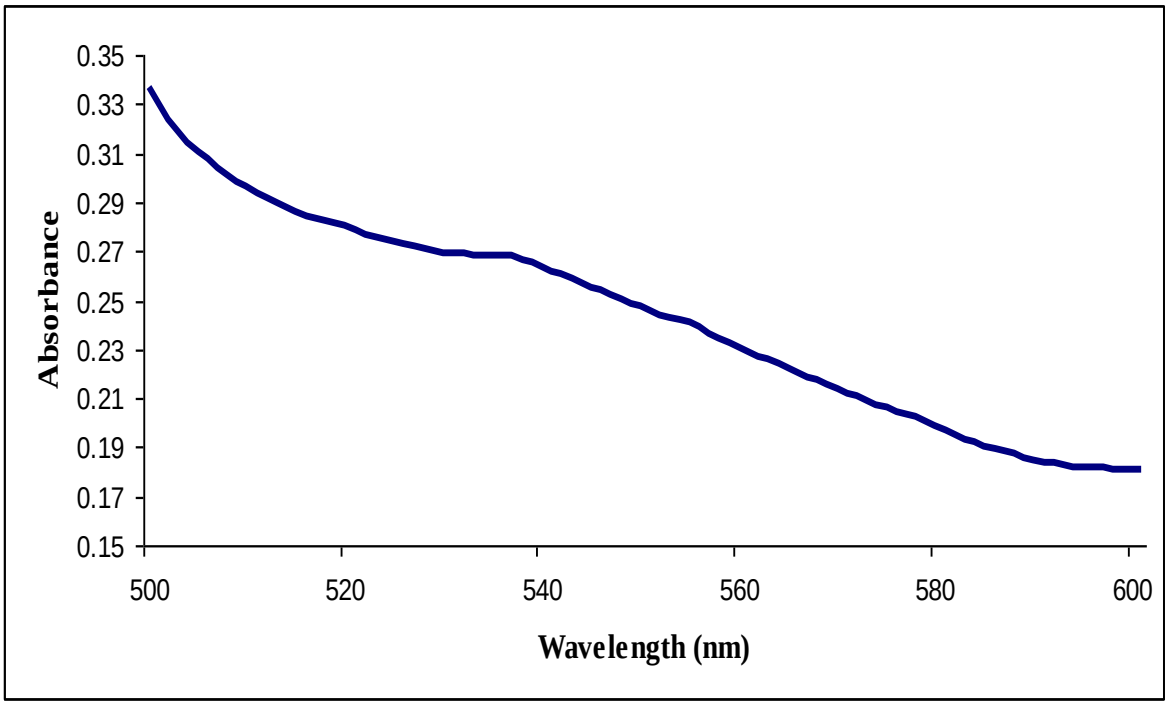


Figure 5.6: UV-visible spectroscopy reading of anthocyanin from *in vitro* petiole at the ratio of extracts to acidified acetone is 1:12.5.

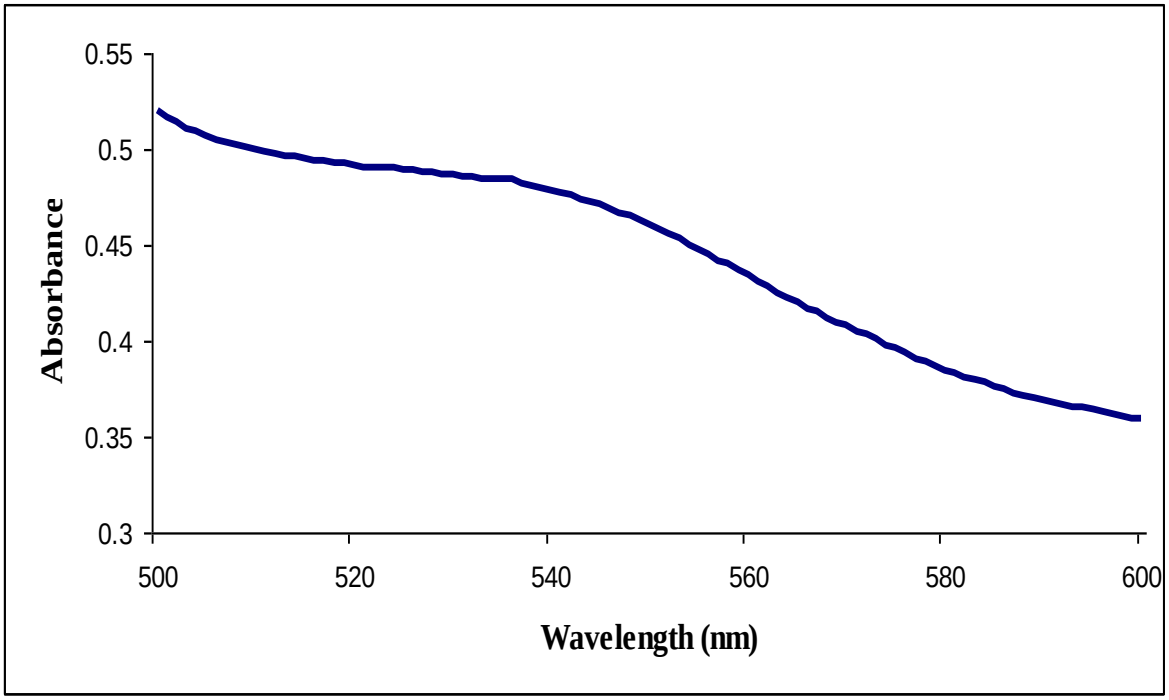


Figure 5.7: UV-visible spectroscopy reading of anthocyanin from *in vivo* petiole at the ratio of extracts to acidified methanol is 1:5.

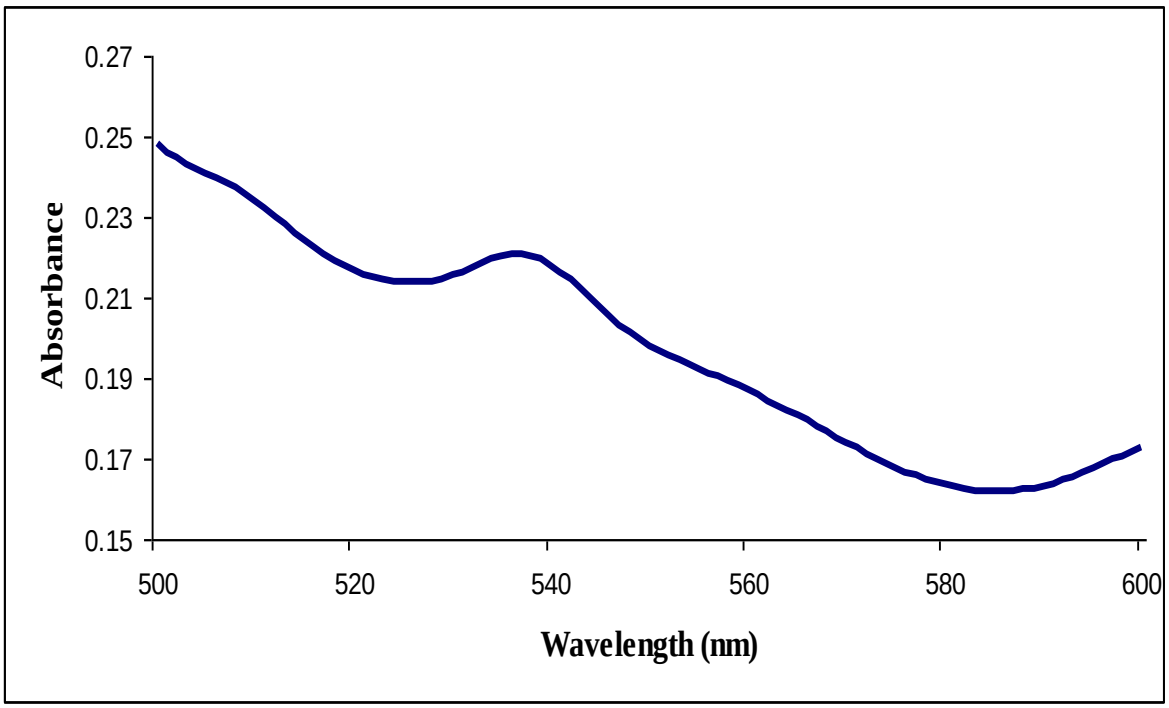


Figure 5.8: UV-visible spectroscopy reading of anthocyanin from *in vivo* petiole at the ratio of extracts to acidified methanol is 1:10.

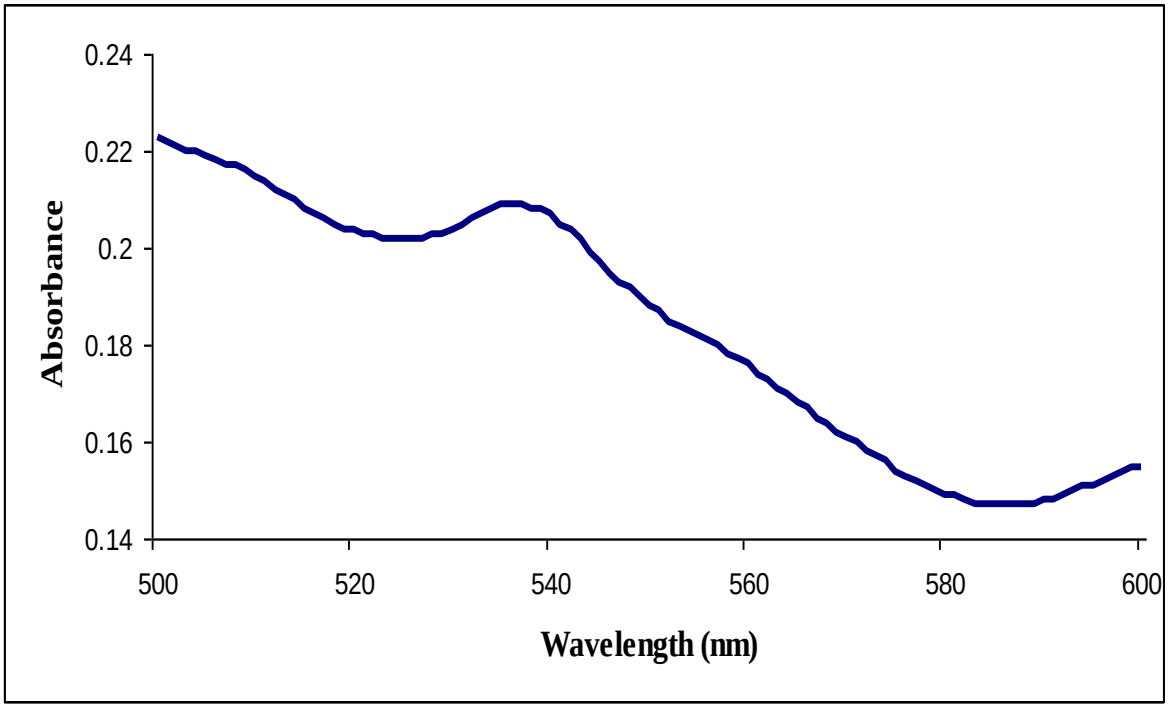


Figure 5.9: UV-visible spectroscopy reading of anthocyanin from *in vivo* petiole at the ratio of extracts to acidified methanol is 1:12.5.

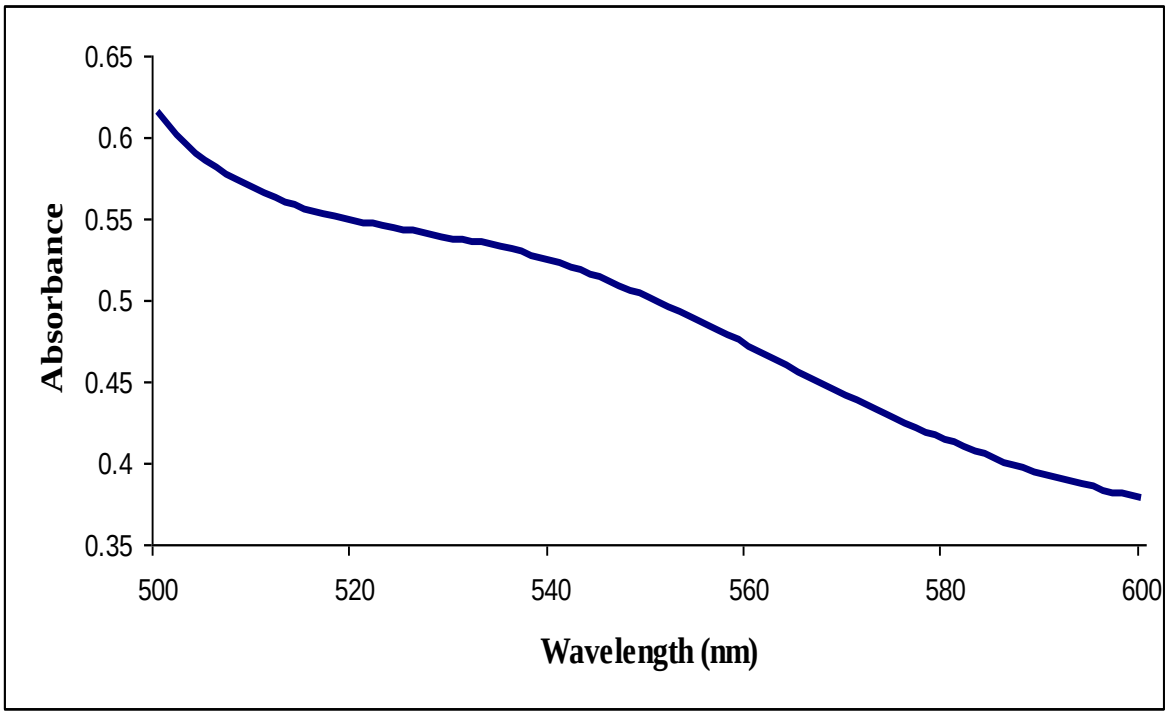


Figure 5.10: UV-visible spectroscopy reading of anthocyanin from *in vitro* petiole at the ratio of extracts to acidified methanol is 1:5.

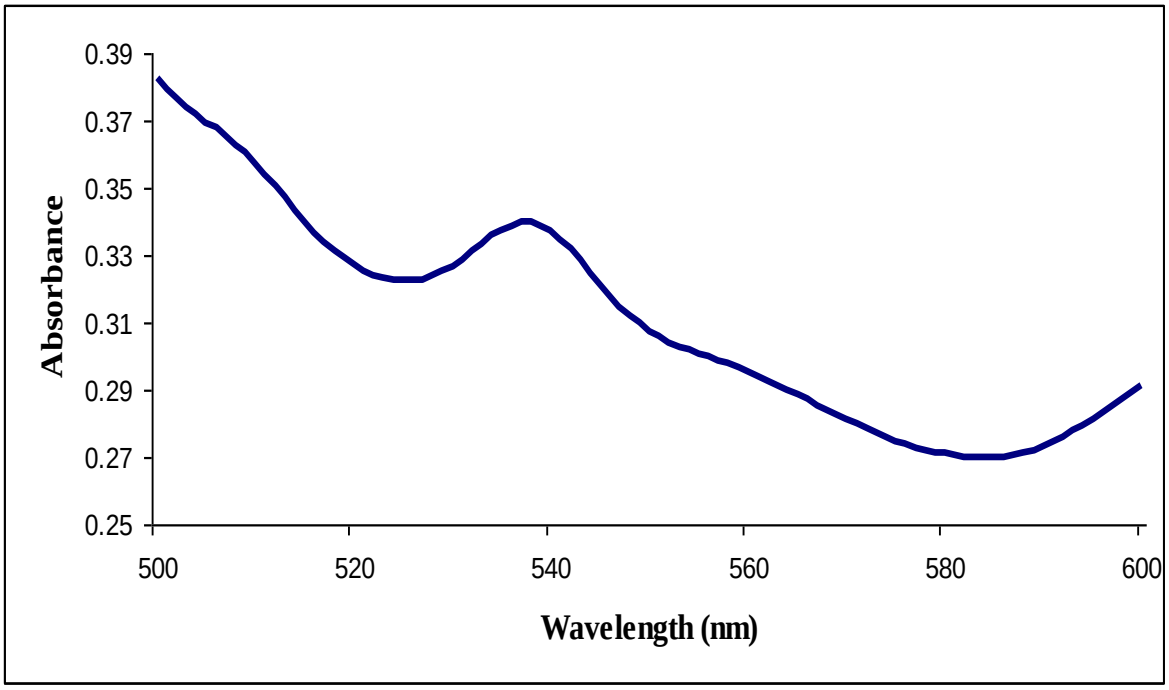


Figure 5.11: UV-visible spectroscopy reading of anthocyanin from *in vitro* petiole at the ratio of extracts to acidified methanol is 1:10.

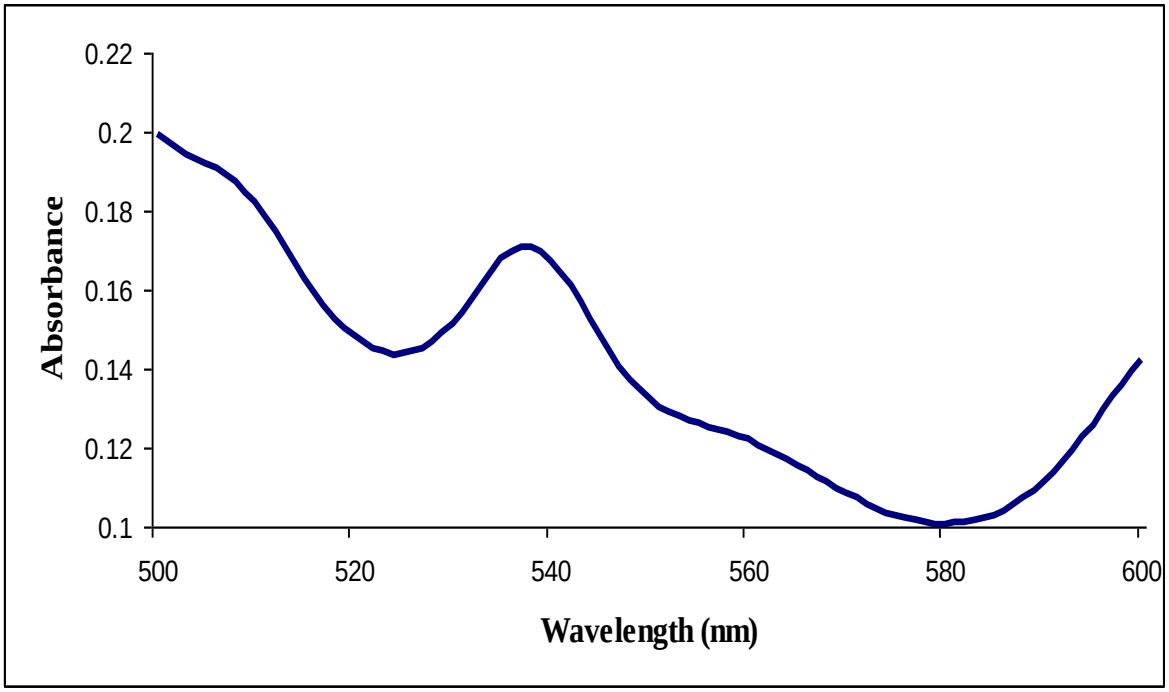


Figure 5.12: UV-visible spectroscopy reading of anthocyanin from *in vitro* petiole at the ratio of extracts to acidified methanol is 1:12.5.

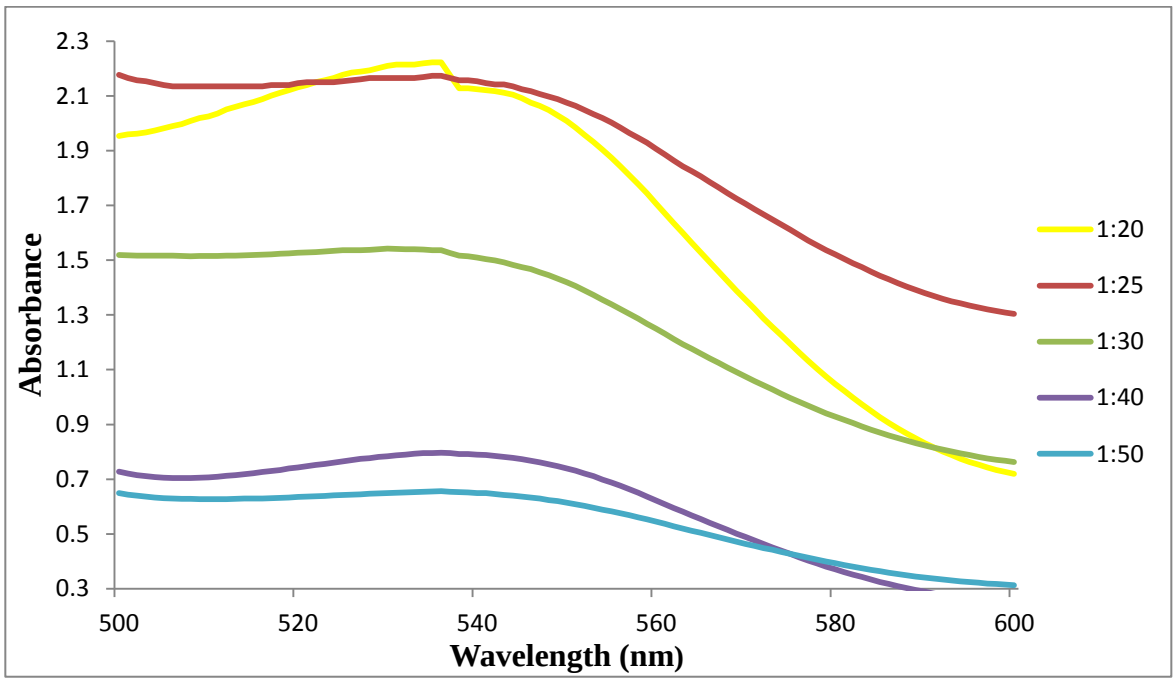


Figure 5.13: UV-visible spectroscopy reading of anthocyanin from *in vivo* leaf extracted using acidified acetone solvent at different concentrations.

