STUDIES ON MICROPROPAGATION, CELLULAR BEHAVIOR, PHOTOSYNTHETIC AND BIOLOGICAL ACTIVITIES OF RED CLOVER (TRIFOLIUM PRATENSE L.)

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ABSTRACT

Tissue culture studies of a temperate forage crop, *Trifolium pratense* L. were investigated in the current project. *In vitro* regeneration of this species was successfully achieved in this study using nodal explants cultured on Murashige and Skoog (MS) media supplemented with different hormones at various concentrations and also on MS hormone free media as a control. Complete plant regeneration of *T. pratense* was best achieved when the nodal explants were cultured on MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA, with mean number of 6.05 ± 0.28 shoots per explant, and 100% of the explant samples produced shoots. On the other hand, the best root formation was obtained on MS media supplemented with 1.5 mg/l BAP and 0.75 mg/l IBA, with the mean number of 3.3 ± 0.21 roots per explant. However, the nodal explants cultured on MS hormone free medium failed to produce any shoots or roots. Callus formation was successfully achieved when the nodal explants were cultured on MS medium containing different types of plant hormones. MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l 2,4-D was the most responsive, whereby 100% of the explants managed to produce callus.

Adaptation process to the natural environment or acclimatization, i,e. the transfer of *in vitro* grown plants to the ex *vitro* condition was successfully undertaken, with very high survival rates of plantlets (93.71 ± 4.64 %) when they were transferred to the combination of red soil and black soil with the ratio of 1:1.

Subsequently, the extracts of *in vivo* and *in vitro* grown plants as well as callus tissues of *T. pratense* were tested for their antioxidant activities, using different extraction solvents and different antioxidant assays. The total flavonoid and phenolic contents as well as extraction yield of the extracts were also investigated to determine their correlation with the antioxidant activity of the extracts. Among all the tested extracts, the highest amount of total phenolic and total flavonoids content were found in methanol

extract from *in vivo* grown plants. The antioxidant activity of tested samples followed the order; *in vivo* plant extract > callus extract > *in vitro* extract. The highest reducing power, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging and chelating power were found in methanol extracts of *in vivo* grown *T. pratense*. Whilst the chloroform fraction of *in vivo* grown plants showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, superoxide anion radical scavenging and hydrogen peroxide scavenging compared to the other tested extracts. A significant correlation was found between the antioxidant activity of extracts and their total phenolic and total flavonoid content.

The cytotoxicity of the plant extracts were examined against two human cancer cell lines (human breast carcinoma (MCF-7) and human colon carcinoma (HCT-116)), using MTT assay. Four different extraction solvents were used to examine the effect of the solvent on cytotoxic activity of the extracts. Two cancer cell lines were treated with the extracts for 24, 48 and 72 hours. All of the examined extracts exhibited toxicity on the tested cell lines in a time dependent increase, but in a lower potency than doxorubicin (positive control). The chloroform fraction of *in vivo* grown plants showed the highest cytotoxic activity against the MCF-7 cell line (IC_{50} = 66.44 ± 2.05 µg/ml) but it was not significantly different with the cytotoxic activity of chloroform fraction of callus tissue (IC_{50} = 69.48 ± 2.66 µg/ml). The highest cytotoxic activity against the HCT-116 cell line was shown by the chloroform fraction of callus tissue (IC_{50} = 79.53 ± 2.00 µg/ml).

The antimicrobial efficiency of extract derived from *T. pratense* (*in vivo* and *in vitro* grown plants, including callus) were examined using ethanol and methanol as solvents for extraction and tested against four bacterial pathogens (two gram negative and two gram positive) and three fungal pathogens. The antimicrobial activity of the methanol extract was found to give higher inhibition zone when compared with ethanol extract.

Among the callus, *in vitro* and *in vivo* grown plants, the callus extract showed better antimicrobial activity, thus revealing a new potential use of *T. pratense* callus.