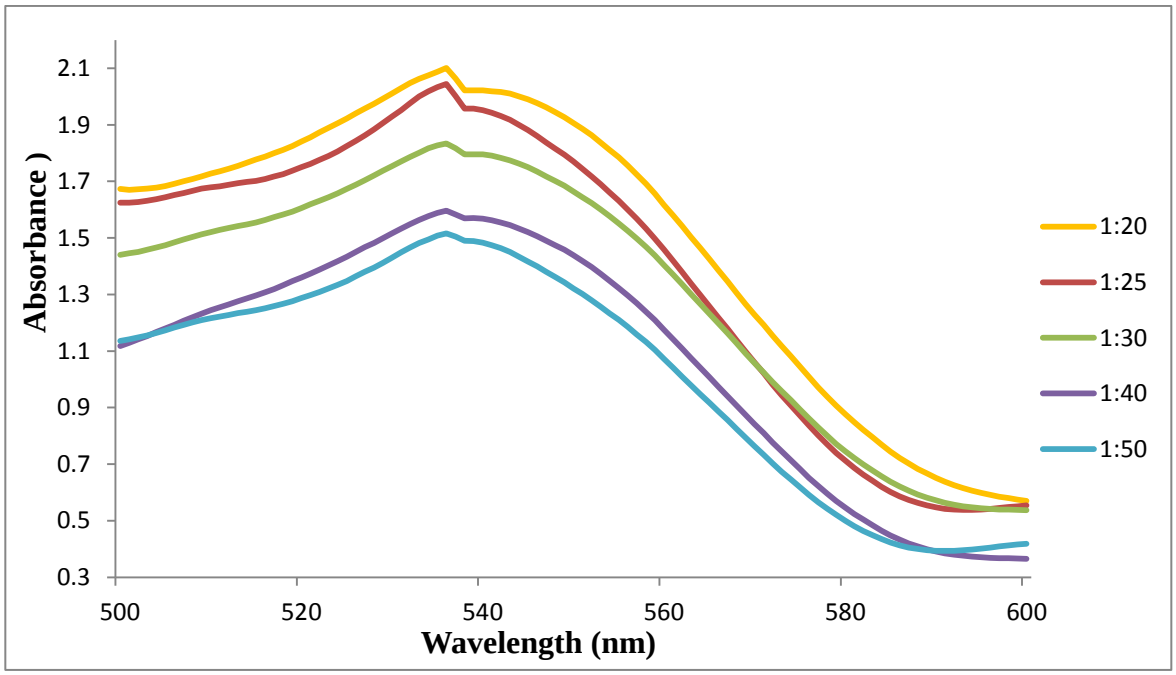


Figure 5.14: UV-visible spectroscopy reading of anthocyanin from *in vivo* leaf extracted using acidified methanol solvent at different concentrations.

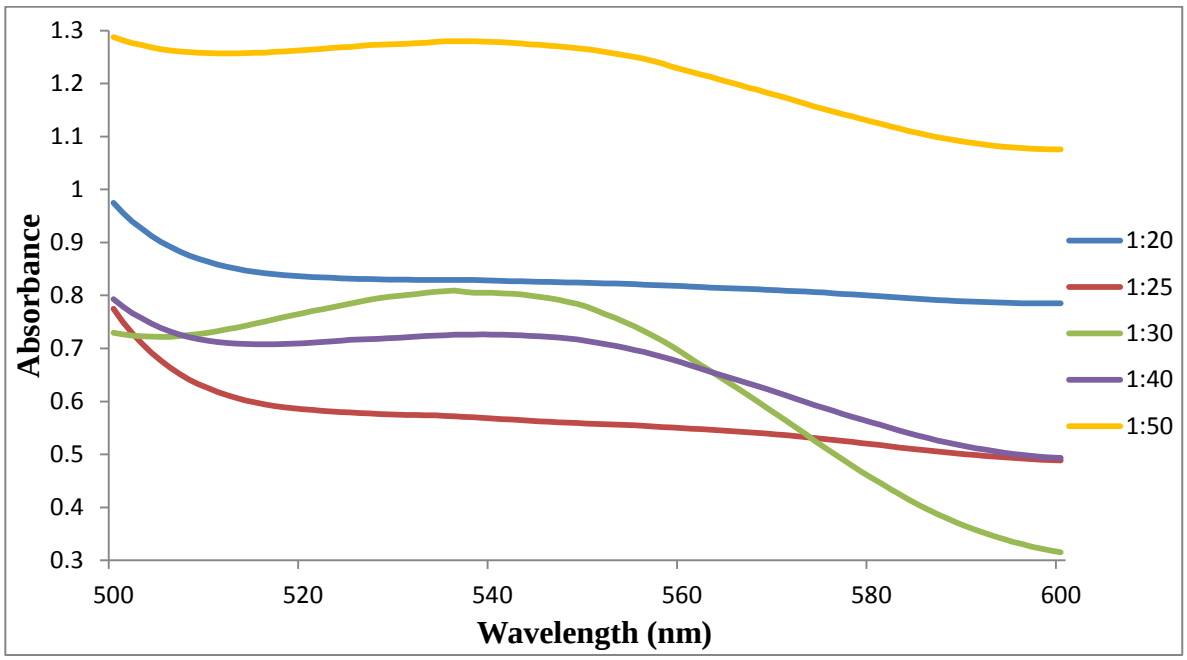


Figure 5.15: UV-visible spectroscopy reading of anthocyanin from *in vitro* leaf extracted using acidified acetone solvent at different concentrations.

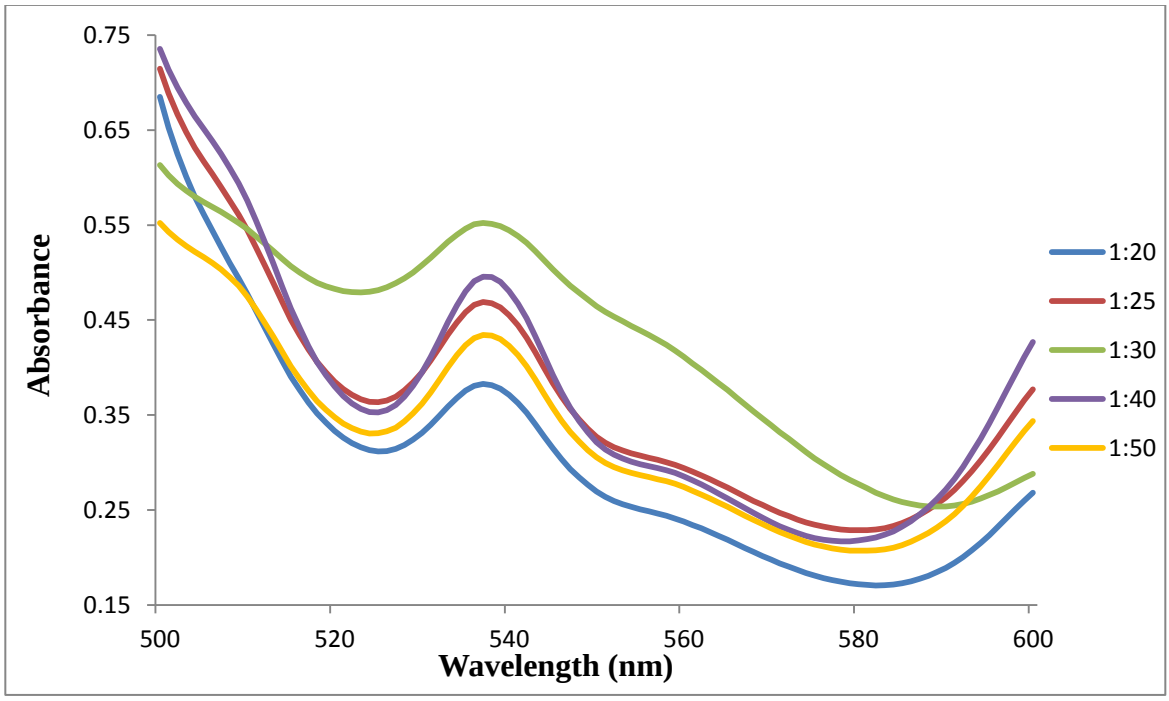


Figure 5.16: UV-visible spectroscopy reading of anthocyanin from *in vitro* leaf extracted using acidified methanol solvent at different concentrations.

5.3.2 Natural Pigment from *In Vitro* Extract for Paint Production

In this experiment, extraction was further studied using *in vitro* leaf extracts since the extracted purple colour was denser compared to *in vitro* petiole extracts as can be seen from Figure 5.17. Two methods (were used for colour extraction and both were mixed with polyvinyl alcohol (PVA) before being coated on glass slides and left to dry for another 24 hours prior to the study of weathering tests (salt and heat tests). As the results, only extraction after 24 hours evaporated at room temperature were further studied because the purple colour became denser while *in vitro* leaf soaked in acidified methanol for three days without evaporating changed into yellow colour (Figure 5.18). Degradation is due to anthocyanins was a light sensitive.

Based on the salt test results (Figure 5.19), it was shown that exposure of anthocyanin coated on glass slide in variable concentrations of salt solutions caused the degradation of anthocyanin. Sodium chloride at a concentration of 1.5% gave a greater degradation rate compared to the other concentrations. Concentration of sodium chloride was also proportional to the degradation of anthocyanin.

For heat test results, as can be seen in Figure 5.20, continuous exposure of *in vitro* anthocyanin coated on glass slide at consistence temperature of 40°C and at room temperature also caused the degradation of the coating. Temperature at 40°C resulted in much degradation rate compared to room temperature.

Figure 5.17: a) Purple colour extraction from *in vitro* leaf explants with a ratio of dried leaf to methanolic acid is 1:5.
b) Very light purple colour extraction from *in vitro* petiole explants with a ratio of dried petiole to methanolic acid is 1:5.

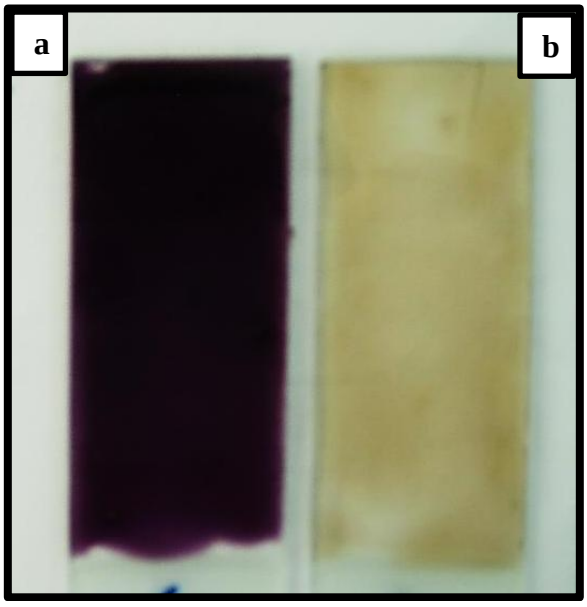


Figure 5.18: a) Purple colour extracts of *in vitro* leaf were taken after evaporation for 24 hours at room temperature and coated on glass slide.
b) Yellow colour extracts of *in vitro* leaf were taken after being soaked in acidified methanol for 3 days without evaporating and coated on glass slide.

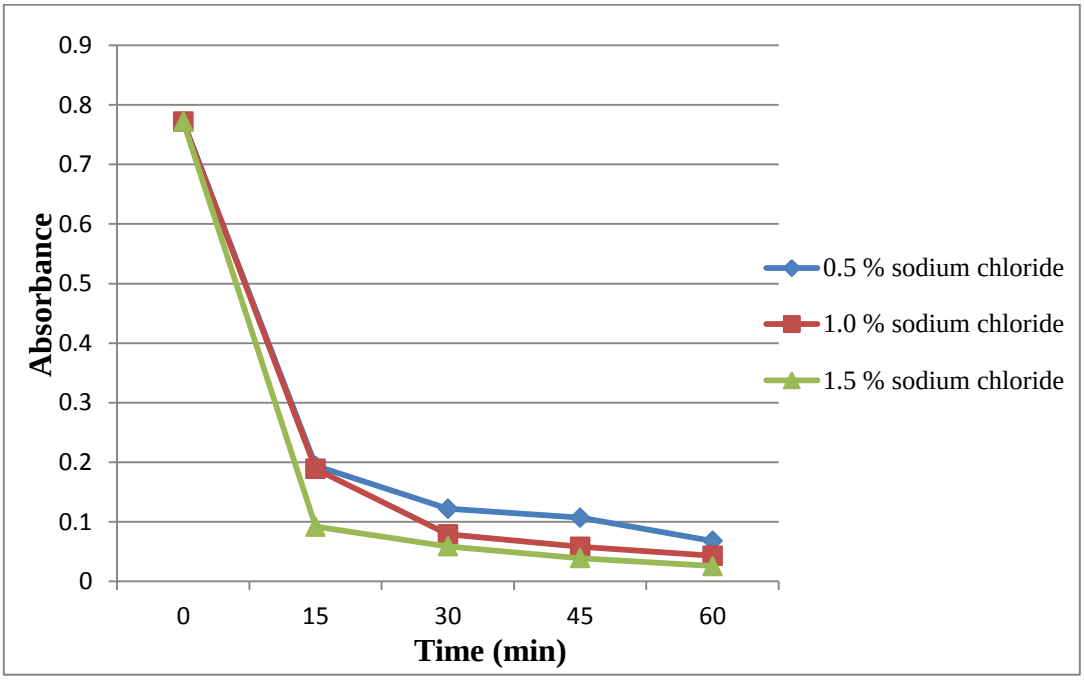


Figure 5.19: Exposure of *in vitro* anthocyanin coated on glass slide at different concentrations of sodium chloride for 60 minutes.

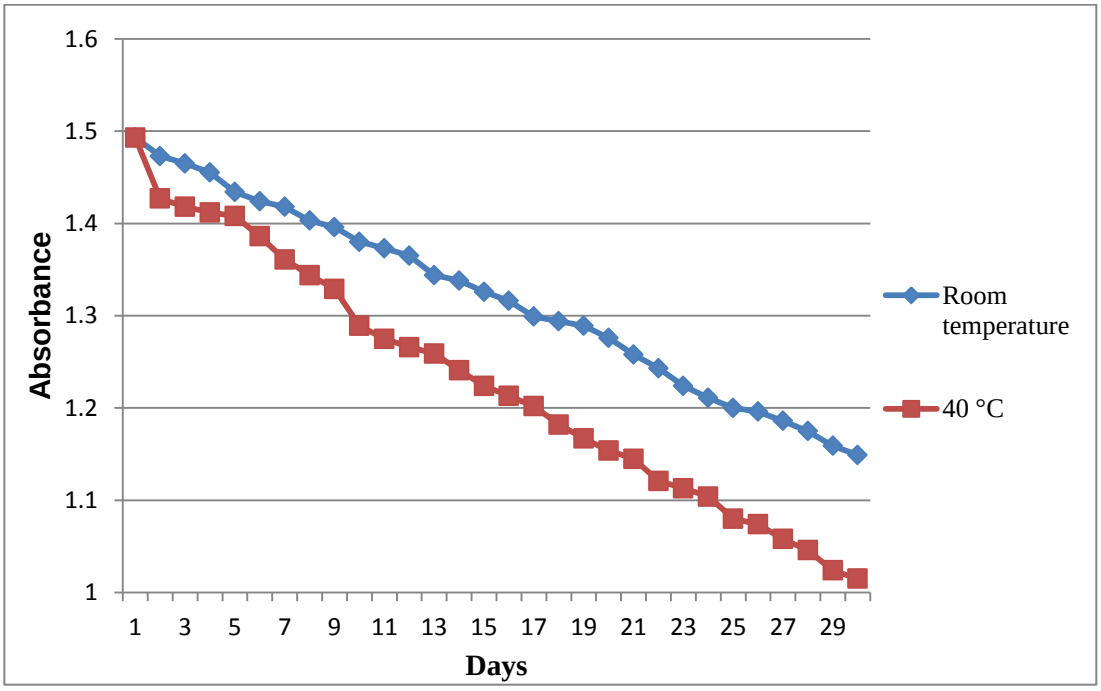


Figure 5.20: Exposure of *in vitro* anthocyanin coated on glass slide at different temperatures for 30 days.

5.4 SUMMARY OF RESULTS

1) Anthocyanin from petiole and leaf samples of *Oxalis triangularis* can be extracted by acidified acetone and acidified methanol with different absorption peak at wavelength 536 nm using UV-visible spectrophotometer.

2) Highest reading of anthocyanin were recorded for petiole from intact plants (*in vivo*) and explants grown *in vitro* of *Oxalis triangularis* extracted in acidified acetone, while *in vivo* leaf extracts recorded highest anthocyanin content in both acidified acetone and acidified methanol.

3) Natural pigment for paint production study showed a degradation reading of anthocyanin for both weathering tests (salt and heat tests).

CHAPTER 6

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITIES OF *Oxalis triangularis*

6.1 EXPERIMENTAL AIMS

Plants are potent biochemical factories which are rich in a variety of secondary metabolites such as tannins, saponins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides and volatile oils. It is necessary to identify the phytochemical components of plants especially medicinal plants because it can be manipulated as an alternative treatment of diseases. The aim of this study was to identify the phytochemical components presence in the *Oxalis triangularis.* Antioxidant activities of *Oxalis triangularis* have not yet been pharmacologically evaluated. Hence, the present study was also undertaken to measure and compare antioxidant activities of *in vivo* and *in vitro* plants for both petiole and leaf extracts of *Oxalis triangularis*.

6.2 MATERIALS AND METHODS

6.2.1 Source of Explants

Petiole and leaf samples were used to screen phytochemical compounds from *in vivo* and *in vitro* samples. Both samples conditions were taken from the same developmental stages. *In vitro* explants were obtained from optimum regeneration of *Oxalis triangularis* which were obtained in CHAPTER 2: *IN VITRO* PLANT REGENERATION OF *Oxalis triangularis* (Table 2.3).

6.2.1.1 Explants Extraction

In this study, four solvents (methanol, ethanol, chloroform and distilled water) were used to extract petiole and leaf of *Oxalis triangularis* grown *in vivo* and *in vitro*. 2 g of petiole from *in vivo* and *in vitro* grown plants were homogenized with 10 ml of methanol using mortar and pestle under low light conditions, separately. The extracts were then centrifuged at 5000 rpm for 5 minutes. After centrifuge, only supernatant were pipetted into cuvette. The procedure was repeated with the solvents of ethanol, chloroform and distilled water and leaf samples grown *in vivo* and *in vitro*.

6.2.2 Phytochemical Screening of Extracts

Phytochemical screening for both plant extracts (petiole and leaf) of *Oxalis triangularis* was performed using standard procedures (Sofowora, 1993; Trease and Evans, 1989).

6.2.2.1 Test for Reducing Sugars (Fehling's Test)

Fehling's solution A and B were mixed in a beaker. The aqueous ethanol extract (0.5 ml in 5 ml of water) was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction. Test was repeated with aqueous methanol, chloroform and distilled water extracts.

6.2.2.2 Test for Terpenoids / Steroids (Salkowski Test)

To 0.5 ml of the aqueous ethanol extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. Test was repeated with aqueous methanol, chloroform and distilled water extracts.

6.2.2.3 Test for Saponins

To 0.5 ml of ethanol extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion. Test was repeated with aqueous methanol, chloroform and distilled water extracts.

6.2.2.4 Test for Tannins

About 0.5 ml of the ethanol extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration. Test was repeated with aqueous methanol, chloroform and distilled water extracts.

6.2.2.5 Test for Cardiac Glycosides (Keller-Killiani Test)

To 0.5 ml of ethanol extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayered with 1 ml of concentrated sulphuric acid. Test was repeated with aqueous methanol, chloroform and distilled water extracts.

6.2.3 Antioxidant Activities

Antioxidant activities of petiole and leaf extracts of *Oxalis triangularis* both for *in vivo* and *in vitro* were determined using the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay using Spectrometer at 517 nm (Hasan *et al*., 2006; Koleva *et al*., 2002; Lee *et al*., 2003). The DPPH solution (0.004 % w/v) was prepared in 95% methanol.

The methanolic extracts of petiole and leaf of *Oxalis triangularis* both for *in vivo* and *in vitro* were mixed with 95% methanol to prepare stock solution (10 mg/100 ml or 100 µg/ml). From the stock solution 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of this solution were taken in five test tubes and serial dilution with same solvent were made to the final volume of each test tube up to 10 ml whose concentration was then 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml, respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing methanolic extracts of *Oxalis triangularis* (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml) and after 120 minutes, the absorbance were taken at 517 nm using spectrophotometer.

Ascorbic acid was used as a reference standard. It was dissolved in distilled water to make stock solution with the same concentration (10 mg/100 ml or 100 µg/ml) of

methanolic extracts of *Oxalis triangularis*. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as a blank. Scavenging percentage of the DPPH free radical was measured using the following equation:-

$$\text{Percentage of DPPH radical scavenging} = \left(\frac{\text{(Absorbance of Control – Absorbance of Test Sample)}}{\text{(Absorbance of Control)}} \right) \text{X } 100$$

Subsequently, the percentages of inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated.

6.2.3.1 Experimental Outline

DPPH solution (0.004 % w/v) was prepared in 95% methanol.

↓

For stock solution, methanolic extracts of petiole and leaf of *Oxalis triangularis* (*in vivo* and *in vitro*) were mixed with 95% methanol.

↓

From the stock solution; 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of this solution were taken in five test tubes and by serial dilution with same solvent were made the final volume of each test tube up to 10 ml.

↓

Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing methanolic extracts of *Oxalis triangularis.*

↓

Absorbance were taken at 517 nm using a spectrophotometer after were kept in dark condition for 120 minutes.

↓

Ascorbic acid was used as reference standard and 95% methanol was used as blank.

6.2.4 Data Analysis

Analysis of phytochemical screening used standard procedures by Sofowora (1993) and Trease and Evans (1989). Antioxidant activities of petiole and leaf extracts of *Oxalis triangularis* both grown *in vivo* and *in vitro* were determined using spectrophotometer at 517 nm.

6.3 RESULTS

6.3.1 Phytochemical Screening of Extracts

In phytochemical screening studies, four different solvents were used to detect the presence of terpenoids/steroids, tannins, saponins, reducing sugar, and glycosides in petiole and leaf extracts of *Oxalis triangularis* grown *in vivo* and *in vitro*. Four different solvents used were methanol, ethanol, chloroform and distilled water.

A reddish brown colouration of the interface indicates the presence of terpenoids but if the interface colour is green bluish, it indicates the presence of steroids. For tannins, observed for brownish green or blue-black colouration were observed. Saponins were detected with formation of an emulsion after shaking the mixture vigorously. Presence of reducing sugar was indicated by formation of red precipitate after boiling the mixture. Glycosides were observed by the formation of brown ring at the interface, a violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

From Tables 6.1 and 6.2, both *in vivo* and *in vitro* petiole and leaf extracts of *Oxalis triangularis* tested gave negative results for the presence of tannins, reducing sugar and glycosides. Instead, both *in vitro* petiole and leaf extracts revealed the presence of steroids. In contrast with *in vitro* petiole and leaf extracts, i*n vivo* extracts for both petiole and leaf in all solvents showed negative results for steroids. Observations on saponins test, only chloroform solvent for both extracts, *in vivo* and *in vitro* showed the negative results.

Table 6.1: Phytochemical screening of *in vivo* petiole and leaf extracts of *Oxalis triangularis.*

Tests	Observations							
	Extracts							
	Petiole				Leaf			
	Solvents				Solvents			
	Methanol	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**
Terpenoids/ Steroids	Cloudy white ↓ Formed two layer (cloudy white and colourless)	Cloudy white ↓ Formed two layer (cloudy white and colourless	Colourless ↓ Formed two layer (cloudy white and colourless)	Formed two layer (light green and colourless) ↓ No reaction	Red ↓ A reddish brown colouration of the interface	Red ↓ A green bluish colouration of the interface	Light green ↓ A green bluish colouration of the interface	Formed two layer (reddish brown and colourless) ↓ A green bluish colouration of the interface
Tannins	Light yellow ↓ Cloudy green	Light yellow ↓ No reaction	Cloudy white precipitate ↓ No reaction	Colourless ↓ No reaction	Red ↓ Cloudy yellow	Red ↓ No reaction	Green precipitate ↓ No reaction	Orange brownish ↓ No reaction

‘**Table 6.1**, continued’

Tests	Observations							
	Extracts							
	Petiole				Leaf			
	Solvents				Solvents			
	Methanol	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**
Saponins	Colourless ↓ Formed emulsion at the surface of mixture	Colourless ↓ No reaction	Cloudy white precipitate ↓ Formed emulsion at the surface of mixture	Colourless ↓ Formed emulsion at the surface of mixture	Light red ↓ Formed emulsion at the surface of mixture	Light red ↓ Formed emulsion at the surface of mixture	Green precipitate ↓ No reaction	Light green ↓ Formed emulsion at the surface of mixture
Reducing Sugars	Colourless ↓ Light red	Light yellow ↓ Cloudy yellow	Cloudy white precipitate ↓ Reddish brown	Colourless ↓ Cloudy yellow	Light red ↓ Cloudy green	Red ↓ Cloudy green	Green precipitate ↓ Reddish brown	Orange brownish ↓ Reddish brown
Glycosides	Colourless ↓ No reaction	Colourless ↓ No reaction	Cloudy white precipitate ↓ No reaction	Colourless ↓ No reaction	Light red ↓ Red	Light red ↓ Red	Green precipitate ↓ No reaction	Light green ↓ Light red

Table 6.2: Phytochemical screening of *in vitro* petiole and leaf extracts of *Oxalis triangularis.*

Tests	Observations							
	Extracts							
	Petiole				Leaf			
	Solvent				Solvents			
	Methanol	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**
Terpenoids/ Steroids	Light green ↓ Formed three layer (red, cloudy white and colourless)	Cloudy white ↓ Formed three layer (light red, cloudy white and colourless)	Colourless ↓ Formed two layer (cloudy white and colourless)	Formed two layer (cloudy white and colourless) ↓ No reaction	Green ↓ A green bluish colouration of the interface	Muddy green ↓ A green bluish colouration of the interface	Light green ↓ A green bluish colouration of the interface	Formed two layer (light red and colourless) ↓ A green bluish colouration of the interface
Tannins	Light green ↓ No reaction	Light green ↓ No reaction	Cloudy white precipitate ↓ No reaction	Colourless ↓ No reaction	Light green ↓ No reaction	Reddish brown ↓ No reaction	Green precipitate ↓ No reaction	Light red ↓ No reaction

‘**Table 6.2**, continued’

Tests	Observations							
	Extracts							
	Petiole				Leaf			
	Solvents				Solvents			
	Methanol	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**
Saponins	Colourless ↓ Formed emulsion at the surface of mixture	Colourless ↓ Formed emulsion at the surface of mixture	Cloudy white precipitate ↓ No reaction	Colourless ↓ Formed emulsion at the surface of mixture	Light yellow ↓ Formed emulsion at the surface of mixture	Light green ↓ Formed emulsion at the surface of mixture	Green precipitate ↓ No reaction	Light red ↓ Formed emulsion at the surface of mixture
Reducing Sugars	Light green ↓ Cloudy yellow	Light green ↓ Cloudy yellow	Cloudy white precipitate ↓ Reddish brown	Colourless ↓ Muddy green	Light yellow ↓ Cloudy green	Reddish brown ↓ Muddy green	Green precipitate ↓ Reddish brown	Light red ↓ Muddy green
Glycosides	Colourless ↓ Colourless	Colourless ↓ No reaction	Cloudy white precipitate ↓ No reaction	Colourless ↓ No reaction	Light yellow ↓ Light red	Light green ↓ Light red	Green precipitate ↓ No reaction	Colourless ↓ Light red

Table 6.3: Summary of phytochemical screening of petiole and leaf extracts of *Oxalis triangularis* grown *in vivo* and *in vitro*.

Tests	**Extracts**															
	Petiole								**Leaf**							
	In vivo				***In vitro***				***In vivo***				***In vitro***			
Solvents	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**	**Methanol**	**Ethanol**	**Chloroform**	**Distilled water**
Terpenoids / Steroids	- -	- -	- -	- -	+ + (steroids)	+ + (steroids)	+ + (steroids)	+ + (steroids)	- -	- -	- -	- -	+ + (steroids)	+ + (steroids)	+ + (steroids)	+ + (steroids)
Tannins	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Saponins	+ +	+ +	- -	+ +	+ +	+ +	- -	+ +	+ +	+ +	- -	+ +	+ +	+ +	- -	+ +
Reducing sugars	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Glycosides	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

Key:
+ + = Positive (present)
- - = Negative (absent)

6.3.2 Antioxidant Activities

Antioxidant activities of petiole and leaf of *Oxalis triangularis* grown *in vivo* and *in vitro* were investigated by using DPPH scavenging test. The scavenging radical activity was compared with standard ascorbic acid. Figure 6.1 shows the antioxidant activities of methanolic extracts of *Oxalis triangularis* petiole and leaf grown *in vivo* and *in vitro.* Ascorbic acid was used as positive control. The results of lC_{50} are presented in Table 6.4. Percentage of scavenging activity was plotted against log concentration and from the graph lC_{50} (inhibition concentration 50) value was calculated by linear regression analysis (Figure 6.1). Value of reference standard (ascorbic acid) was found to be 39.63 µg/ ml. Among all the extracts, methanolic extracts of petiole grown *in vivo* showed the lowest value of lC_{50} (IC_{50} = less than 20.00 µg/ml) indicating higher free radical scavenging activity.

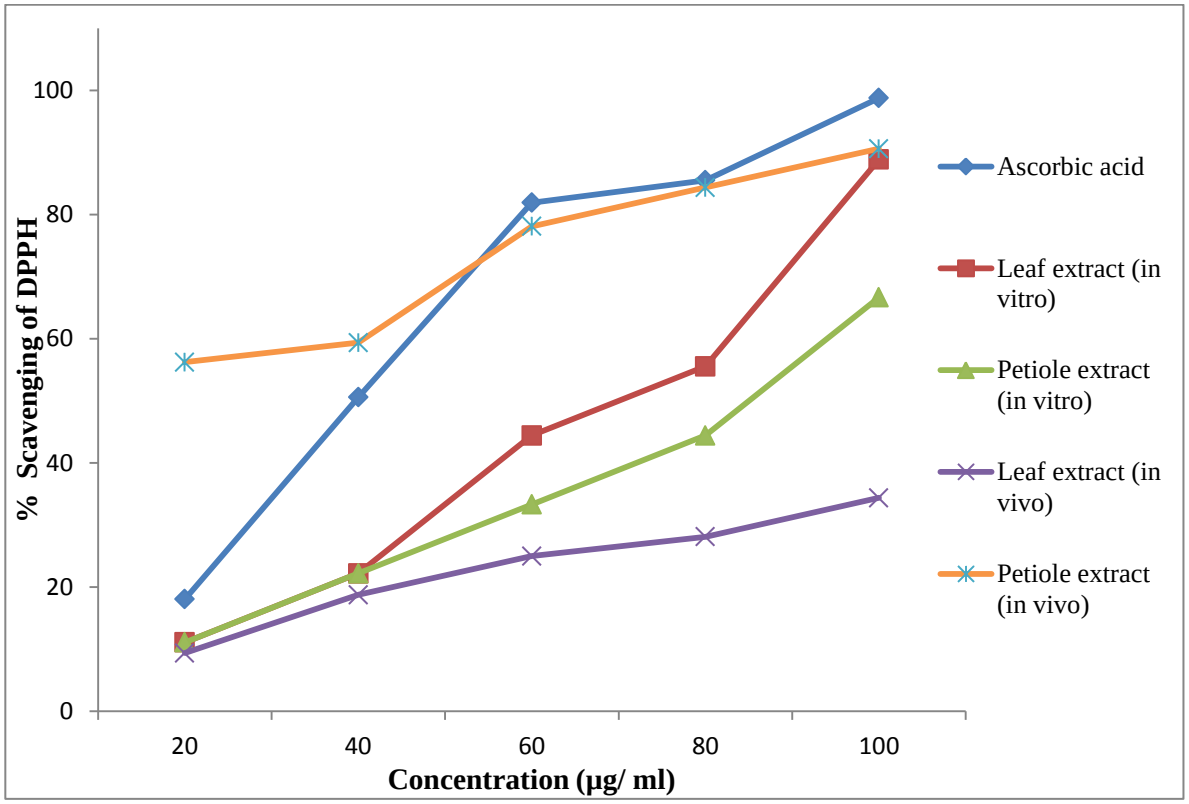


Figure 6.1: DPPH radical scavenging activity of methanolic extracts of *Oxalis triangularis* added to methanolic solution of DPPH and radical scavenging activity measured at 517 nm as compared to standard ascorbic acid.

Table 6.4: lC_{50} value of DPPH assay of methanolic *Oxalis triangularis* leaves and petioles extracts grown *in vivo* and *in vitro*.

Extracts of *Oxalis triangularis*	Concentration (µg/ ml)	Scavenging activity (%)	lC_{50} (µg/ ml)
Ascorbic acid	20	18.07	lC_{50} = 39.63
	40	50.60	
	60	81.93	
	80	85.54	
	100	98.8	
Petiole extracts (*in vivo*)	20	56.25	lC_{50} = less than 20.00
	40	59.38	
	60	78.13	
	80	84.38	
	100	90.63	
Petiole extracts (*in vitro*)	20	11.11	lC_{50} = 85.00
	40	22.22	
	60	33.33	
	80	44.44	
	100	66.67	
Leaf extracts (*in vivo*)	20	9.38	lC_{50} cannot be determined
	40	18.75	
	60	25.00	
	80	28.13	
	100	34.38	
Leaf extracts (*in vitro*)	20	11.11	lC_{50} = 70.00
	40	22.22	
	60	44.44	
	80	55.56	
	100	88.89	

6.4 SUMMARY OF RESULTS

1) Phytochemical screening was performed using standard procedures (Sofowora, 1993; Trease and Evans, 1989).

2) Phytochemical analysis of four solvents tested revealed the presence of steroids and saponins.

3) Tannins, reducing sugars and glycosides were absence in both extracts (petiole anf leaf) of *Oxalis triangularis* grown *in vivo* and *in vitro*.

4) Steroids were only presence in leaf extracts of *Oxalis triangularis* grown *in vivo* and *in vitro*. Saponins were detected in all solvents tested in both extracts grown *in vivo* and *in vitro* except chloroform solvent (shown negative feedback).

5) The antioxidant activities of petiole and leaf of *Oxalis triangularis* grown *in vivo* and *in vitro* in methanolic extracts were evaluated by DPPH free radical scavenging activity.

6) Methanolic extracts of petiole grown *in vivo* of *Oxalis triangularis* showed the lowest lC_{50} value (IC_{50} = less than 20.00µg/ml) among all the extracts which means that the inhibition rate was the highest.

CHAPTER 7

ACCLIMATIZATION OF *Oxalis triangularis*

7.1 EXPERIMENTAL AIMS

Oxalis triangularis regenerated from tissue culture technique need to proceed with the acclimatization procedure to complete the whole process or cycle of plant regeneration study. *Oxalis triangularis* were being acclimatized from cultured bottles (jam jar) to greenhouse and lastly to open field. Hence, the aim of this topic was to establish the plantlets of *Oxalis triangularis* from *in vitro* conditions which provided complete nutrients and under fully supervised conditions to the outside natural environment. Acclimatized *Oxalis triangularis* were tested with different substrates (types of soil) to identify the most suitable substrate for this species to survive and grow normally like those obtained from conventional methods.

7.2 MATERIALS AND METHODS

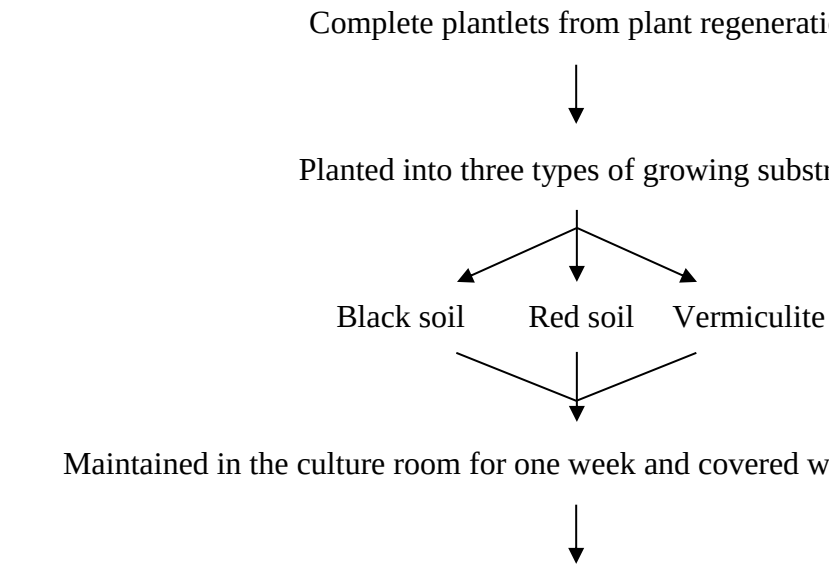
7.2.1 Source of *In Vitro* Plantlets

The optimum *in vitro* regeneration of *Oxalis triangularis* was obtained from leaf explants (Figure 2.1) when the explants were cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP after 8 weeks observation. Eight-week-old adventitious shoots of *Oxalis triangularis* were subcultured into fresh medium with same combinations and concentrations of hormones for further growth. After 4 months in the culture vessels, plantlets were ready to proceed with acclimatization before being transferred to greenhouse. 30 replicates of samples were used.

7.2.2 Acclimatization of *Oxalis triangularis* on Various Growing Substrates

Plantlets were carefully taken out from the jam jar and washed under running tap water until all agar were removed especially at the root parts to prevent from being infected by microorganisms. Plantlets were then planted on three types of substrates; black soil, red soil and vermiculite. All acclimatized *Oxalis triangularis* plantlets were covered with transparent plastic and placed in the culture room for one week. The plantlets were watered every day. After one week, the plants were transferred to a shaded place and transparent plastic were removed. Lastly, the plants were transferred to bigger pots where they began to adapt to natural environment. Percentage of survival was observed upon transfer acclimatized plants to greenhouse. Shoots length (cm) was monitored every 2 weeks.

7.2.2.1 Experimental Outline



7.2.3 Data Analysis

Data obtained were analysed using Duncan's Multiple Range Test (DMRT). The mean with different letters in the same column differ significantly at $p = 0.05$.

7.3 RESULTS

Multiple shoots of *Oxalis triangularis* which obtained from the optimum *in vitro* regeneration in previous study was maintained and subcultured for acclimatization study (Figure 7.1). In acclimatization study, four-month-old *Oxalis triangularis* rooted plantlets from culture vessels (Figure 7.2) were transferred to pots containing three different types of substrates; black soil, red soil and vermiculite. Table 7.1 indicates the pH and moisture contents in the different substrate. From Table 7.2, the highest percentages of survival *in vitro* plants were recorded as 93.00 ± 0.05 % in black soil followed by red soil (83.00 ± 0.07 %) and lastly in vermiculite (67.00 ± 0.09 %). Among them, maximum shoot length (18 cm) was observed with plant grown in black soil while minimum shoots length (0.5 cm) was obtained with plant grown in vermiculite. The Duncan's multiple range test (Table 7.2), revealed the comparison of black soil (10.75 ± 0.74) between red soil (4.83 ± 0.69) and vermiculite (4.62 ± 0.88) was very significant. Figure 7.3 and Figure 7.4 showing *Oxalis triangularis* grew healthy in the greenhouse after acclimatization.

Table 7.1: Condition of growing substrates.

Type of soil	Soil pH	Soil moisture content (%)
Black soil	5.40	60.67
Red soil	6.84	0.80
Vermiculite	8.01	3.13

Table 7.2: Comparison of acclimatization process of *Oxalis triangularis* on various growing substrates.

Type of soil	Percentage of survival (%)	Shoot length (cm) Mean ± SE	Flowering (%)	No. of flowers per plant Mean ± SE	Observations
Black soil	93.00 ± 0.05 b	10.75 ± 0.74 b	83.00 ± 0.07 b	12.40 ± 2.19 b	Plants which survived grown healthy and only a few plants did not survive when being transferred into field.
Red soil	83.00 ± 0.07 a	4.83 ± 0.69 a	23.00 ± 0.08 a	1.60 ± 0.75 a	Plants moderately survived with healthy growth. Red soil need to regularly watering since the soil moisture content is very low.
Vermiculite	67.00 ± 0.09 a	4.62 ± 0.88 a	30.00 ± 0.09 a	2.27 ± 0.78 a	Plant survived with healthy growth. Vermiculite did not need watering excessively because it tended to be contaminated by fungi and hence, inhibited plant growth.

Each value represents the mean ± SE of 30 replicates.
The mean with different letters in the same column differ significantly at 0.05 level.

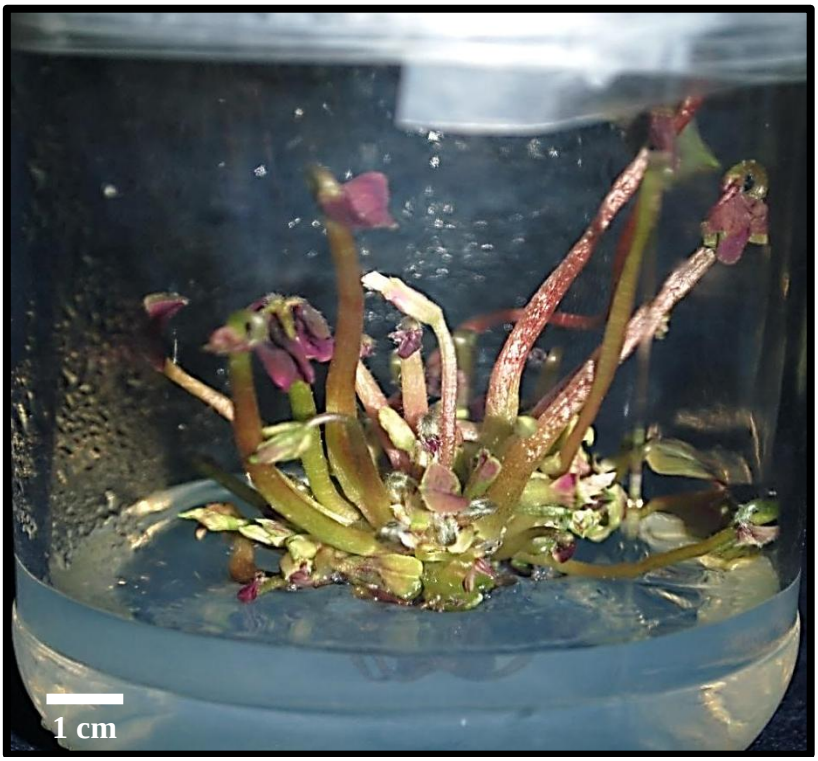


Figure 7.1: Six-week-old *in vitro* multiple shoots of *Oxalis triangularis* cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP.

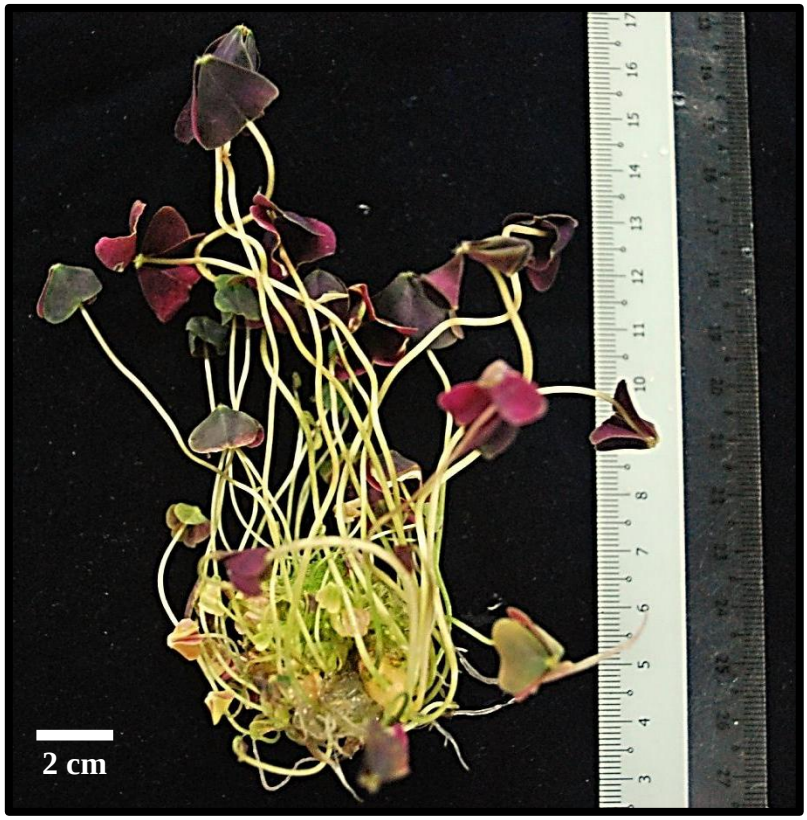


Figure 7.2: *Oxalis triangularis* plantlet after being transferred from culture vessel.

Figure 7.3: Eight-week-old *Oxalis triangularis* plants from *in vitro* regeneration showing healthy growth in the greenhouse.

Figure 7.4: Flowers of *Oxalis triangularis* after plantlets being acclimatized in the greenhouse for 8 weeks.

7.4 SUMMARY OF RESULTS

1) The successful acclimatization of *Oxalis triangularis* is considered achieved when the plants survived and had successfully grown into healthy plant in the field without support of growth hormones and synthetic media.

2) The survival percentage of regenerated *Oxalis triangularis* was affected by different types of growing substrates used.

3) The highest percentage of survival of regenerated plants was obtained in black soil with the percentage of 93.00 %.

4) Maximum shoot length (10.75 ± 0.74 b) and flowering rate (83.00 ± 0.07 b) was also obtained in a plant grown in black.

CHAPTER 8

DISCUSSION

In the last decade, with the emerging and advanced technologies entire plants can develop from any part of plant and also a mass of undifferentiated cells growing in culture in the laboratory. It is now possible to regenerate plants from single cells and protoplasts of numerous species (Narayanaswamy, 1977). Plants bearing specific mutations can be obtained by mutagenising cultured cells (King, 1984). New species have been created by interspecific fusion of protoplasts (Harms, 1983). Foreign genes can be introduced into cultured cells where they are expressed (Fraley *et al*., 1983; Murai *et al*., 1983). Plant tissue culture techniques for ornamental as well as herbaceous plants have been well established (Vijayakumar *et al*., 2010).

In vitro propagation technique for *Oxalis triangularis* involves various steps, for instance selection of explants, its sterilisation procedures, tissue culture techniques and establishment of *in vitro* cultures. Beside the growth regulators, the culture conditions namely temperature, relative humidity and photoperiod also influence the growth and development process of *in vitro* cultures (Hussey and Stacey, 1981). Most important factor for successful micropropagation is aseptic conditions which is free from any microorganisms like fungi, bacteria and virus. Great care should be taken to ensure that the cultures are free from contamination. In addition to maintain an aseptic environment, all culture glassware, media and instruments used in handling tissues, as well as explants itself must be sterilised properly. All operations should be carried out in laminar airflow sterile

cabinet (Chawla, 2003). That is the simplest way to provide aseptic conditions. Various sterilisation agents were used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to the various sterilants, the sequences of using these sterilants has to be standardised to minimise explants injury and achieve better survival (CPRI, 1992). In this study, household bleach (chlorox) was selected as a pretreatment to disinfect intact plants with the addition a few drops of Tween 20, a wetting agent to minimise contamination in the *in vitro* cultures.

In plant regeneration study deals with the role of determination in culture initiation and regeneration of *Oxalis triangularis*. General purpose of culturing is to isolate a group of cells, tissues or organs from plants. Micropropagation is used mainly for getting disease-free plants of superior vigor and productivity (Singh, 1997). Starting plant material or known as explants were cultured under suitable laboratory conditions. As explants, very diverse parts of a plant can be used. The selection will depend on the particular species that is employed and the kind of response desired. Types of explants frequently used are leaf parts, meristems, hypocotyls, root tips, seeds among others. In short, any part of the plant that is able to respond to the culture medium and the growing conditions can be used to propagate in a large scale.

For this study, explants for induction of plant regeneration were taken from six-month-old intact plants which were grown from bulbs in pots with commercial soil in the greenhouse. Two types of explants were used in this study; petiole and leaf. Explants went through the sterilisation procedure prior to culture on the surface of a solid culture medium to make sure the culture did not get contaminated from microorganisms. Plant surfaces

usually contain microflora, bacteria and fungi. Normally microflora are not dangerous to plant tissue but should be eliminated because the microorganisms can grow faster in the culture medium and eventually disrupt growth and development of explants. A good clean explant, once established in aseptic conditions, can be multiplied several times; hence, explant initiation in aseptic conditions should be regarded as a critical step in micropropagation (Ahloowalia *et al*., 2004). Explants were placed on the MS medium which is generally composed of macronutrients, micronutrients, vitamins and PGRs. Gelling agents is added to solidify the medium so the plant cells or tissues can be grown on the surface of medium. Both explants were induced in 22 combinations of hormones (Table 2.1) to identify the optimum culture conditions for plant regeneration of *Oxalis triangularis* in tissue culture technique under aseptic conditions.

Plants can be regenerated from cultured materials either by organogenesis, usually the formation of shoots or by somatic embryogenesis (Krikorian, 1982). From the observation, complete plantlets were obtained directly from both explants in culture media by direct organogenesis. Direct organogenesis is a term referring to the formation of organs directly on the surface of cultured intact explants. The process does not involve intervening of callus formation. The medium for successful organogenesis of *Oxalis triangularis* was the Murashige and Skoog medium supplemented with various combinations of hormones. Results obtained show that, both explants which are petiole and leaf were able to produce multiple shoots in solid medium, while root explants did not show any response after 8 weeks being cultured even after being subcultured into fresh medium.

The most suitable part of the plant for starting tissue cultures will depend on the species and the type of shoot proliferation that is considered the most appropriate. Shoots may be regenerated directly on explants from numerous species; examples are given by Murashige (1974) and by Conger (1981). Most plants can be readily cultured when explants from actively growing plants are used, but others may produce shoots only from plants that are dormant. Robb (1957), for example, reported that bulb scales of *Lilium speciosum* regenerated bulbils freely when taken from plants during spring and autumn periods of growth, but not when taken during summer or winter. Wright and Alderson (1980) on the other hand, found that flower stem explants of *Tulipa* regenerated shoots only when taken from unsprouted bulbs. Other example is regeneration from flower and stem explants in tulip (Kuijpers and Langens, 1997). The formation of flowers or floral parts is rare, occurring only under special circumstances. They are formed for, example, flower stem tissues (Van *et al.,* 1984a,b).

Based on the present study, the regeneration of *Oxalis triangularis* from different explants was significantly affected by different concentrations of hormones. Of the many factors that influence organogenesis *in vitro,* examples, the sources of nitrogen and carbon, pH of the medium and physical conditions of culture, the most important single factor seems to be the plant hormones (Halperin, 1969; Gresshof, 1978; Tran, 1981; Harms, 1982; Krikorian, 1982). In their classical experiments with cultured stem tissue of tobacco, Skoog and Miller (1957) showed that different types of organogenesis can be obtained by varying the concentrations of cytokinin and auxin; when cytokinin are high relative to auxin, shoots are induced; when the concentrations of cytokinin are low relative to auxin, roots are induced; and at intermediate concentrations of cytokinin and auxin, callus are induced. This

basic approach has been used to regenerate a wide variety of dicotyledonous plants (Murashige, 1974; Narayanaswamy, 1977). In general, monocotyledonous species do not show a pronounced response to cytokinins and require high concentrations of potent auxins such as 2,4-D to achieve changes in the development of cultured tissues (Gresshof, 1978; Harms, 1982). Other plant hormones, particularly gibberellins and abscisic acid, sometimes have dramatic effects on organogenesis. In most cases, however, these plant growth regulators do not appear to be essential for plant regeneration (Gresshof, 1978).

The number of shoots produced per explant increased with increasing concentrations of hormones for both explants. However, after the best concentrations and combinations of hormones NAA and BAP was selected in both explants studies, they were further being tested in MS medium supplemented with different PGRs but at the same concentrations. From the Table 2.2 and Table 2.3, the best concentration of hormones which produced the maximum numbers of shoots was obtained on a MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP whereby 14.02 ± 0.84 shoots were produced in leaf explants compared to petiole explants where the maximum numbers of shoots (12.07 ± 0.64) was elicited on MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l KIN. Multiple shoots formation in an *in vitro* culture is more advantageous over a single shoot formation for rapid clonal multiplication as well as for its conservation (Saritha and Naidu, 2007). Addition of cytokinin in *in vitro* shoots regeneration has been studied in various kinds of plant species. Various reports have shown that *in vitro* shoots regeneration could be successfully induced by using the combinations of hormones NAA and BAP. These findings were similar to the results reported in the medicinal plant, *Withania somnifera*, tissue culture on MS medium supplemented with 8.8 µM BAP and 0.5

µM NAA showed multiple shoots regeneration (Saritha and Naidu, 2007). Also, in an *in vitro* multiple shoots regeneration protocol of *Boerhaavia diffusa* by Roy (2008) showed that maximum frequency (90.00 ± 0.07 %) was possible to obtain only when cultured in MS medium containing 6.6 µM BAP and 2.6 µM NAA.

Comparison of explants types in term of excellent maximum shoots formation within 8 weeks showed that leaf explants produced 14 shoots per explant compared to petiole explants where only 12 shoots per explant regenerated. From Table 2.3, it can be seen that combinations of NAA and BAP on shoots formation from leaf explants gave better results compared with other hormones. There are several reports (Shrivastava and Rajani, 1999; Nirmal *et al*., 2000) which suggest that the leaves are the best sources of explants for the induction of multiple shoots. Previous study reported by Varotto *et al*. (2000) on plant regeneration from leaf protoplasts derived callus has been obtained in Italian red chocory (*C. intybus*). Earlier reports on *Cichorium intybus*, plant regeneration from the leaf explants were reported to be better for callus formation and shoot development (Eung *et al*., 1999). In addition, Rehman *et al*., (2003) developed a protocol for the regeneration of plantlets from leaf explants of witloof chicory. These previous findings support the current findings that leaf explants cultured on MS medium supplemented with 1.0 mg/l NAA and 1.5 mg/l BAP treatment generated the maximum frequency of *in vitro* multiple shoots regeneration in *Oxalis triangularis.*

Beyond the best selected combination of hormones on MS medium for both explants, when higher combination of hormones was used on MS medium, number of shoots formed per explant started to decrease. When higher concentration of hormones was

used, both explants managed to grow but with lower number of shoots produced. However, the number of shoots produced was lowest for both explants cultured on MS medium without addition of PGRs. Little is known about how hormones evoke a particular pattern of morphogenesis or the mechanism of induction at the cell and molecular level (Thorpe, 1980). The various hypotheses fall into two broad categories, depending upon how much emphasis is placed on the developmental state of the responding cell (Halperin, 1969; Gresshof, 1978; Henshaw *et al*., 1982; Krikorian, 1982). Acclimatization of *Oxalis triangularis in vitro* plantlets will further be discussed later.

Microscopic studies of the structures trichomes and stomata were done to observe the differences between petiole and leaf explants of *Oxalis triangularis*, both grown under *in vivo* and *in vitro* conditions using scanning electron microscope. Morphologically, there were no differences between *in vivo* and *in vitro* leaf and petiole explants of *Oxalis triangularis*. This result correlated with the previous research on *Murraya paniculata* (Taha and Haron, 2008) whereby there were no differences in vegetative and reproductive characters between *in vivo* and *in vitro* plant observed. It was observed that both *in vivo* and *in vitro* petiole and leaf explants of *Oxalis triangularis* contained same structure of trichomes and stomata.

Trichomes are single or multicellular outgrowths of the plant epidermis and collectively constitute the hairiness of the plant surface. They help to increase the rate of water absorption. It has been well documented that trichomes affect transpiration rate by influencing the water diffusion boundary layer of the transpiring surface (Fahn, 1990). Furthermore, trichomes also influence the water uptake of the plants through temperature

regulation, by either reducing the absorption of radiant energy or by enhancing its dissipation once the water has been absorbed (Johnson, 1975). In other words, it is reducing the rate of water loss due to evaporation in order to keep the leaf surface cool.

Stoma is a minute aperture or tiny pore found on the outer leaf and petiole skin, also known as the epidermis. They consist of two specialised cells, called guard cells surrounding the tiny pore and they are responsible for the opening and closing of the stoma. Function of stoma is for gas exchange in plants. As stomata open in the presence of sunlight, carbon dioxide is taken into the plant to be used in photosynthesis, while oxygen (product of photosynthesis) and water vapour escape from the stomata to the surrounding atmosphere through the process of transpiration. The outer periclinal walls of epidermal cells of the leaf of *Oxalis triangularis* grown *in vivo* and *in vitro* were raised and convex above the epidermal walls (Figures 2.7(c) and 2.7(e)). Similar structures were reported by *Gossypium hirsutum* (Bondada and Oosterhuis, 2000). Other than that, both leaf and petiole samples grown *in vivo* and *in vitro* contained same types of trichomes (long and short trichomes) when observed under scanning electron microscope (Figures 2.6 (e and f) and 2.7 (b and c)).

In Chapter 3, for studies on *in vitro* flowering of *Oxalis triangularis*, explants were taken from optimum *in vitro* regeneration of *Oxalis triangularis* (Table 2.3) to investigate the role of PGRs on inducing *in vitro* flowering. Flowering is an important process which it represent one of the largest groups of primary producers. Their contribution to the production of oxygen as well as that to the nutriment of animals and man is consequently very large. Flowering is a part of plants as their reproductive structures. They come in a

great variety of shapes sizes and colours. Flowers often, though not always, have brightly coloured petals to highlight their natural beauty.

Flowering is considered as a complex process regulated by a combination of environmental and genetic factors, including plant growth regulators (PGRs), carbohydrates, light, and pH of the culture medium (Heylen and Vendrig, 1988). The mechanism that regulates the transition in plants from the vegetative state to reproductive development is poorly understood because the flowering physiology is difficult to study using traditional methods (Zhang, 2007). *In vitro* flowering is not a widespread phenomenon. It occurs spontaneously or deliberately in several herbaceous and geophyte species (de Fossard, 1974; Scorza, 1982; Dickens and van Staden, 1988; Rastogi and Sawheny, 1989; Taixeira da Silva, 2003). Advantage of *in vitro* flowering, is to reduce the influence of environmental factors and, meanwhile, allow for precise control of environment factors and the application of PGRs (Zhang, 2007). It may also offer a means for studying the mechanism of flowering (Zhang, 2007). In addition, *in vitro* flowering may be used for rapid breeding of distant varieties by synchronization of flowering (Zhang, 2007).

In the present study, plantlets derived from leaf explants were subcultured on freshly prepared Murashige and Skoog (MS) medium containing various combinations and concentrations of hormones (Table 3.2). Subculture of plants regenerated on the same medium composition showed continuous growth without any sign of necrosis. The *in vitro* flowers appeared within 8 weeks of culture on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP, MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP,

MS medium supplemented with 0.5 mg/l NAA and 1.5 mg/l BAP, MS medium supplemented with 0.5 mg/l NAA and 2.0 mg/l BAP, MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BAP, MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP and MS medium supplemented with 1.5 mg/l NAA and 1.5 mg/l BAP. Plant growth regulators influence various developmental processes, ranging from seed germination to root, shoot and flower formation (McCourt, 1999).

Plantlets subcultured on MS medium without hormone which served as a control, did not show any response for flowering. Similar results were observed in other combinations of hormones apart from combinations of α-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP). They are plantlets subcultured in MS medium supplemented with Kinetin (Kin) and Indole-3-acetic acid (IAA), Gibberellic acid (GA_3) and BAP, Adenine sulphate (ADS) and BAP and IAA, Ammonium nitrate (NH_4NO_3) and Indole-3-butyric acid (IBA) and also MS medium supplemented with silver nitrate ($AgNO_3$) and MS medium supplemented with Cobalt Chloride ($CoCl_2$) (Table 3.2). In some cases, *in vitro* flowering could be obtained when explants were cultured on a PGRs-free medium, examples, *Celosia cristata* (Taha and Wafa, 2011) and *Murraya paniculata* (Taha and Haron, 2008). Kintzyios and Michaelakis (1999) stated that Kin inhibited the *in vitro* induction of flowers in *Chamomile*. The above statement was proven in the present study, where *in vitro* grown plantlets subcultured on MS medium supplemented with Kin and IAA did not show any response of *in vitro* flowering.

As can be seen in Table 3.2, only combinations of NAA and BAP had shown some response for flowering after 8 weeks of culture. In general, the floral organs of *in vitro*

flowers were smaller in size than those produced in *in vivo* flowers. Earlier studies also stated that *in vitro* flowers and inflorescences are often smaller than normal *in vivo* ones (Rastogi and Sawheny 1989; Nadgauda *et al*., 1990, 1997; Chang and Chang, 2003; Naor *et al*., 2004). This is because the size of plantlets depend on size of glasswares used for *in vitro* regeneration. Hence, the size of *in vitro* flowers will be smaller than *in vivo* flowers, possibly due to limitation of space, gaseous and nutrient supplies. The flowers produced *in vitro* appeared morphologically normal, pale purple in colour (Figure 7.4). Based on the results shown in Table 3.2, high frequency and maximum number of *in vitro* flowering were induced on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP (2.20 ± 0.43), followed by MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP (1.15 ± 0.45). Similar findings were reported in plants like tobacco, bamboo, *Lemna* and maize, where BAP was found to promote floral bud formation in regenerated plantlets (Sridhar and Naidu, 2011). Even though, BAP can be used to induce *in vitro* flowering, from the present results, it is recorded that at the higher concentration of BAP, the percentage of *in vitro* flowering was reduced. However, not all the explants in the successful treatment of *in vitro* flowering responded well. Only MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP had shown some *in vitro* flowering with 90% of flowering were obtained, while, the other successful treatment did not achieve more than 50% of *in vitro* flowering rate.

In *in vitro* flowering study, combinations of hormone auxin (NAA) and cytokinin (BAP) are essential for the induction of flowering. Joshi and Nadganda (1997) made similar observations in *Bambusa arundinacea*. Some researchers demonstrated the essentiality of auxins and cytokinins for the initiation of flowering in *Spathoglottis plicata*

(Rama Murthy *et al.*, 2006). Cytokinin is a common requirement for *in vitro* flowering (Scorza, 1982). Studies on the use of cytokinins for *in vitro* flowering in a number of species like *Murraya paniculata* (Jumin and Nito, 1996), *Fortunella hindsii* (Kanchanapoom *et al*., 2009), *Gentiana triflora* (Ziv and Naor, 2006), *Pharbitis nil* (Galoch *et al*., 2002) and *Ammi majus* (Pande *et al*., 2002) have been reported. Previous studies also have indicated the beneficial effects of BAP on the induction of *in vitro* flowering for other plants such as *Withania somnifera*, *Rauvolfia tetraphylla* and *Anethem graveolens* (Anitha and Ranjitha Kumari, 2006; Jana and Shekhawat, 2011; Saritha and Naidu, 2007). BAP was found to play an important role not only as a growth regulator, but also as a factor regulating floral organ formation of regenerated plantlets (Mandal *et al*., 2000). Not only BAP, effect of other cytokinins on *in vitro* flowering have also been noted by Meeks-Wagner *et al.*, (1989) who showed that in *Nicotiana tabacum*, Kin promoted flower formation.

The combined effect of auxin and cytokinin on *in vitro* flower induction was also reported in a number of previous studies (Ammar *et al*., 1987; Wang *et al*., 2002). Similarly, Taylor *et al*., (2005) reported that phytohormones affected flowering by mediating growth changes within the apical meristem and that cytokinins, in particular, played a key role in the initiation of mitosis and the regulation of cell division and organ formation. PGRs influence various developmental processes, ranging from seed germination to root, shoot and flower formation (McCourt, 1999). The important role of auxin in flower induction and development has been reported in green pea (Franklin *et al*., 2000), *Vigna radiata* (Avenido and Haulea, 1990) and *Vigna mungo* (Ignacimuthu *et al*.,

1997). However, in some species, auxin is either ineffective (Rastogi and Sawhney, 1987) or inhibitory (Deaton *et al.,* 1984) for floral development.

Although *in vitro* flowering has been reported for many plant species, for example, *Ocimum basilicum* (Sudhakaran and Sivasankari, 2002) and *Panax ginseng* (Tang, 2000), reports on *in vitro* flowering are still limited for *Oxalis triangularis*. *In vitro* flowering was observed on MS medium with BAP in *Dendrocalamus hamiltonii* (Chambers *et al.,* 1991). Floral induction in date palm was observed in seedlings grown *in vitro* (Ammar *et al*., 1987). In *Arabidopsis thaliana,* isopentenyl adenosine (2iP) induced floral bud formation (He and Loh, 2002). In roses, the combination of Thidiazuron (TDZ) or Zeatin (Zn) and NAA induced flowering *in vitro* (Wang *et al*., 2002). In tobacco, *in vitro* flowering was observed after the addition of growth regulators such as BAP and IAA (Peeters *et al.,* 1991). *In vitro* flowering of *Capsicum fruitescens* was observed in liquid MS medium without growth regulators (Tisserat and Galletta, 1995). *In vitro* flowering and pod formation were observed in *Arachis hypogea* on MS medium with KIN or BAP and IAA or NAA (Narasimhulu and Reddy, 1984). All these findings were also in agreement with the present study with induction of *in vitro* flowering on MS medium supplemented with auxin (NAA) and cytokinin (BAP), eventhough, hormones combinations required different concentrations (Table 3.2). Although *in vitro* flowering of *Oxalis triangularis* are still limited, the obtained knowledge might help seek a better understanding to improve flowering under controlled conditions.

Optimum regeneration of *Oxalis triangularis* from Chapter 2 were further studied for the production of synthetic seeds. Five-week-old multiple shoots of *Oxalis triangularis*

were used as explants. Shoot tips contain meristematic tissues which are dividing actively throughout the life of the plant. Shoot tips contain meristematic tissues with high capacity for cell division, thus synthetic seed developed from shoot tips generally yield good response than other non-embryogenic vegetative propagules (Verma *et al*., 2010). The reason for such a difference in response is not clear, but it may be related to mitotic activity being greater in the meristem of the shoot tips than in lateral buds, which are subject to apical dominance (Ballester *et al.,* 1997).

The microshoots were carefully isolated from the culture containers and were encapsulated in the 3% w/v sodium alginate complexing with calcium chloride ($CaCl_2$). Sodium alginate at 3% was found to be the most effective for encapsulation of the explants as the beads formed were firm and could withstand breakage. At lower concentrations, beads were fragile and too difficult to handle, while at high concentrations of alginates, the beads were hard and this might hinder or prevent the emerging shoots. Similar results were reported by Saiprasad and Raghuveer (2003) for the encapsulation of protocorm-like bodies in *Dendrobium, Oncidium* and *Cattleya*. In order to get optimum bead hardness and rigidity, the beads were hardened in the solution of 0.2 M $CaCl_2$ for about 30 minutes. The encapsulation matrix serves as a reservoir and supplies the essential nutrients to encase explants (Mohanraj *et al*., 2009).

Effect of storage duration on encapsulated beads was studied for 7 and 30 days. Day 0 was considered as control. Encapsulated beads were stored at 4°C ± 1°C in dark condition before being cultured on MS basal medium. Storage at low temperature (4°C ± 1°C) was preferable probably due to least desiccation that might have occurred under low

temperature because lower dessication inhibits the embryo respiration. At low temperature, no metabolism occurs. Sharma *et al*., (1992) made similar observation on *Dendrobium wadianum*. The observation of plantlets regeneration from synthetic seeds showed that the plantlets growth were healthy and free from any morphological variations. Roots started to emerge from encapsulated beads on Day 20, while shoots started to emerge on Day 4. From Table 4.2, all the synthetic seeds of *Oxalis triangularis* were able to produce or protruding roots from beads.

As stated in Table 4.2, plant regenerated from encapsulated beads at different period of culture showed varying percentages of conversion frequency. Results of the present study showed that conversion frequency on Day 7 (96.67%) gave maximum regrowth percentage compared to the encapsulated beads cultured immediately after the encapsulation of microshoots (Day 0). On Day 30, the germination rate was reduced (90%). Similar findings were recorded from previous studies. Ikhlaq *et al*., (2010) stated that germination percentage of synthetic seeds of olive cv. 'Moraiolo' stored at 4°C for 15 days increased compared to synthetic seeds cultured immediately after encapsulation (control). This indicate that, the percentage of conversion frequency increased with increasing storage days up to certain extent after which the viability of encapsulated beads reduce.

A decline in germination percentage with increased storage is probably due to an anaerobic environment in the capsule. This could be due to the fact that embryos are not developmentally arrested and they continue active respiration (De, 2007). Nevertheless, storage of encapsulated somatic embryos at low temperature for longer periods resulted in a sudden drop in the conversion percentage to less than 6% after 130 days and after 140 days

(Pintos *et al*., 2008). Redenbaugh *et al*., (1987a) proposed that a decline in the conversion frequency observed among encapsulated propagules stored at low temperatures may be due to inhibited respiration of plant tissues, perhaps due to alginate cover.

The number of shoots produced from encapsulated beads were also higher from synthetic seeds stored on Day 7 (4.57 ± 0.72) in comparison to control (Day 0) treatments (2.10 ± 0.57). On Day 30, the number of shoots was recorded as 3.97 ± 0.65 shoots. This may be because the beads contained plant growth regulators (PGRs) where 3% sodium alginate were added with 1.0 mg/l NAA and 1.5 mg/l BAP hormones. Presence of PGRs in the beads helped to promote shoot growth of the cultivated synthetic seeds of *Oxalis triangularis*. Similar experiment was conducted for encapsulation of adventitious buds of Mulberry by Machii (1992) and he observed that synthetic seeds grew vigorously when nutrient component and phytohormones were added during encapsulation. Soneji *et al*., (2002) reported that germination of synthetic seeds to plantlets appeared to depend on the hormonal concentrations in bead medium. In *Morus alba* it was found that BAP in seed beads gave desirable result in synthetic seeds germination (Machii, 1992). The effects of various auxins on seed germination process include direct and indirect evidences indicating the involvement of auxins in seed germination (Chiwocha *et al*., 2005).

Studies were also carried out with comparison of synthetic seeds of *Oxalis triangularis* sown directly on MS basal medium, black soil and sterile black soil. The present study suggested that conversion frequency of synthetic seeds of *Oxalis triangularis* into complete plantlets were better under *in vitro* conditions compared to *in vivo* conditions. Synthetic seeds of *Oxalis triangularis* have the capability to response when cultured on MS

basal medium, resulting in a high rate of plantlets regeneration. More than 60% successful regeneration from synthetic seeds of *Oxalis triangularis* was observed (Table 4.3). Successful plant regeneration from synthetic seeds of *Oxalis triangularis* on MS basal medium was probably because the beads were cultured under aseptic conditions. So, probability for the beads to get contaminated was much lower compared to seeds directly sown on *in vivo* substrates.

Observations after 7 days of encapsulated beads sowing on the substrates both in black soil and sterile black soil gave negative results which failed to survive and finally died. On black soil substrate moistened with distilled water, the encapsulated beads during the first 7 days showed shrinking signs where they failed to germinate and eventually became contaminated with fungal infection. While synthetic seeds of *Oxalis triangularis* which were sown on sterile black soil moistened with distilled water did not show any sign of contamination but with no germination response observed. However, the beads eventually shrunk. Both synthetic seeds of *Oxalis triangularis* sown in black soil and sterile black soil moistened with liquid MS without sucrose gave similar observations where all encapsulated beads were contaminated with fungal infection after 7 days.

The potential of synthetic seeds to be sown directly on *in vivo* substrates has received limited attention. From the previous studies, Preece and West (2006) were successful in sowing *Hibiscus moscheutos* directly into a greenhouse hydroponic system, both with and without light pretreatment. Bapat and Rao (1990) conducted research on *in vivo* growth of encapsulated axillary buds of mulberry (*Morus indica*) and reported that addition of a fungicide to the alginate beads prevented contamination of the bud and

increased survival of the buds when sown in soil. Ramakrishnappa (1998) also observed that addition of fungicide (0.1% carbendazim) and bacteriocide (0.1% streptomycin) into the encapsulating gel brought down the incidence of fungal and bacterial contamination to a minimum level and nearly 95% of the capsules remained healthy. A lot of researches need to be done to increase germination percentage of synthetic seeds to be directly sown on non-sterile environment, such as in greenhouse or directly in the field.

After successful and promising results in the studies of *in vitro* flowering and synthetic seeds production of *Oxalis triangularis* where source of explants were obtained from optimum adventitious shoots regeneration of *Oxalis triangularis* (Chapter 2, Table 2.3), studies were continued with the anthocyanin extraction to identify suitable solvents for extraction. Anthocyanin is water-soluble flavonoid compounds that are widely distributed in plants. They are responsible for blue, purple, violet, magenta, red and orange plant colouration (Jackman and Smith, 1996). Petiole and leaf of *Oxalis triangularis* were used as samples to study the presence of anthocyanin at different types and concentrations of solvents. Absorbance measurements were taken using UV-visible spectroscopy at the wavelength of 536 nm. This is possible because anthocyanin have a typical absorption band in the 490 nm to 550 nm region of the visible spectra. This band is far from the absorption bands of other phenolics, which have spectral maxima in the UV range (Fuleki and Francis, 1968).

From all the graphs (Figure 5.1 until Figure 5.16), it can be seen that the absorption peaks were present at the range of anthocyanin typical absorption band of the visible spectra (490 nm to 550 nm). Table 5.1 and Table 5.2 showed the absorbance of

anthocyanin taken at the wavelength of 536 nm. Absorbance reading of anthocyanin in petiole samples of *Oxalis triangularis* was higher when extracted by acidified acetone solvents compared with methanol solvents. Both petioles samples obtained from *in vivo* and *in vitro* plants gave higher value of absorption peaks of anthocyanin pigment at the lower concentrations of extract (400mg/mlml). Similar observations were recorded for the leaf samples of *Oxalis triangularis*. When extracted by acidified acetone solvents these samples showed better absorbance reading of anthocyanin pigment. However, leaf samples for both *in vivo* extractions gave higher absorption peaks of anthocyanin pigment at the lower concentrations of extract (100mg/ml). In contrast with *in vitro* leaf explants, the anthocyanin increased with the increasing of solvent concentrations.

Extractions were done under dark conditions because light exposure could promote pigment destruction. Extraction procedures have generally involved the use of acidic solvents, which denatured the membranes of cell tissue and simultaneously dissolve pigments. Fuleki and Francis (1968) noted that 100% of alcohol (methanol or acetone alone) is not preferable as an extracting solvent, because the presence of a little water is required for the extraction of hydrophilic anthocyanins. Anthocyanins are soluble in polar solvents, and they are normally extracted from plant materials by using methanol that contains small amounts of hydrochloric acid or formic acid, which favour the formation of the stable flavylium ion. The acid lowers the solutions pH value and prevents the degradation of the non-acylated anthocyanin pigments. However, as hydrochloric acid or formic acid is concentrated during the evaporation of the methanol-hydrochloric acid or methanol-formic acid solvent, pigment degradation occurs (Kong, 2003).

Acetone has also been used to extract anthocyanins from several plant sources (Giusti *et al.*, 1998; Garcia-Viguera *et al.*, 1998). In comparison with acidified methanol, this technique allows an efficient and more reproducible extraction, avoids problems with pectins, and permits a much lower temperature for sample concentration (Garcia-Viguera *et al.*, 1998). Several authors reported that aqueous acetone was better than various alcoholic solvents for fruit procyanidins, anthocyanins and other phenols (Garcia-Viguera *et al.*, 1998; Kallithraka *et al.*, 1995). However, more recently Lu and Foo (2001) observed significant anthocyanin interaction with aqueous acetone to form pyrano-anthocyanidins which significantly lowered quantities of detectable anthocyanins. This reaction was significantly affected by time of anthocyanin-acetone interaction and temperature.

Anthocyanin can be used for paint production from natural colour. Basically, there are two purposes in making paint; to decorate and to protect which makes investigating the properties of paint very important in producing high quality paint while maintaining the colour of the paint. High demands of natural paint are increasing for healthy living and also as an environmental friendly product. Therefore, weathering tests in the early process of paint making is a very important step. In this study, the factors that can affect the quality of natural paint colour were identified.

In this study, the extraction of colour was chosen either from petiole or leaf explants of *Oxalis triangularis* grown *in vitro*. The purple colour extracted from leaf explants were chosen based on the most concentrated purple colour and ignoring the reading of anthocyanin content. The purple colour of the extracts started to fade with increasing of solvent concentrations.

Based on the salt test results (Figure 5.19), the degradation of *in vitro* anthocyanin was proportional to concentration of sodium chloride. It was also observed in heat test; both purple colour of *in vitro* anthocyanin coated on glass slides placed in an oven (40°C) and at room temperature were reduced with increasing time up to 30 days (Figure 5.20). The purple colour faded quickly as the temperature increased. The temperature of 40°C in the oven was mimic sunlight source while sodium chloride was mimic rain water. Polyvinyl acetone (PVA) was used as a binder because it can act as a glue which bind the colour extracted from leaf explants of *Oxalis triangularis* on the glass slides.

Both weathering tests gave similar observations, whereby the purple colours of *in vitro* anthocyanin faded time. This study was important to analyse the early conditions that promoted colour fading and poor adhesions. Salt test is a common test for testing the protective coatings.

There are a few reasons why paint coating becomes discoloured day by day. According to Steel Structures Painting Council, Causes and Prevention of Paint Failure, Good Painting Practice, Steel Structures Painting Manual (1982), discolouration is due to chemical reaction and improper pigmentation. So to solve the problems there is a need to use stable binder and stable pigments (Bayer and Zamanzadeh, 2004). Addition of stabilizer also can help in stabilizing or maintaining the colour for longer periods. Observations from the salt test also showed that coating started to erode when immersed in salt water (sodium chloride) after some time and resulted in the surface becoming rough. This is due to lack of protection against water and aqueous systems. This is a serious problem with aqueous systems containing corrosive compounds such as chlorides (Bayer

and Zamanzadeh, 2004). In addition, Bayer and Zamanzadeh (2004) also mentioned that improper coating selection, as well as incompatibility of coating layers could be attributed to erosion of the primer with the substrate surface. The problem can be solved by using coatings with strong adhesion and proper drying conditions. However, further research need to be done in order to have a stable results and to produce high quality of end products.

Phytochemical screening of *Oxalis triangularis* are presented in Table 6.3. Phytochemical screening is a process of tracing plant constituents and is an important step in the chemical and pharmacological study of a medicinal plant. Evaluation of phytochemical screening of *Oxalis triangularis* in this study was carried out according to Sofowora (1993) and Trease and Evans (1989) by application of qualitative tests; terpenoids/ steroids, tannins, saponins, reducing sugars and glycosides. Investigations on the phytochemical screening tested on petiole and leaf extracts of *Oxalis triangularis* grown *in vivo* and *in vitro* on various solvents revealed the presence of steroids and saponins. These results are also comparable to phytochemical analysis of *Oxalis corniculata* (Raghavendra *et al*., 2006).

Triterpenoids are the most abundant plant terpenes, they include plant steroids and are components of saponins and steroidal glycosides (Harborne, 1998; Bruneton, 1999). Bruneton (1999), Heinrich *et al*., (2004) and Gurib-Fakim (2005) reported that triterpenes contain anti-inflammatory and hemolytic properties. Presence of steroids in plants also enhanced intestinal absorption of natrium ion and water which good for anti-diarrheal properties.

Another secondary metabolite compound observed in leaves and petioles extracts of *Oxalis triangularis*, both *in vivo* and *in vitro* in all solvents tested (except chloroform solvent) was saponins. The presence of saponins was confirmed by the formation of emulsion at the surface of mixture due a lipophilic portion in its chemical structure, called aglycone or sapogenin, and a hydrophilic portion, formed by one or more sugars that provide detergent properties (Farnsworth, 1966; N'guessan *et al*., 2009).

In the antioxidant activities, DPPH test provides information in the reactivity of the test compounds with a stable free radical. DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) radical scavenging method is rapid, sensitive, simple, cheap and independent of sample polarity (Koleva *et al.*, 2002). Radical scavenging activity of plant extracts against stable DPPH was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, the concentration of DPPH decreased due to the scavenging ability of the extracts and standard (ascorbic acid) (Wei *et al.*, 2010). The changes in colour from purple to light yellow were measured at 517 nm through UV-visible spectrophotometer. Rapidly the DPPH obtained electron donated by an antioxidant compound, the DPPH was decolourized, which can be quantitatively measured from the changes in absorbance, the more effective the antioxidant activity of the compound to prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Fang *et al.*, 2002; Yen and Duh, 1994,).

The lower value of lC_{50} indicated higher free radical scavenging activity, which means strong antioxidant activities in the samples. The present study, showed that *in vivo* petiole of *Oxalis triangularis* had a lower reading compared to the other extracts with the value of lC_{50} = less than 2µg/ml (Table 6.4). The high scavenging property of petiole

extracts of *Oxalis triangularis* may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as a radical scavenger. Ascorbic acid which was used as a standard antioxidant in this study, acts as a chain breaking scavenging agent that impairs the formation of free radicals in the process of intracellular substances formation throughout the body including collagen, bone matrix and tooth (Kanatt *et al*., 2007; Beyer, 1994).

The crucial success in tissue culture studies of *Oxalis triangularis* is dependent on the last stage of plant regeneration which is acclimatization process. Plant regeneration was achieved successfully through the establishment of explants taken from intact mother plants. Explants from petiole and leaf of *Oxalis triangularis* could be induced to produce complete plantlets under sterile conditions when complete nutrients and plant growth regulators (PGRs) were provided. Further growth of the adventitious shoots from plant regeneration study were obtained when being subcultured in large sterile containers (jam jars) and maintained under the same incubation conditions of light and temperature as before.

After the successful growth of plantlets in *in vitro* conditions, study was continued with the acclimatization process to complete the plant regeneration process. *In vitro* plantlets were introduced to the environmental conditions where the plantlets needed to adapt and to survive in their own for their further growth. However, all these plantlets need some adjustment with the outdoor conditions to adapt physiological changes. It cannot be done suddenly because the plantlets might not adapt with the harsh conditions and subsequently plants wither in the process.

During *in vitro* growth, plants developed under controlled conditions comprising of enclosed environments, with limited gases exchanges, with high moisture in the container, low light intensity and the use of sugars from the medium as a source of carbon and energy (Preece and Sutter, 1991; Sciutti and Morini, 1993; Pospísilová *et al*., 1999). Thus, the transplanting of *in vitro* grown plantlets and the complete establishment in the greenhouse can be complicated for some species (Van Huylenbroeck and Debergh, 1996; Ross-Karsten *et al.*, 1998). *In vitro* plantlets usually cannot survive well if transferred directly to the field. It needs to be done step by step to transfer them to the open environments, so they can slowly successfully adapt with the outside environment.

There are few reasons why plantlets need to undergo acclimatization procedure. In culture jam jars, the humidity is high and plantlets cannot efficiently carry out photosynthesis process. When acclimatized plants are transferred to the fields, they will wither due to loss of water. Causes for acclimatized plants mortality are partially due to water stress effects. Tissue culture plantlets have a divergent anatomy compared to non-cultured plants which includes reduced quantities of epicuticular wax (Grout, 1975; Grout and Aston, 1977), reduced cuticular development (Wetzstein and Sommer, 1982), extensive intercellular spaces (Brainerd *et al.*, 1981; Grout and Aston, 1978; Wetzstein and Sommer, 1982), and stomata which are raised, larger, of higher density (Wetzstein and Sommer, 1983), and with reduced stomatal closure (Brainerd and Fuchigami, 1981). Despite continuous supply of nutrients and PGRs but can result in the formation of plantlets of abnormal morphology, anatomy and physiology (Kozai, 1991; Pospíšilová *et al.*, 1992, 1997; Buddendorf-Joosten and Woltering, 1994; Desjardins, 1995; Kozai and Smith 1995). Therefore, after *in vivo* transplantation plantlets usually need some weeks of

acclimatization with gradual lowering in air humidity (Preece and Sutter, 1991; Kadleček, 1997; Bolar *et al.*, 1998).

Microprapagated plantlets are first hardened before they ready for acclimatization. Acclimatization is a process where complete *in vitro* plantlets adjust to a gradual change in the environment, allowing them to maintain performance or adapt well with the surrounding across a range of environmental conditions. In this present study, *Oxalis triangularis* plantlets were being acclimated from *in vitro* cultured conditions to field conditions. Even though the plantlets already have roots they were still not yet capable of supporting themselves in the soil. This is because in *in vitro* regeneration, explants were cultured on provided medium that contains complete nutrients for plants growth. In addition, some of the species or cultivar needs to be subcultured onto rooting medium to induce good sturdy well-rooted plants before being transferred to soil. It is essential to induce rooting for high survival rate at the outside environments.

Plantlets were transferred out from sterile containers and roots were washed gently under running tap water to remove any leftover agar to prevent microbial attack. Subsequently, *Oxalis triangularis* plants were planted into pots containing soil and covered with transparent plastic to maintain high relative humidity. Plants were then placed in the culture room for two weeks and were watered every day. After two weeks in the culture room, *Oxalis triangularis* became more sturdy and firm and yet can be ready to be placed under shade area with indirect sunlight and also need to water the plants every day.

In acclimatization study, three different growing substrates were tested; black soil, red soil and vermiculite. Study was done to compare the best growing substrates to plant the *Oxalis triangularis* without much of plants end up dying after transferred into *in vivo* conditions.

Plants grow on soils with a wide range of pH. The pH condition of soil is one of environmental conditions that affect the quality of plant growth and nutrients availability. For most plant species, there is an optimum pH where most plants grow best within a pH range of pH 5 to 6. However desirable pH requirement for various plant growths can vary depending on plant species. When soil is acidic, minerals such as zinc, aluminum, manganese, copper and cobalt are soluble and available for plant uptake. However, in highly acidic soils, calcium, phosphorus and magnesium become tied up and are unavailable, and manganese and aluminum can reach toxic level. Therefore, extreme pH can be toxic to plant and affect plant growth. Alkaline soil on the other hand, may contain a higher quantity of bicarbonate ions, and this can affect optimum growth in plants by interfering with the normal uptake of other ions. In highly alkaline soils, micronutrients such as iron, zinc, copper and manganese become chemically tied up and are sparingly available for plant use.

As seen in Table 7.1, each type of soil has different pH, whereby the black soil is strongly acidic (pH 5.4), red soil is very slightly acidic (pH 6.84) and vermiculite is slightly alkaline (pH 8.01). Despite different pH range (pH 5.4 to pH 8.01) *Oxalis triangularis* could adapt well with the outside environments after being acclimatized. Moisture content

in black soil was also high with 60.67% compared to red soil and vermiculite with 0.80 % and 3.13 % respectively.

The survival percentage of *in vivo Oxalis triangularis* after acclimatization was affected by different growing substrates used. By referring to Table 7.2, black soil recorded high percentage of plants survival (93.00 ± 0.05%,) after acclimatization followed by red soil (83.00 ± 0.07%) and lastly vermiculite (67.00 ± 0.09%). After 2 weeks being transferred to greenhouse, all the acclimatized plants started to grow normally like conventional method of planting. After 4 weeks, petiole of *Oxalis triangularis* started to become firmer and stronger. The leaves also began to grow wider and the colours of leaves were darker compared to *in vitro* cultured plants. Previous studies have stated that the growth of tissue cultured plants may seem to be slow during the initial weeks after transplanting. This is due to residual presence of growth regulators that they were exposed to during micropropagation (Perez and Hooks, 2008).

The highest percentage of survival for *Oxalis triangularis* were achieved in black soil with the average shoot length recorded as 10.75 cm (Table 4.12). Even though average of shoot length of survival acclimatized plants in red soil and vermiculite were about the same, the percentage of survival in red soil (83.00 ± 0.07%) was higher compared to acclimatized in vermiculite (67.00 ± 0.09%). Vermiculite showed a low capacity of aggregation with the roots, which negatively affected plant development, in agreement with Hoffman *et al*., (2001) who worked with apple tree rootstocks and Silva *et al*., (2003) with gloxinia plantlets (Rodrigues *et al*., 2005). Flowering percentage of regenerated *Oxalis triangularis* plants was also high in black soil with respect to flowering rates (83.00 ±

0.07%), followed by vermiculite and red soil at 30.00 ± 0.09% and 23.00 ± 0.08%, respectively. Twelve flowers (12.40 ± 2.19) per plant were observed in black soil and the morphological charactristics of the flowers were no different with flowers produced from conventional method of planting of *Oxalis triangularis.* The colour of flower was pale purple with five petals (Figure 7.4).

It is hoped that the present studies on *in vitro* plant regeneration, *in vitro* flowering, synthetic seeds, pigment extraction, phytochemical and antioxidant activities and acclimatization of *Oxalis triangularis* will add to the existing knowledge of this plant.

CHAPTER 9

CONCLUSION

The present studies deals with *in vitro* plant regeneration, *in vitro* flowering, synthetic seeds, pigment extraction, phytochemical screening and antioxidant activities and acclimatization of *Oxalis triangularis*. The purpose was to develop an approach of multiplication of *Oxalis triangularis* in a bulk quantity under sterile conditions and to evaluate the potential of biological activities studies in the industry. Acclimatization of *Oxalis triangularis* in greenhouse was also established.

Explants were taken from intact plants which were grown at the greenhouse of Institute of Biological of Sciences and washed under series of sterilisation process before being cultured. Both explants; petiole and leaf were able to regenerate on Murashige and Skoog (MS) medium supplemented with various combinations and concentrations of plant growth regulator (PGRs). Among the various combinations and concentrations of PGRs being tested, treatment with combinations of hormone α-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) at the concentrations of 1.0 mg/l NAA and 1.5 mg/l BAP was the best treatment to produce adventitious shoots and healthy trifoliate leaves for leaf explants, meanwhile for petiole explants, best treatment was obtained at the combination of NAA and Kinetin (KIN) at the concentrations of 0.5 mg/l NAA and 1.0 mg/l KIN. For the root formation, both explants gave 100% root formation on all combinations of hormones. The successful of plant regeneration through tissue culture technique is very useful for establishment an efficient protocol for rapid multiplication in a large scale industry. Scanning Electron Microscopy (SEM) was used to analyse *in vivo* and *in vitro* grown

samples which resulted in no morphological difference between *in vitro* cultured plant and intact plant.

In the current study on *in vitro* flowering of *Oxalis triangularis* some positive response were observed. *In vitro* flowering was achieved when young adventitious shoots of *Oxalis triangularis* were cultured on MS medium supplemented with PGRs. From the present study, the best combination of hormones to induce *in vitro* flowering of *Oxalis triangularis* was on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP. From the morphological observations, there were no differences between *in vivo* and *in vitro* flowers except that the flowers produced from tissue culture technique were smaller in size due to the size of container used to culture which could be the limiting size factor. Not all flowering plant species can easily flowers under *in vitro* conditions. Hopefully, this study can be used as a platform or background for more research to give deeper understanding in physiology of *in vitro* flowering. This can be very useful in ornamental plant industry.

Synthetic seeds have many applications and advantages such as easy handling especially during storage, transportation and planting, as these are of small size. It can be applied to endangered species to preserve or protect species from extinction. So, it is important in germplasm conservation for future research. The most important is, it can be applied for those species which are unable to produce viable seeds. The best survival rate for synthetic seeds of *Oxalis triangularis* within 4 weeks observed were recorded at Day 7 with 96.67% of frequency conversion. The number of shoots per encapsulated bead was 4.57 ± 0.72 with 100% showed the emergence of roots. Besides that, all regenerated

plantlets from synthetic seeds of *Oxalis triangularis* showed no morphological variations when compared with the mother plant and they showed healthy growth. Synthetic seeds of *Oxalis triangularis* were successfully germinated on MS medium supplemented with PGRs but when it was sown on black soil and sterile black soil, the synthetic seeds gave negative results with fungal infection. More research needs to be done in this part where the synthetic seeds can be directly planted in the field.

For extraction study, anthocyanin could be extracted from petiole and leaf of *Oxalis triangularis* using acidified acetone and acidified methanol solvents and analysed using UV-visible spectroscopy to obtain anthocyanin readings. The choice of an extraction method is of great importance in the analysis of anthocyanin and largely depends on the purpose of the extraction, the nature of the anthocyanin and the source material. For natural pigment from *in vitro* extracts for paint production study, both weathering tests, salt and heat tests showed some degradation of *in vitro* anthocyanin. Further study needs to be done to improve the results which can be applied in industry especially in textile and food colouring.

In the phytochemical study, it is very important to determine the chemical constituents in plants where it can be manipulated as an alternative to treat disease. Phytochemical analysis revealed the presence of steroids and saponins in *Oxalis triangularis*. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Methanolic extracts of *in vivo* petiole samples showed the lowest value of lC_{50} (IC_{50} = less than 20 µg/ml) indicated higher free radical scavenging activity.

However, potential antioxidant activities in *Oxalis triangularis* need more research for industrial purposes.

The plant regeneration studies were completed with the acclimatization process, whereby *in vitro* plantlets were transferred to different commercial soils; black soil, red soil and vermiculite. Frequency of rooting was 100% recorded in all various combinations and concentrations of hormone tested for plant regeneration studies. Rooting was very important in the process of plant adaptation from cultured conditions to field conditions and also to improve survival rate. Black soil with pH of 5.4 was recorded as high percentage of survival prior to acclimatize (93.00 ± 0.05%) compared to red soil (83.00 ± 0.07%) and vermiculite (67.00 ± 0.09%). After a few weeks, all the acclimatized *Oxalis triangularis* grew like normal plants. The leaf became wider and darker, the petiole started to harden and became firm and flowering was obtained after 8 weeks being transferred to the greenhouse.

REFERENCES

Abreu, I.N., Azevede, M.T.A., Solferini, V.M. and Mazzafera, P. (2003). *In vitro* propagation and isozyme polymorphism of the medicinal plant *Hypericum brasiliense*. *Biol. Plant*. **47(4)**: 629-632.

Ahloowalia, B.S., Prakash, J., Savangikar, V.A. and Savangikar, C. (2004). Low cost options for tissue culture technology in developing countries. Plant Breeding and Genetics Section International Atomic Energy Agency. Austria.

Ammar, S., Benbadis, A. and Tripathi, B.K. (1987). Floral induction in date palm seedling (*Phoenix dactylifera* L. var. Deglet Nour) cultured *in vitro*. *Canadian Journal of Botany*. **65**: 137-142.

Ammirato, P.V. (1983). Embryogenesis. In: Evans, D., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.). *Handbook of Plant Cell Culture*, MacMillan. New York. pp 82-123.

Anitha, S. and Ranjitha Kumari, B.D. (2006). *In vitro* flowering in *Rauvolfia tetraphylla* L. *Pakistan Journal of Biological Sciences*. **9** (3): 422-424.

Attree, S.M. and Fowke, L.C. (1993). Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. *Plant Cell Tissue Organ Cult*. **35**: 1-35.

Avenido, R.A. and Haulea, D.M. (1990). *In vitro* organogenesis and flowering in mungbean (*Vigna radiata* L.). *Phill. J. Crop. Sci*. **15**: 169-173.

Bajaj, Y.P.S. (1995). Somatic Embryogenesis and Synthetic Seed. In: *Biotechnology in Agriculture and Forestry. Vol. 30,* Springer-Verlag. Berlin.

Ballester, A., Janeiro, L.V. and Vieitez, A.M. (1997). Storage of shoot cultures and alginate encapsulation of shoot tips of *Camellia japonica* and *C. reticulata* Lindley. *Scientia Horticulturae*. **71**: 67-78.

Bapat, V.A. and Rao, P.S. (1990). *In vitro* growth of encapsulated axillary buds of mulberry (*Morus indica* L.). *Plant Cell Tissue Org. Cult.* **20**: 69–70.

Bayer, G.T. and Zamanzadeh, M. (2004). Failure Analysis of Paints and Coating. Matco Associates, Inc. Pittsburgh, Pennsylvania.

Bernier, G. (1988). The control of floral evocation and morphogenesis. *Ann. Rev. Plant. Physiol. Plant Mol. Biol*. **39**: 175-219.

Beyer, R.E. (1994). The role of ascorbate in antioxidant protection of biomembranes: interaction with vit-E and coenzyme Q. *J. Bioen Biomemb*. **24**: 349-358.

Bhojwani, S.S., and Razdan, M.K. (1983). Plant Tissue Culture: Theory and Practice. *Elsevier Science Pub*. Amsterdam.

Bolar, J.P., Norelli, J.L., Aldwinckle, H.S. and Hanke, V. (1998). An efficient method for rooting and acclimation of micropropagated apple cultivars. *HortScience*. **37**: 1251-1252.

Bondada, B.R. and Oosterhuis, D.M. (2000). Comparative epidermal ultrastructure of cotton (*Gossypium hirsutum* L.) leaf, bract and capsule wall. *Annals of Botany*. **86**: 1143-1152.

Bonner, J. (1936). Plant tissue cultures from a hormone point of view. *Proc. Nat. Acad. Sci.* **22**: 426-430.

Brainerd, K.E. and Fuchigami, L.H. (1981). Acclimatization of aseptically cultured apple plants to low relative humidity. *J Am Soc Hortic Sci*. **106**: 515-518.

Brainerd, K.E., Fuchigami, L.H., Kwiatkowski, S. and Clark, C.S. (1981). Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments. *Hortic Sci*. **16**: 173-175.

Bruneton, J. (1999). Pharmacognosy, Phytochemistry and Medicinal Plants. *Intercept. Ltd*. England, U.K.

Buddendorf-Joosten, J.M.C. and Woltering, E.J. (1994). Components of the gaseous environment and their effects on plant growth and development *in vitro*. *Plant Growth Regul*. **15**: 1-16.

Carman, J.G. (1989). The in ovulo environment and its relevance to cloning wheat via somatic embryogenesis. *In Vitro Cell Dev Biol*. **25**: 1155-1162.

Caruso, J.L. (1971). Bud formation in excised stem segments of *Verbascum thapsus*. *Amer. J. Bot.* **58**: 429-31.

Central Potato Research Institute, Shimla (1992). Tissue culture technique for potato health, conservation, micro propagation and improvement. CPRI, Shimla. pp 1-23.

Chambers, S.M., Heuch, J.H.R. and Pirrle, A. (1991). Micropropagation and *in vitro* flowering of the bamboo *Dendrocalamus hamiltonii Munro*. *Plant Cell Tiss Org. Cult*. **27**: 45–48.

Chang, C. and Chang, W.C. (2003). Cytokinin promotion of flowering in *Cymbidium ensifoium* var. *misiricors in vitro*. *Plant Growth Regulation*. **39**: 217-221.

Chawla, H.S. (2003). Plant Biotechnology: Laboratory Manual for Plant Biotechnology. Oxford & IBH Publishing Co. Pvt. Ltd. New Delhi.

Chittendon, F. (1956). RHS Dictionary of Plants plus Supplement. Oxford University Press 1951.

Chiwocha, S.D.S., Cutler, A.J., Abrams, S.R., Ambrose, S.J., Yang, J., Ross, A.R.S. and Kermode, A.R. (2005). The etrl-2 mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellins metaboli pathways during maintainance of seed dormancy, moist-chilling and germination. *Plant J*. **42**: 35-48.

Cutter, E.G. (1965). Recent experimental studies of the shoot apex and shoot morphogenesis. *Bot. Rev*. **31**: 7-113.

Cyr, D.R. (2000). Seed substitutes from the laboratory. In: Black, M. and Bewley, J.D. (Eds.). *Seed Technology and its Biological Basis*, Sheffield Acad. Press. Sheffield. pp 326-372.

Conger, B.V. (Ed.). (1981). Cloning Agricultural Plants via *In Vitro* Techniques. *CRC Press*, Boca Raton, Florida.

de Fossard, R.A. (1974). Flower initiation in tissue and organ cultures. *In*: Street, H.D. (Ed.). *Tissue culture and plant science*. Academic Press, New York: 193-211.

de Fossard, R.A. (1976). Tissue culture for plant propagators. Armidale (Australia): University of New England.

de Fossard, R.S. (1977). Tissue Culture for Plant Propagators. University of New England Printing, Armidale.

de Langhe, E. and de Bruijne, E. (1976). Continuous propagation of tomato plants by means of callus cultures. *Scientia Horticulturae*. **4**: 221-7.

Danso, K.E. and Ford-Lloyd, B.V. (2003). Encapsulation of nodal cuttings and shoot tips for storage and exchange of cassava germplasm. *Plant Cell Rep*. **21**: 718-725.

Davey, M.R., Kumar, V. and Hammart, N. (1994). *In vitro* culture of legumes. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 313-329.

De, K.K. (2007). Plant Tissue Culture. New Central Book Agancy (P) Ltd., India, pp: 232.

Deaton, M.H., Buxton, J.W. and Hamilton-Kemp, T.R. (1984). The effects of growth regulators on development of Nicotiana affinis flowers *in vitro*. *Hort Science*. 19: 509-511.

Debergh, P. (1994). *In vitro* culture ofornamentals. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 561-573.

Dekhuijzen, H.M. (1971). Sterilization of cytokinins. In Bragt, . J.V., Mossel, D.A.A., Pierik, R.L.M. and Veldstra, H. (Eds.), In: *Effects of sterilization on components in nutrient media*. Wageningen, The Netherlands: Kniphorst Scientific. pp. 12-9-132.

Desjardins, Y. (1995). Photosynthesis *in vitro* - on the factors regulating CO_2 assimilation in micropropagation systems. *Acta Hort.* **393**: 45-61.

Dickens, C.W.S. and Van Staden, J. (1988). The induction and evocation of flowering *in vitro*. *South African Journal of Botany*. **54**: 325-344.

Dobrev, P., Motyka, V., Gaudinová, A., Malbeck, J., Trávnílková, A., Kamínek, M. and Valková, R. (2002). Transient accumulation of *cis*- and *trans*-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. *Plant Physiol. Biochem*. **40**: 333-337.

Du Plessis, N. and Duncan, G. (1989). Bulbous Plants of Southern Africa. A Guide to their Cultivation and Propagation. Tafelberg Publishers Limited, Cape Town. pp. 1952.

Dunlap, J.R. and Robacker, K.M. (1988). Nutrient salts promote light-induced degradation of indole-3-acetic acid in tissue culture media. *Plant Physiology*. **88**: 379-382.

Eung, J.P., Lim, H.T., Part, E.J., Lim, H.T. and Lee, J.M. (1999). Establishment of an efficient *in vitro* plant regeneration system in Chicory (*Cichorium intybus* L.). *Acta Horticulturae*. **483**: 367-370.

Fahn, A. (1990). Plant anatomy. New York: Pergamon Press.

Fang, Y., Yang, S. and Wu, G. (2002). Free radicals, antioxidants and nutrition. Nutrition. **18**: 872-879.

Farnsworth, N.R. (1966). Biological and phytochemical screening of plants. *J. Pharm. Sci*. **55**: 225-276.

Floh, E.I.S. and Handro, W. (2001). Effect of photoperiod and chorogenic acid on morphogenesis in leaf discs of *Streptocarpus nobilis* cultured *in vitro*. *Biologia Plantarum*. **44**: 615-618.

Francis, J.F. (1982). Analysis of anthocyanins. In: P. Markakis (Ed.), Anthocyanin as Food Color. Academic Press, New York. pp: 181-201.

Francis, F.J. (1989). Food colorants: anthocyanins. *Crit. Rev. Food. Sci. Nutr*. **28**: 273-314.

Franklin, G., Pius, P.K. and Ignacimuthu, S. (2000). Factors affecting in vitro flowering and fruiting of green pea (Pisum sativum L.). Euphytica. **115**: 65-73.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L.

and Woo, S.C. (1983). Expression of bacterial genes in plant cells. *Proc. Nat. Acad. Sci. USA.* **80**: 4803-7.

Fuleki, T. and Francis, F.J. (1968). Quantitative methods for anthocyanins: extraction and determination of total anthocyanin in cranberries. *J. Food Sci*. **33:** 72-83.

Fujii, J.A., Slade, D.T, Redenbaugh, K. and Walker, K.A. (1987a). Artificial seeds for plant propagation. *Trends Biotechnol*. **5**: 335-340.

Fujii, J.A., Slade, D.T., Redenbaugh, K. and Walker, K.A. (1987b). How APV puts the technology into biotechnology. *BioTechnology*. **5**: 335-339.

Fujii, J.A, Slade, D.T. and Redenbaugh, K. (1989). Maturation and greenhouse planting of alfalfa artificial seeds. *In vitro Cell Dev Biol*. **25**: 1179-1182.

Fujioka, T., Fujita, M. and Miyamoto, Y. (1999). Flowering and pod setting of non – symbiotically germinated pea. *J. Jap. Soc. Hort. Sci*. **68**: 117-123.

Galoch, E., Czaplewska, J., Burkacka-Laukajtys, E. and Kopcewicz, J. (2002). Induction and stimulation of *in vitro* flowering of *Pharbitis nil* by cytokinin and gibberellin. *Plant Growth Regulator*. **37**: 199-205.

Ganapathi, T.R., Suprasnna, P., Bapat, V.A. and Rao, P.S. (1992). Propagation of banana through encapsulated shoot tips. *Plant Cell Rep*. **11**: 571-575.

Garcia-Falcon, M.S., Perez-Lamela, C., Martinez-Carballo, E. and SimalGandara, J. (2007). *Food Chemistry*. **105**: 248–259.

Garcia-Luis, A., Santamarina, A. and Guardiola, A. (1989). Flower Formation from *Citrus unshiu* Buds cultured *In vitro*. *Ann. of Bot*. **64**: 515-519.

Garcia-Viguera, C., Zafrilla, P. and Thomás-Barberán, F.A. (1998). The use of acetone as an extraction solvent for anthocyanins from strawberry fruit. *Phytochemical Analysis*. **9:** 274-277.

Gautheret, R.J. (1934). Culture du tissues cambial. *C. R. Hebd. Seances Acad. Sc.* **198**: 2195-2196.

Gautheret, R.J. (1935). *Recherches sur la culture des tissues végétaux*. Ph.D. Thesis, Paris.

Gautheret, R.J. (1939). Sur la possibilité de réaliser la culture indéfinie des tissus de tubercules de carotte. *C. R. Hebd. Seances Acad. Sc.* **208**: 118-120.

Gautheret, R.J. (1983). Plant Tissue Culture: A History. Bot. Mag. Tokyo. **96**: 393-410.

George, E.F. (1993). Plant Propagation by Tissue Culture. Part 1. The Technology. Exegetics Ltd., Edington.

George, E.F., and Debergh, P.C. (2008). Micropropagation: Uses and Methods. In: George, *et al*., (eds.). *Plant Propagation by Tissue Culture 3rd Edition*, Springer. Dordrecht, The Netherlands. pp 1–28.

Giusti, M.M., Luis, E.R.-S., James, R., *et al*. (1998). Anthocyanin pigments composition of red radish cultivars as potential food colorant. *Journal of Food Science*. **63:** 219-224.

Good painting practice. Causes and prevention of paint failure, Chapter 23, Steel structures painting manual (1982). Steel structures painting council. Pittsburgh, PA.

Gray, D.J. (1987a). Introduction to the symposium. In: Proc Symp Synthetic seed technology for the mass cloning of crop plants: problems and perspectives. *HortScience*. **22**: 796-797.

Gray, D.J. (1990a). Synthetic seed for clonal production of crop plants. In: Taylorson, R.D. (ed). *Recent advances in the development and germination of seeds*, Plenum Press. New York. pp 29-45.

Gray, D.J. (1990b). Synthetic seed for clonal production of crop plants. In: Taylorson, R.D. (ed). *Recent advances in the development and germination of seeds*, Plenum Press. New York. pp 29-45.

Gray, D.J., Conger, B.V. and Hanning, G.E. (1984). Somatic embryogenesis in suspension and suspension-derived callus cultures of *Dactylis glomerata*. *Protoplasma*. **122**: 196-202.

Gray, D.J. and Mortensen, J.A. (1987). Initiation and maintenance of long-term somatic embryogenesis from anthers and ovaries of *Vitis longii* 'Microsperma'. *Plant Cell Tissue Organ Cult*. **9**: 73-80.

Gray, D.J., Trigiano, R.N. and Conger, B.V. (1992). Liquid suspension culture of orchardgrass somatic embryos and their potential for the breeding of improved varieties. In: Redenbaugh, K. (Ed). *Synseeds: applications of synthetic seeds to crop improvement*, CRC Press. Boca Raton. pp 351-366.

Gresshoff, P.M. (1978). Phytohormones and growth and differentiation of cells and tissues cultured *in vitro*. In: Letham, D.S., Goodwin, P.B. and Higgins, T.J.V. (Eds.). *Phytohormones and Related Compound – A Comprehensive Treatise*, Vol. II. Elsevier/ North-Holland, Amsterdam. pp. 1-29.

Grosser, J.W. (1994). *In vitro* culture of tropical fruits. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 475-496.

Grout, B.W.W. (1975). Wax development on leaf surfaces of *Brassica oleracea* var. Currawong regenerated from meristem culture. *Plant Sci Lett*. **5**: 401- 405.

Grout, B.W.W. and Aston, M.J. (1977). Transplanting of cauliflower plants regenerated from meristem culture: I. Water loss and water transfer related to changes in leaf wax and xylem regeneration. *Hortic Res.* **17**: 1-17.

Grout, B.W.W. and Aston, M.J. (1978). Transplanting of cauliflower plants regenerated from meristem culture. 11. Carbon dioxide fixation and the development of photosynthetic activity. *Hortic Res*. **17**: 65-71.

Guri, A.Z. and Patel, K.N. (1998). Compositions and methods to prevent microbial contamination of plant tissue culture media. U. S. Patent No. 5,750,402.

Gurib-Fakim, A., (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*. **27**: 1–93.

Haberlandt, G. (1902). Kulturversuchemit isolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien. Math. Naturwiss. Kl. *Abt. J*. **111**: 69–92.

Halliwell, B. (1996). Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radical Research.* **25**: 1-32.

Halliwell, B., Aeschbach, R., Löliger, J. and Aruoma, O.I. (1995). The characterization of antioxidants. *Food Chemistry and Toxicology*. **33**: 601-617.

Halperin, W. (1969) Morphogenesis in cell cultures. *Ann. Rev. Plant Physiol*. **20**: 395-417.

Hanning, G.E. and Conger, B.V. (1986). Factors influencing somatic embryogenesis from cultured leaf segments of *Dactylis glomerata*. *J. Plant Physiol*. **123**: 23-29.

Harborne, J.B. (1988). Introduction to Ecological Biochemistry (3rd ed.). Academic Press, London.

Harbone, J. (1998). Phytochemical methods: a guide to modern techniques of plant analysis. *Springer, Nature*. pp. 302.

Harborne, J.B. and Williams, C.A. (1995). Anthocyanins and other flavonoids. *Natural Product Reports*. **12**: 639-657.

Harms, C.T. (1982). Maize and cereal protoplasts – facts and perspectives. In Sheridan,W.F. (Ed.) *Maize for Biological Research*. University of North Dakota Press, grand forks, ND, USA. pp. 373-448

Harms, C.T. (1983). Somatic hybridization by plant protoplast fusion. *Experientia Suppl.* **46**: 69-84.

Harry, I.S. and Thorpe, T.A. (1994). *In vitro* culture of forest trees. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture,* Kluwer. Dordrecht, The Netherlands. pp 539-560.

Havelange, A., Lejeune, P. and Bernier, G. (2000). Sucrose/cytokinin interaction in *Sinapis alba* at floral induction: a shoot-to-root-to-shoot physiological loop. *Physiol. Plant*. **109**: 343-350.

He, Y.W. and Loh, C.S. (2002). Induction of early bolting in Arabidopsis thaliana by triacontanol, cerium and lanthanum is correlated with increased endogenous concentration of isopentenyl adenosine (iPAdos). *J. Exp. Botany*. **53**: 505–512.

Hedin, P.A., Jenkins, J.N., Collum, D.H., White, W.H., Parrot, W.L. and MacGown, M.W. (1983). Cyanidin-3-glucoside, a newly recognized basis for resistance in cotton to the tobacco budworm *Heliothis virescens* (Fab.) (lepidoptera: Noctuidae). *Experentia*. **39**:799-801.

Heinrich, M., Barnes, J., Gibbons, S. and Williamson, E.M. (2004). Fundamentals of Pharmacognosy and Phytotherapy. Churchill Livingstone, Elsevier Science Ltd., UK.

Henshaw, G.G., O'Hara, J.F. and Webb, K.J. (1e982). Morphogenetic studies in plant tissue cultures. *Symp. Brit. Soc. Biol*. **4**: 231-51.

Heylen, C. and Vendrig, J.C. (1988). The influence of different cytokinins and auxins on flower neoformation in thin cell layers of *Nicotiana tabacum* L. *Plant Cell Physiol*. **29**: 665-671.

Hoffmann, A., Pasqual, M., Chalfun, N.N.J. and Fráguas, C.B. (2001). Efeito de substratos na aclimatização de plantas micropropagadas do porta-enxerto de macieira 'MARUBAKAIDO'. *Ciência Agrotecnológica*. **25**: 462-467.

Holdgate, D.P and Zandvoort, E.A. (1997). Strategic considerations for the establishment of microorganism free tissue cultures for commercial ornamental micropropagation. In: Cassells, A.C. (Ed.). *Pathogen and Microbial Contamination Management in Micropropagation*, Kluwer Acad. Pub. Dordrecht. pp 15-22.

Huda, A.K.M.N., Rahman, M. and Bari, M.A. (2007). Effect of carbon source in alginate bead on synthetic seed germination in eggplant (*Solanum melongena* L.). *J. Plant Science*. **2**: 538- 544.

Hussey, G. (1976). Plantlet regeneration from callus and parent tissue in *Ornithogalum thyrsoides*. *J. exp. Bot*. **27**: 375-82.

Hussey, G. (1978a). The application of tissue culture to the vegetative propagation of plants. *Science progress.* **65**: 185-208.

Hussey, G. (1980a). *In vitro* propagation. In: Ingram, D.S. and Helgeson, J.P. (Eds.). *Tissue Culture for Plant Pathologists,* Blackwell Scientific Publications. Oxford. pp 51-61.

Hussey, G. (1983). *In vitro* propagation of horticultural and agricultural crops. In: Mantell, S.H. and Smith, H. (Eds.). *Plant Biotechnology,* Cambridge University Press. Cambridge. pp 111-38.

Hussey, G. and Stacey N.J. (1981). *In vitro* propagation of potato (*Solanum tuberosum* L.). *Ann. Bot.* **48(6)**: 787-796.

Huxley, A. (1992). *The New RHS Dictionary of Gardening*. MacMillan Press.

Ignacimuthu, S., Franklin, G. and Melchias, G. (1997). Multiple shoot formation and *in vitro* fruiting of *Vigna mungo* (L.). *Hepper. Curr. Sci.* **73**: 733-735.

Ikhlaq, M., Ishfaq, A.H., Maurizio, M., Touqeer, A., Nadeem, A.A. and Alvaro, S. (2010). *In vitro* storage of synthetic seeds: Effect of different storage conditions and intervals on their conversion ability. *African Journal of Biotechnology*. **9(35)**: 5712-5721.

Jackman, R.L. and Smith, J.L. (1996). Anthocyanins and Betalaines. In: Hendry, G.A.F. and Houghton, J.D. (Eds.). *Natural Food Colourant* (2nd ed). Blackie Academic and Proffesional. pp: 244-309.

Jana, S. and Shekhawat, G.S. (2011). Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and *in vitro* flowering of *Anethum graveolens*. *Acta Physiologiae Plantarum*. **33**: 305-311.

Janick, J., Kitto, S.L. and Kim, Y.H. (1989). Production of synthetic seed by desiccation and encapsulation. *In Vitro Cell Dev Biol*. **25**: 1167-1172.

Jayasankar, S., Bondada, B.R., Li, Z. and Gray, D.J. (2002). A unique morphotype of grapevine somatic embryos exhibits accelerated germination and early plant development. *Plant Cell Rep*. **20**: 907-911.

Johnson, H.B. (1975). Plant pubescence: An ecological perspective. *Botanical Review*. **41**: 233-258.

Jones, M.G.K. (1994). *In vitro* culture of potato. In: Vasil, I.K. and Thorpe, T.A. (Eds.). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 363-378.

Joshi, M. and Nadgauda (1997). Cytokinin and *in vitro* induction of flowering in bamboo: *Bambusa arundinacea* (Reitz) Wild. *Curr Sci*. **73**: 523-526.

Juan, R., Fernandez, I. and Pastor, J. (1999). Morphological and anatomical studies of Linaria species from South-west Spain: Fruits. *Annals of Botany*. **84**: 21-23.

Jumin, H.B. and Ahmad, M. (1999). High-frequency *in vitro* flowering of *Murraya paniculata* (L.) Jack. *Plant Cell Rep*. **18**: 764-768.

Jumin, H.B. and Nito, N. (1996). *In vitro* flowering of *Fortunella hindsii* (Champ.). *Plant Cell Reports*. **15**: 484-488.

Kadleček, P. (1997). Effect of pretreatment by irradiance and exogenous saccharose under *in vitro* conditions on photosynthesis and growth of tobacco (*Nicotiana tabacum* L.) plants during acclimatization after transfer to soil. Diploma Thesis, Charles University, Department of Plant Physiology, Praha, Czech.

Kallithraka, S., Garcia-Viguera, C., Bridle, P. and Bakker, J. (1995). Survey of solvents for the extraction of grape seed phenolics. *Phytochemical Analysis*. **6:** 265-267.

Kanatt, S.R., Chander, R. and Sharma, A. (2007). Antioxidant potential of mint (*Mentha spicata* L.) in radiation-processed lamb meat. *Food Chem*. **100**: 451-458.

Kanchanapoom, K., Posayapisit, N. and Kanchanapoom, K. (2009). *In vitro* flowering from cultured nodal explants of rose (*Rosa hybrida* L.). *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*. **37** (2): 261-263.

Kartha, K.K. and Engelmann, F. (1994). Cryopreservation and germplasm storage. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture,* Kluwer. Dordrecht, The Netherlands. pp 195-230.

Kim, Y.H. and Janick, J. (1989a). Origin of somatic embryos in celery tissue culture. *HortScience.* **24**: 671-673.

Kim, Y.H. and Janick, J. (1989b). ABA and polyox-encapsulation of high humidity increases survival of desiccated somatic embryos of celery. *HortScience*. **24**: 674-676.

King, P.J. (1984). Selection for plant variation using cultured cells. In: Lange, W., Zeven, A.C. and Hogenboom, N.G. (Eds.). *Efficiency in Plant Breeding*. Proc. 10th Congr. Eucarpia. Pudoc, Wageningen. pp. 199-214.

Kintzios, A.S and Michelakis (1992). Induction of somatic embryogesis and *in vitro* flowering from inflorescence of chamomile (*Chamomilla reticulate* L.). *Plant Cell Rep*. **18**: 684-690.

Kitto, S.L. and Janick, J. (1985a). A citrus embryo assay to screen water-soluble resins as synthetic seed coats. *HortScience.* **20**: 98-100.

Kitto S.L. and Janick, J. (1985b). Production of synthetic seeds by encapsulating asexual embryos of carrot. J Am Soc *Hortic Sci*. **110**: 277-282.

Kitto, S.L. and Janick, J. (1985c). Hardening treatments increase survival of synthetically coated asexual embryos of carrot. *J Am Soc Hortic Sci*. **110**: 283-286.

Kitzinger, E.M., Jansen Van Vuuren, R., Woodward, B., Rong, I.H., Spreth, M.H.and Slabbert, M.M. (1997). Elimination of external and internal contaminants in rhizomes of *Zantedeschia aethiopica* with commercial fungicides and antibiotics. In: Cassells, A.C. (Ed.). *Pathogen and Microbial Contamination Management in Micropropagation*, Kluwer Acad. Pub. Dordrecht. pp 61-167.

Koleva, I.I., van Beek, T.A., Linssen, J.P.H., de Groot, A. and Evstatieva, L.N. (2002). Screening of Plant Extracts for Antioxidant Activity: a Comparative Study on Three Testing Methods. *Phytochemical Analysis*. **13**: 8-17.

Kong, J.-M., Chia, L.-S., Goh, N.-K., Chia, T.-F. and Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry*. **64:** 923-933.

Kozai, T. (1991). Micropropagation under photoautotrophic conditions. In: Debergh, P.C. and Zimmerman, R.H. (ed.). *Micropropagation. Technology and Application*, Kluwer Academic Publishers. Dordrecht - Boston – London. pp 447-469.

Kozai, T. and Smith, M.A.L (1995). Environmental control in plant tissue culture - general introduction and overview. In: Aitken-Christie, J., Kozai, T. and Smith, M.L. (Ed.). *Automation and Environmental Control in Plant Tissue Culture*, Kluwer Academic Publishers. Dordrecht - Boston – London. pp 301-318.

Krikorian, A.D. (1982). Cloning higher plants from aseptically cultured tissues and cells. *Biol. Rev., Cambridge.* **57**: 151-218.

Krikorian, A.D. (1994a). *In vitro* culture of root and tuber crops. In: Vasil, I.K. and Thorpe, T.A. (Eds.). *Plant cell and tissue culture*, Kluwer Dordrecht, The Netherlands. pp 379-411.

Krikorian, A.D. (1994b). *In vitro* culture of plantation crops. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 497-537.

Kuijpers, A.-M. and Langens, M.-G. (1997). Propagation of tulip *in vitro*. *Acta Hortic.* **430**: 321-332.

Lee, E.K, Cho, D.Y. and Soh, W.Y. (2001). Enhanced production and germination of somatic embryos by temporary starvation in tissue cultures of *Daucus carota*. *Plant Cell Rep*. **20**: 408-415.

Lee, N., Wetzstein, H.Y. and Sommer, H.E. (1985). Effects of Quantum Flux Density on Photosynthesis and Chloroplast Ultrastructure in Tissue-Cultured Plantlets and Seedlings of *Liquidambar styraciflua* L. towards Improved Acclimatization and Field Survivall. *Plant Physiol.* **78**: 637-641.

Leifert, C. and Cassells, A.C. (2001). Microbial hazards in plant tissue and cell cultures. *In vitro* cellular and developmental biology. *Planta*. **37**: 133-138.

Leifert, C. and Woodward, S. (1998). Laboratory contamination management: the requirement for microbiological quality assurance. *Plant Cell Tiss. Org. Cult*. **52**: 83-88.

Lin, C.S., Lin, C.C. and Chung, W.C. (2003). *In vitro* flowering of *Bambusa edulis* and subsequent plantlet survival. *Plant Cell Tiss. Org. Cult*. **72**: 71-78.

Lin, C.S., Lin, C.C. and Chung, W.C. (2004). Effect of thidiazuron on vegetative tissue-derived somatic embryogenesis and flowering of bamboo (*Bambusa edulis*). *Plant Cell Tissue Organ Culture*. **76**: 75-82.

Lioa, K.L. and Yin, M.C. (2000). Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems: Importance of the partition coefficient. *Journal of agricultural and food chemistry*. **48**: 2266-2270.

Lu, Y. and Foo, L.Y. (2001). Unusual anthocyanin reactions with acetone leading to pyranoanthocyanin formation. *Tetrahedron Letters*. **42:** 1371-1373.

Machii, H. (1992). *In vitro* growth of encapsulated adventitious buds in Mulberry, *Morus alba* L. *Jap. J. Breed*. **42**: 553-559.

Mohanraj, R., Ananthan, R. and Bai, V.N. (2009). Production and storage of synthetic seeds in *Coelogyne breviscapa* Lindl. *Asian Journal of Biotechnology*. **1 (3)**: 124-128.

Mamiya, K. and Sakamoto, Y. (2001). A method to produce encapsulatable units for synthetic seeds in *Asparagus officinalis*. *Plant Cell Tiss. Org. Cult*. **64**: 27-32.

Mandal, A.B., Maiti, A. and Elanchezhian, R. (2000). *In vitro* flowering in maize (*Zea mays* L.). *Asia Pacific Journal of Molecular Biology and Biotechnology*. **8**: 81-83.

Markakis, P. (1982). Introduction. In: *Anthocyanin in fruits, vegetables, and grain* (Chapter 1). Boca Roton,FL: CRC Press. pp. 1-28

Mazza, G. and Minitiati, E. (1993). Introduction. In: *Anthocyanin in Fruits, vegetables, and grains* (Chapter 1). Boca raton, FL: CRC Press. pp: 1-28

McCourt, P. (1999). Genetic analysis of hormone signaling. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**: 219-243.

McKersie, B.D. and Bowley, S.R. (1993). Synthetic seeds of alfalfa. In: Redenbaugh, K. (Ed.). *Synseeds. Applications of Synthetic Seeds to Crop Improvement*, CRC Press. Boca Raton, Florida. pp 231-255.

Mckersie, B.D., Senaratna, R., Bowley, S.R., Brown, D.C.W., Krochko, J.E. and Bewley, J.D. (1989). Application of artificial seed technology in the production of hybrid alfalfa (*Medicago sativa* L.). *In Vitro Cell Dev Biol*. **25**: 1183-1188.

Medford, J.I. (1992). Vegetative apical meristems. *Plant Cell*, **4**: 1029-1039.

Meeks-Wagner, D.R., Dennis, E.S., Tran Tranth Van, K. and Peacock, W.J. (1989). Tobacco genes expressed during *in vitro* floral initiation and their expressed during normal plant development. *Plant Cell*. **1**: 25-35.

Micheli, M., Hafiz, I.A. and Standardi, A. (2007). Encapsulation of *in vitro*-derived explants of olive (*Olea europaea* L. cv. Moraiolo). II. Effects of storage on capsule and derived shoots performance. *Sci. Hortic.* **113**: 286-292.

Micheli, M., Mencuccini, M. and Standardi, A. (1998). Encapsulation of *in vitro* proliferated buds of olive. *Adv. Hort. Sci*. **12**: 163-168.

Murai, N., Sutton, D.W., Murray, M.G., Slightom, J.L., Merlo, D.J., Reichert, N.A., Sengwpta-Gopalan, C., Stock, C.A., Barker, R.F., Kemp, J.D. and Hall, T.C. (1983). Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. *Science*. **222**: 476-482.

Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. *Science*. **222**: 476-82.

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

Murashige, T. (1974). Plant propagation through tissue cultures. *Ann. Rev. Plant Physiol*. **25**: 135-66.

Murashige, T. (1977). Manipulation of organ culture in plant tissue cultures. *Botanical Bulletin Academic Sinica*. **18**: 1-24.

Murashige, T. (1978). The Impact of plant tissue culture on agriculture. In: Thorpe, T.A. (Ed.). *Frontiers of plant tissue culture*. International Association for Plant Tissue Culture, University of Calgary, Calgary, Alberta. pp 15-26.

N'guessan, K., Tiébré, M.S., Aké, A.E. and Zirihi, G.N. (2009). Ethnobotanical study of plants used to treat arterial hypertension, in traditional medicine, by Abbey and Krobou Populations of Agboville (Côted'Ivoire). *Eur. J. Sci. Res*. **35**: 85-98.

Nadgauda, R.S, Parasharami, V.A. and Mascarenhas (1990). Precautious flowering and seedling behavior in tissue cultured Bamboos. *Nature*. **344**: 335-336.

Nadgauda, R.S., John, C.K., Parasharami, V.A., Joshi, M.S. and Mascarenhas, A.F. (1997). A comparison of *in vitro* with *in vivo* flowering in bamboo: *Bambusa arundinacea*. *Plant Cell, Tissue and Organ Culture*. **48**: 181-188.

Naik, S.K. and Chand, P.K. (2006). Nutrient-alginate encapsulation of *in vitro* nodal segments of pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. *Scientia Horticulturae*. **108**: 247-252.

Naor, V., Kigel, J. and Ziv, M. (2004). Hormonal control of inflorescence development in plantlets of Calla lily (*Zantedeschia* spp) grown *in vitro*. *Plant Growth Regulation*. **42**: 7-14.

Narasimhulu, S.B. and Reddy, G.M. (1984). *In vitro* flowering and pod formation from cotyledons of groundnut (*Arachis hypogea* L.). Theor. Appl. Genet. 69: 87–91.

Narayanaswamy, S. (1977). Regeneration of plants from tissue cultures. In: Reinert, J. and Bajaj, Y.P.S. (Eds.). *Plant Cell, Tissue, and Organ Culture*. Springer-Verlag, Berlin. pp.179-248

Nieves, N., Martínez, M.E., Castillo, R., Blanco, M.A. and González-Olmedo, J.L. (2001). Effect of abscisic acid and jasmonic acid on partial desiccation of encapsulated somatic embryos of sugarcane. *Plant Cell Tiss. Org. Cult*. **65**: 15-21.

Nirmal, B.K., Anu Ramashree, A.B. and Praveen, K. (2000). Micropropagation of curry leaf trees. *Plant cell Tiss. Org. Cult*. **61**: 199-203.

Nobécourt, P. (1939a). Sur la pérennité et l'augmentation de volume des cultures de tissues végétaux. *C. R. Seances Soc. Biol. Ses Fil.* **130**: 1270-1271.

Nobécourt, P. (1939b). Sur les radicelles naissant des cultures de tissues végétaux. *C. R. Seances Soc. Biol. Ses Fil.* **130**: 1271-1272.

Nyende, A.B., Schittenhelm, S., Wagner, G.M. and Greef, J.M. (2003). Production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum* L.) encapsulated in calcium alginate hallow beads. *In Vitro Cell. Dev. Biol.: Plant*. **39**: 540-544.

Ozden-Tokatli, Y., Carlo, A.D., Fumusel, F., Pignattelli, S. and Lambardi, M. (2008). Development of encapsulation techniques for the production and conservation of synthetic seeds in ornamental plants. *Prop of Ornamental Plants*. **8**: 17-22.

Palmer, C.E. and Keller, W.A. (1994). *In vitro* culture of oilseeds. In: Vasil, I.K. and Thorpe, T.A. (Eds.). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 413-455.

Pande, D., Purohit, M. and Srivastava, P.S. (2002). Variation in xanthotoxin content in *Ammi majus* L. cultures during *in vitro* flowering and fruiting. *Plant Science*. **162**: 583-587.

Peeters, A.J.M., Gerarda, W., Barendse, G.W.M. and Wullems, G.J. (1991). *In vitro* flower bud formation in tobacco: Interaction of hormones. *Plant Physiol*. **97**: 402–408.

Perez, E.A. and Hooks, C.R. (2008). Preparing Tissue-Cultured Banana Plantlets for Field Planting. Biotechnology. College of Tropical Agriculture and Human Resources (CTAHR).

Phillips, D.A. and Cleland, C. (1972). Cytokinin activity from the phloem sap of *Xanthium strumarium* L. *Planta.* **102**: 173-178.

Phillips, R. and Rix, M. (1998). Conservatory and Indoor Plants. Volumes 1 & 2. Pan Books, London. ISBN 0-330-37376-5.

Pierik, R.L.M. (1979). *In Vitro Culture of Higher Plants*. Ponsen en Looijen, Kniphorst. Scientific Bookshop, PO Box 67, Wageningen.

Pierik, R.L.M. (1989). *In Vitro* Culture of Higher Plants. Martinus Nijhoff Pub. Dordrecht.

Pintos, B., Bueno, M.A., Cuenca, B. and Manzanera, J.A. (2008). Synthetic seed production from encapsulated somatic embryos of cork oak (*Quercus suber* L.) and automated growth monitoring. *Plant Cell Tissue and Organ Culture*. **95 (2)**: 217-225.

Pospíšilová, J., Čatský, J. and Šesták, Z. (1997). Photosynthesis in plants cultivated *in vitro*. In: Pessarakli, M. (Ed.). *Handbook of Photosynthesis*, Marcel Dekker .New York - Basel - Hong Kong. pp 525-540.

Pospíšilová, J., Solárová, J. and Čatský, J. (1992). Photosynthetic responses to stresses during *in vitro* cultivation. *Photosynthetica*. **26**: 3-18.

Pospísilová, J., Tichá, I., Kadlecek, P., Haisel, D. and Plzáková, S. (1999). Acclimatization of micropropagated plants to *ex vitro* conditions. *Biologia Plantarum*. **42**: 481-497.
Preece, J.E. and Sutter, E.G. (1991). Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh, P.C., Zimmerman, R.H. (ed.). *Micropropagation. Technology and Application*, Kluwer Academic Publishers. Dordrecht - Boston – London. pp 71-93.

Preece, J.E. and West, T.P. (2006). Greenhouse growth and acclimatization of encapsulated *Hibiscus moscheutos* nodal segments. *Plant Cell Tiss Organ Clut*. **87**: 127-138.

Quak, F. (1977). Meristem culture and virus free plants. In: Reinert, J. and Bajaj, Y.P.S. (Eds.). *Applied and Fundamental aspects of Plant Cell, Tissue and Organ Culture*, Springer- Verlag. Berlin. pp 598-615.

Raghavendra, M.P., Satish, S. and Raveesha, K.A. (2006). Phytochemical analysis and antibacterial activity of *Oxalis corniculata*; a known medicinal plant. *mySCIENCE*. **1(1)**: 72-78.

Rai, M.K., Jaiswal, V.S. and Jaiswal, U. (2008a). Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange and distribution. *Sci. Hortic*. **118**: 33-38.

Ramakrishnappa (1998). Studies on somatic embryogenesis and production of synthetic seeds in Gladiolus cultivars. Ph. D. Thesis, Univ. Agric. Sci., Bangalore.

Rama Murthy, K., Ramulu, S., Rao, D.R., Chandrashekhar, J., Emmanuel, S. and Pullaiah, T. (2006). *In vitro* flowering of *Spathiglottis plicata* Bl (Orchidaceae). *Phytomorphology*. **56**: 117-120.

Rastogi, R. and Sawhney, V.K. (1987). The role of plant growth regulators, sucrose and pH in the development of floral buds of tomato (*Lycopersicon esculentum* Mill.) cultured *in vitro*. *J. Plant Physiol*. **128**: 285-295.

Rastogi, R. and Sawheny, V.K. (1989). *In vitro* development of angiosperm floral buds and organs. *Plant Cell, Tissue and Organ Culture*. **16**: 145-174.

Redenbaugh, K. (1990). Application of artificial seed to tropical crops. *HortScience*. **25**: 251-255.

Redenbaugh, K. (1993). Synseeds: Applications of synthetic seeds to crop improvement. *CRC Press*. Boca Raton, Florida.

Redenbaugh, K., Fujii, J.A. and Slade, D. (1988). Encapsulated plant embryos. In: Mizrahi, A. (ed). *Biotechnology in agriculture*. Liss, New York. pp 225-248.

Redenbaugh, K., Fujii, J.A. and Slade, D. (1993). Hydrated coatings for synthetic seeds. In: Redenbaugh, K. (ed). *Synseeds application of synthetic seeds to crop improvement*, CRC Press. Boca Raton. pp 35-45.

Redenbaugh, K., Fujii, J.A., Slade, D., Viss, P. and Kossler, M. (1991). Artificial seed – encapsulated somatic embryos. In: Bajaj, Y.P.S. (Ed). *Biotechnology in agriculture and forestry, vol 17*. High-tech and micropropagation I, Springer. Berlin Heidelberg, New York. pp 395-416.

Redenbaugh, K., Fujii, J.A.A. and Slade. D. (1993). Hydrated coating for synthetic seeds. In: Redenbaugh, K. (Ed.). *Synseeds. Applications of Synthetic Seeds to Crop Improvement*, CRC Press. Boca Raton, Florida. pp 35-46.

Redenbaugh, K., Paasch, B.D., Nichol, J.W., Kossler, M.E., Viss, P.R. and Walker, K.A. (1986). Somatic seeds: encapsulation of asexual plant embryos. *BioTechnology*. **4**: 797-801.

Redenbaugh, K., Slade, D., Viss, P. and Fujii, J.A. (1987a). Encapsulation of somatic embryos in synthetic seed coats. *HortScience*. **22**: 803-809.

Redenbaugh, K., Viss, P., Slade, D. and Fujii, J. (1987b). Scaleup: artificial seeds. In: Green, C., Somers, D., Hackett, W. and Biersboer, D. (Eds). *Plant tissue culture*. Liss, New York. pp 473-493.

Rehman, R.U., Israr, M., Srivastava, P.S., Bansal, K.C., and Abidin, M.Z. (2003). *In vitro* regeneration of witloof chicory (*Cichorium intybus* L.) from leaf explants and accumulation of esculin. *In vitro Cellular and Developmental Biology-Plant*. **39**: 142-146.

Reynolds, J.F. (1994). *In vitro* culture of vegetable crops. In: Vasil, I.K. and Thorpe, T.A. (Eds). *Plant cell and tissue culture,* Kluwer. Dordrecht, The Netherlands. pp 331-362.

Robb, S.H. (1957). The culture of excised tissue from bulb scales of *Lilium speciosum*. *J. exp. Bot*. **8**: 348-52.

Rodrigues, P.H.V., Lima, A.M.L.P., Ambrosano, G.M.B. and Dutra, M.F.B. (2005). Acclimatization of micropropagated *Heliconia bihai* (Heliconiaceae) plants. *Sci. Agric. (Piracicaba, Braz.)*. **62(3)**: 299-301.

Rolando, G.G., Karla, Q., Basilio, C. and Peter, C. (2010). Plant tissue culture: Current status, opportunities and challenges. *Cien. Inv. Agr*. **37(3):** 5-30.

Roldan, M., Gomez-Mena, C., Ruiz-Garcia, L., Salinas, J. and Martinez-Zapater, J.M. (1999). Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of *Arabidopsis* in the dark, *Plant J*. **20**: 581-590.

Ross-Karstens, G.S., Ebert, G. and Ludders, P. (1998). Influence of *in vitro* growth conditions on stomatal density, index and aperture of grape, coffee and banana plantlets. *Plant Tissue Culture and Biotechnology*. **4**: 21-27.

Roy, P.K. (2008). Rapid multiplication of *Boerhaavia diffusa* L. through *in vitro* culture of shoot tip and nodal explants. *Plant Tissue Culture and Biotechnology*. **181(1)**: 49-56.

Saiprasad, G.V.S. and Raghuveer, P. (2003). Propagation of three orchid genera using encapsulated protocorm-like bodies. *In vitro Cell. Dev. Biol. Plant*. **39**: 42-48.

Sarkar, D. and Naik, P.S. (1998). Synseeds in potato: An investigation using nutrient-encapsulated *in vivo* nodal segments. *Scientia Horticulturae*. **73**: 179- 184.

Saritha, K.V. and Naidu, C.V. (2007). *In vitro* flowering of *Withania somnifera* Dunal.- An important antitumor medicinal plant. *Plant Science*. **172 (4)**: 847-851.

Schuller, A., Kirchner-Ness, R. and Reuther, G. (2000). Interaction of plant growth regulators and organic C and N components in the formation and maturation of *Abies alba* somatic embryos. *Plant Cell Tiss. Org. Cult.* **60**: 23-31.

Scorza, R. (1982). *In vitro* flowering. *Horticulture Review*. **4**: 106-127.

Scowcroft, W.R. (1977). Somatic cell genetics and plant improvement. *Advanc. Agron.* **29**: 39-81.

Sciutti, R. and Morini, S. (1993). Effect of relative humidity in *in vitro* culture on some growth characteristics of a plum rootstock during shoot proliferation and rooting and on plantlet survival. *Advances in Horticultural Science*. **7**: 153-156.

Senaratna, T. (1992). Artificial seeds. *Biotechnol Adv*. **10**: 379-392.

Sharma, A., Tandon, P. and Kumar, A. (1992). Regeneration of *Dendrobium wardianum* Warner (Orchid) from synthetic seeds. *Ind. J. Exp. Biol.* **30**: 747-748.

Shrivastava and Rajani, M. (1999). Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L) peanell. *Plant Cell Rep*. **18**: 918-923.

Sicurani, M., Piccioni, E. and Standardi, A. (2001). Micropropagation and preparation of synthetic seed in M.26 apple rootstock. I. Attempts towards saving labor in the production of adventitious shoot tips suitable for encapsulation. *Plant Cell Tissue Organ Cult*. **66**: 207-216.

Sies, H. (1993). Strategies of antioxidant defense. *European Journal of Biochemistry*. **215**: 213-219.

Singh, S.P. (1997). Principles of Vegetable Production. Agrotech Publishing Academy, Udaipur, India.

Singh, A.K., Rai, M.K., Asthana, P., Pandey, S., Jaiswal, V.S. and Jaiswal, U. (2009). Plant regeneration from alginate-encapsulated shoot tips of *Spilanthes acmella* (L.) Murr., a medicinally important and herbal pesticidal plant species. *Acta Physiol. Plant*. **31**: 649-653.

Singh, A.K., Sharma, M., Varshney, R., Agarval, S.S. and Bansal, K.C. (2006). Plant regeneration from alginate-encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn, A medicinally important plant species. *In Vitro Cell Dev Biol-Plant*. **42**: 109-113.

Singh, A.K., Varshney, R., Sharma, M., Agarwal, S.S. and Bansal, K.C. (2006a). Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *J. Plant Physiol*. **163**: 220-223.

Singh, A.K., Sharma, M., Varshney, R., Agarwal, S.S. and Bansal, K.C. (2006b). Plant regeneration from alginate-encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn, a medicinally important plant species. *In Vitro Cell. Dev. Biol. Plant.* **42**: 109-113.

Silva, A.B., Pasqual, M., Maciel, A.L.R. and Dutra, L.F. (2003). BAP e substrato na aclimatização de plântulas de gloxínia (*Sinningia speciosa* Lood. Hiern.) provenientes de cultura de tecidos. *Ciência Agrotecnológica*. **27**: 255-260.

Skoog, F. and Miller, E.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *J. Exp. Biol*. **11**: 118-131.

Slater, A., Scott, N.W. and Fowler, M.R. (2003). Plant Biotechnology: The Genetic Manipulation of Plants. *Oxford University Press Inc*, New York.

Smulders, M.J.M., Visser, E.J.W., Croes, A.F. and Wullems, G.J. (1990). The dose of NAA determines flower bud formation in tobacco explants at a large range of concentration. *Planta*. **180**: 410 – 415.

Soares, J.R., Dinis, T.C.P., Cunha, A.P. and Almeida, L.M. (1997). Antioxidant activities of some Extracts of Thymus zygis. *Free Radical Research*. **26**: 469-478.

Sofowora, A. (1993). Medicinal plants and traditional medicine in Africa. Spectrum books, Ibadan. pp. 150

Solomon, E.P., Berg, L.R. and Martin, D.W. (2005). Biology. 7th edition, Thompson Brooks/ Cole, United States of America.

Soneji, J.R., Rao, P.S. and Mhatre, M. (2002). Germination of synthetic seeds of pineapple (*Ananas comosus* L. Merr.). *Plant Cell Rep*. **20**: 891-894.

Sridhar, T.M. and Naidu, C.V. (2011). High frequency plant regeneration, *In vitro* flowering of *Solanum nigrum* (L.) – An important antiulcer medicinal plant. *Journal of Phytology. Tissue culture*. **3(2)**: 85-93.

Standardi, A. and Piccioni, E. (1998). Recent perspectives on synthetic seed technology using non-embryogenic *in vitro*-derived explants. *Int J Plant Sci*. **159(6)**: 968-978.

Strack, D. and Wray, V. (1994). The anthocyanins. In: Harborne, J.B. (Ed). *The Flavonoids*: Advances in Research Since 1986. Chapman and Hall.

Street, H.E. (1977). Introduction. In: Street, H.E. (Ed.). *Plant tissue and cell culture*, Blackwell. Oxford, UK. pp 1-10.

Stuart, D.A., Strickland, S.G. and Walker, K.A. (1987). Bioreactor production of alfalfa somatic embryos. In: Proc Symp Synthetic seed technology for the mass cloning of crop plants: problems and perspectives. *HortScience*. **22**: 800-803.

Sudhakaran, S. and Sivasankari, V. (2002). *In vitro* flowering response of *Oscimum basilicum* (L.). *J. Plant Biotechnol*. **4(4)**: 181-183.

Taha, R.M. and Haron, N.W. (2008). Some morhophological and anatomical studies of leaves and flowers of *Murraya paniculata* (Jack) Linn. *in vivo* and *in vitro*. Pakistan Journal of Biological Sciences. **11(7)**: 1021-1026.

Taha, R.M. and Wafa, S.H. (2011). Plant regeneration and cellular behaviour studies in Celosia cristata grown *in vivo* and *in vitro*. *The Scientific World Journal*. **2012**, Article ID 359413, 8 pages.

Tang, W. (2000). High-frequency plant regeneration via somatic embryogenesis and organogenesis and *in vitro* flowering of regenerated plantlets in *Panax ginseng*. *Plant Cell Rep*. **19**: 727–732.

Taixeira da Silva, J.A. (2003). Thin cell layer technology in ornamental plant micropropagation and biotechnology. *African Journal of Biotechnology*. **2**: 683-691.

Taylor, N.J., Light, M.E. and Staden, J.V. (2005). *In vitro* flowering of *Kniphofia leucocephala*: Influence of cytokinins. *Plant Cell Tissue and Organ Culture*. **83**: 327-333.

Thomas, E., King, P.J. and Potrykus, I. (1979). Improvement of crop plants via single cells *in vitro* – an assessment. *Z. Pflanzenzüchtg*. **82**: 1-30.

Thorpe, T.A (2007). History of plant tissue culture. *Molecular Biotechnology*. **37**: 169-180.

Thorpe, T.A. (1980). Organogenesis *in vitro*: Structural, physiological, and biochemical aspects. *Int. Rev. Cytol. Suppl*. **11A**: 71-111.

Tisserat, B. and Galletta, P.D. (1995). *In vitro* flowering and fruiting of *Capsicum fruitescens* L. Am. Soc. *Horticult. Sci*. **30**: 130–132.

Tran Thahn Van, K. M. (1981). Control of morphogenesis in *in vitro* cultures. *Ann. Rev. Plant Physiol*. **32**: 291-312.

Trease, G.E. and Evans, W.C. (1989). Pharmacognosy. 13th edition. Bailliere Tindall, London. pp. 176-180.

Tseng, T.H., Kao, E.S., Chu, C.Y., Chou, F.P., Lin Wu, H.W. and Wang, C.J. (1997). Protective Effects of Dried Flower Extracts of *Hibiscus sabdariffa* L. against Oxidative Stress in Rat Primary Hepatocytes. *Food Chemistry and Toxicology*. **35**: 1159-1164.

Van, D.E.G., Croes, A.F., Kemp, A. and Barendse, G.W.M. (1984a). Development of flower buds in thin-layer cultures of floral stalk tissue from tobacco: Role of hormones in different stages. *Physiol. Plant*. **61**: 114-118.

Van, D.E.G., Croes, A.F, Kemp, A., Barendse, G.W.M. and Kroh, M. (1984b). Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum*. *Physiol. Plant.* **62**: 83-88.

Van Huylenbroeck, J.M. and Debergh, P.C. (1996). Physiological aspects in acclimatization of micropropagated plantlets. *Plant Tissue Culture and Biotechnology*. **2**: 136-141.

Van Wyk, B.E. and Gericke, N. (2000). People's plants: a guide to useful plants of southern Africa. Briza Publications, Pretoria.

Van Wyk, B.E., Van Heerden, F. and Van Oudtshoorn, B. (2002). Poisonous plants of South Africa. Briza Publications, Pretoria.

Varotto, S., Lucchin, M., and Parrin, P. (2000). Immature embryos culture in Italian red Chicory (*Cichorium intybusc*). *Plant Cell Tissue and Organ Culture*. **62**: 75-77.

Vasil, I.K. and Vasil, V. (1980). Clonal propagation. In: Vasil, I.K. (Ed.). Int. Rev. Cytol. Suppl. 11A. *Perspectives in Plant Cell and Tissue Culture*, Academic press. New York. pp 145-73.

Vasil, I.K. and Vasil, V. (1994). *In vitro* culture of cereals and grasses. In: Vasil, I.K. and Thorpe, T.A. (Eds.). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 293-312.

Vaz, A.P.A., de Cassia, L., Riberiro, F. and Kerbauy, G.B. (2004). Photoperiod and temperature effects on *in vitro* growth and flowering of *P. pusilla*, an epiphytic orchid. *Plant Physiol. and Biochem*. **42**: 411-415.

Verma, S.K., Rai, M.K., Asthana, P., Jaiswal, V.S. and Jaiswal, U. (2010). *In vitro* plantlets from alginate-encapsulated shoot tips of *Solanum nigrum* L. *Scientia Horticulturae.* **124**: 517-521.

Vijayakumar, M., Vijayakumar, R., and Stephen, R. (2010). *In Vitro* propagation of *Bacopa monnieri* L. – a multipurpose medicinal plant. *Indian Journal of Science and Technology*. **3**(7): 781-786.

Wang, G.Y., Yuan, M.F. and Hong, Y. (2002). *In vitro* flower induction in roses. *In vitro Cellular and Developmental Biology- Plant*. **38**: 513-518.

Wang, S., Tang, L. and Chen, F. (2001). *In vitro* flowering of bitter melon. *Plant Cell Rep*. **20**: 393-397.

Wei, S.-D., Zhou, H.-C. and Lin, Y.-M. (2010). Antioxidant activities of extract and fractions from the hypocotyls of the mangrove plant *Kandelia candel*. *Int. J. Mol. Sci*. **11**: 4080-4093.

Wetzsstein, H.Y. and Sommer, H.E. (1982). Leaf anatomy of tissue cultured *Liquidambar styraciflua* (Hamamelidaceae) during acclimatization. *Am J Bot*. **69**: 1579-1586.

Wetzstein, H.Y. and Sommer, H.E. (1983). Scanning electron microscopy of *in vitro* cultured *Liquidambar styraciflua* plantlets during acclimatization. *J Am Soc Hortic Sci*. **108**: 475-480.

White, P.R. (1939). Controlled differentiation in a plant tissue culture. *Bulletin of the Torrey Botany Club*. **66**: 507-513.

Wright, N.A. and Alderson, P.G. (1980). The growth of tulip tissues *in vitro*. *Acta Horticulturae*. **109**: 263-70.

Yamakawa, T., Kurahashi, O., Ishida, K., Kato, S., Kodama, T. and Minoda, Y. (1979). Stability of indole-3-acetic acid to autoclaving, aeration and light illumination. *Agriculture & Biochemical Journal*. **43(4)**: 879-880.

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

Zhang, T. (2007). *In vitro* flowering of *Perilla frutescens*. *In Vitro Cell.Dev.Biol.- Plant*. **43**: 91-94.

Zhang, W. and Furusaki, S. (1999). Production of anthocyanins by plant cell cultures. *Biotechnol. Bioprocess. Eng*. **4:** 231-252.

Zhang, Z. and Leung, D.W.M. (2000). A comparison of *in vitro* and *in vivo* flowering in Gentian. *Plant Cell Tissue Organ Culture*. **63**: 223-226.

Zhang, Z. and Leung, D.W.M. (2002). Factors influencing the growth of micropropagated shoots and *in vitro* flowering of Gentian. *Plant Growth Regul*. **36**: 245-251.

Zhong, H., Srinivasan, C. and Sticklen, M.B. (1992). *In vitro* morphogenesis of corn (*Zea mays* L.). *Planta*. **187**: 490-497.

Zhou, J.H., Zhou, J.R., Lin, B.C. and Din, J. (2004). Influences of 6-BA and amino acids on flower formation from cotyledons of cucumber *in vitro*. *Plant Physiol Comm*. **6**: 31-33.

Zimmerman, R.H. and Swartz, H.J. (1994). *In vitro* culture of temperate fruits. In: Vasil, I.K. and Thorpe, T.A. (Eds.). *Plant cell and tissue culture*, Kluwer. Dordrecht, The Netherlands. pp 457-474.

Ziv, M. and Naor, V. (2006). Flowering of geophytes *in vitro*. *Propagation of Ornamental Plants*. **6**(1): 3-16.

APPENDIX I

PAPERS PRESENTED / SEMINARS ATTENDED

1) Taha, R.M., Hasbullah, N.A., Elias, H., Abdullah, S., Rahiman, F.A., Aziz, A.H.A., Wafa, S.N.W.S.T., **Yusoff, A.I.M**., Radzuan, N.H.M., Anuar, N., Mohsan, S. and Rawi, N. (2010). Miniature Garden of *In Vitro* Plantlets Derived from Somatic Embryos of Ornamental Plants. Malaysia Technology Expo 2010. 4-6 February, Kuala Lumpur. (Awarded Bronze Medal).

2) **Yussof, A.I.M.,** Wafa, S.N.A., Taha, R.M. (2010). Plant regeneration and synthetic seeds production of *Brassica oleracea* var. *italica.* ISHS Symposium 'Sustainable Vegetable Production in SE Asia'. 13-17 March 2011. Salatiga, Indonesia.

3) Abdullah, N., Taha, R.M., Mahmood, N.Z., Elias, H., Johari, N.M.K., Bakar, A.A., Rahiman, F.A., **Yussof, A.I.M**., Abdullah, S. and Aziz, A.H.A. (2010). Recycling of Spent Mushroom Compost via Cultivation of Snowpea Sprout (Duo Miao) Artificial Seeds and Vermicomposting. Innovation and Creativity Expo 2010. 1-3 April 2010. University of Malaya, Kuala Lumpur.

4) **Yussof, A.I.M.**, Yaacob, J.S., Hasni, Z. and Taha, R.M. (2010). Effect of different pH and production of paint from natural pigment of anthocyanins extracted from *Oxalis triangularis*. 3rd International Conference on Functional Materials and Devices. 13-17 June 2010. Terengganu, Malaysia.

5) **Yussof, A.I.M.** and Taha, R.M. (2012). Plant Regeneration and *In Vitro* Flowering of *Oxalis triangularis*. The International Symposium on Orchids and Ornamental Plants. 9-12 January 2012. Chiang Mai, Thailand.

APPENDIX II

RESEARCH ARTICLE PUBLISHED

1) Yaacob, J.S., **Yussof, A.I.M**., Abdullah, S., Ramesh, K. and Taha, R.M. (2011). Investigation of pH varied anthocyanin pigment profiles of *Agapanthus praecox* and its potential as natural colourant. *Materials Research Innovations*. **2(15)**: 106-109.

2) Hasni, Z., Yaacob, J.S., **Yusoff, A.I.M**., Taha, R.M., Yahya, R., Bakrudeen Ali Ahmed, A. and Ramesh, K. (2011). Effect of different solvents extraction on recovery of pigments in *Xylocarpus granatum*, endangered medicinal plant. *Materials Research Innovations*. **2(15)**: 141-143.

3) Yaacob, J.S., **Yussof, A.I.M**., Taha, R.M. and Mohajer, S. (2012). Somatic embryogenesis and plant regeneration from bulb, leaf and root explants of African blue lily (*Agapanthus praecox* ssp. *minimus*). *Australian Journal of Crop Science*. **6(10)**: 1462-1470.

4) **Yussof, A.I.M.,** Wafa, S.N.A., Taha, R.M. (2010). Plant regeneration and synthetic seeds production of *Brassica oleracea* var. *italica*. *Acta Horticulturae*. **958**: 179-185.