To compare the photosynthetic parameters, the Stomatal conductance (g_s), Transpiration rate (E) and Net photosynthetic (P_n) were determined for the plants grown under *in vitro* and *in vivo* conditions. A comparison was made for the observed data for the light-saturated photosynthetic among the treatments which revealed that the maximum photosynthetic rate (P_{Nmax}) was 18.3 and 11.3 µmol $(CO_2)/m^2/s$ in *in vivo* and *in vitro* plant leaves, respectively. Respiration (R_d) and Compensation point (CP) were found 1.5-folds and two-folds higher in *in vitro* plants, respectively. On the other hand, the *in vitro* grown plants exhibited higher transpiration rate and also higher stomatal conductance compared with the *in vivo* plants. Consequently, high levels of differentiation in terms of photosynthesis parameters exist among the *in vivo* and *in vitro* samples. Significant direct relation was observed between net photosynthetic rate and total phenolic and flavonoid content of *T. pratense* leaves.

The effect of optimal and supra-optimal concentrations of Sodium chloride (NaCl) on growth and antioxidant defence was also studied in the *in vitro* cultures of *T. pretence*. Seeds of *T. pratense* were germinated in Murashige and Skoog medium (MS) containing different concentrations of NaCl (0, 50, 100, 150, 200 mM). The lengths of roots and shoots as well as percentage of germination, free radical scavenging activity (DPPH) and Superoxide dismutase (SOD) were measured. A significant decrease in germination and growth was observed in the seeds exposed to 100, 150 and 200 mM salt. The highest percentage of germination was found in the MS medium containing 50 mM NaCl, although the highest root and shoot length were found in MS medium without NaCl. The highest antioxidant activity of methanol extract of the plants occurred in *in vitro* plants cultured in MS medium supplemented with 50 mM NaCl. A significant decrease in free

radicals scavenging and superoxide dismutase activities were found in plants grown in media containing 100, 150 and 200 mM salt.

ABSTRAK

Kajian kultur tisu ke atas tumbuhan temperat untuk makanan haiwan ternakan iaitu *Trifolium pratense* L., telah dikaji dalam projek ini. Regenerasi secara *in vitro* telah berjaya dicapai menggunakan eksplan batang bernod yang dikultur dalam media Murashige dan Skoong (MS) yang ditambah dengan pelbagai jenis hormon dan kepekatan, dan juga ke atas media MS tanpa hormon sebagai kawalan. Regenerasi tumbuhan lengkap yang terbaik telah dicapai apabila eksplan batang bernod dikultur ke atas media MS yang ditambah dengan 1.5 mg/l BAP dan 0.5 mg/l IBA, dengan purata bilangan pucuk 6.05 ± 0.28 per eksplan di mana 100% eksplan menghasilkan pucuk. Walaubagaimanapun, eksplan batang bernod yang dikultur ke atas media MS tanpa hormon gagal menghasilkan sebarang pucuk dan akar. Pembentukan kalus telah berjaya dicapai apabila eksplan batang bernod dikultur ke atas media MS yang mengandungi pelbagai jenis hormon tumbuhan. Media MS yang ditambah dengan 1.5 mg/l BAP dan 0.5 mg/l 2,4-D adalah yang paling responsif di mana 100% eksplan berjaya menghasilkan kalus.

Proses penyesuaian kepada persekitaran semulajadi ataupun *acclimatization* iaitu pemindahan tumbuhan *in vitro* kepada keadaan *ex vitro* telah berjaya dilakukan dengan kadar hidup plantlet yang tinggi (93.71 ± 4.64 %) apabila plantlet dipindahkan kepada kombinasi tanah merah dan tanah hitam dengan nisbah 1:1. Seterusnya, aktiviti antioksidan di dalam ekstrak tumbuhan *in vivo* dan *in vitro* dan juga tisu kalus *T. pratense* dikaji dengan menggunakan pelarut pengekstrakan dan ujian antioksidan yang berlainan. Kandungan flavonoid, fenolik dan juga hasil pengekstrakan juga dikaji untuk menentukan hubungan aktiviti antioksidannya. Di antara keseluruhan ekstrak yang dikaji, ekstrak metanol daripada tumbuhan *in vivo* mengandungi kandungan flavonoid dan fenolik tertinggi. Aktiviti antioksidan sampel-sampel kajian adalah mengikut urutan berikut, ekstrak tumbuhan *in vivo* > ekstrak kalus > ekstrak *in vitro*. Kemampuan memerangkap

radikal 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonik asid) (ABTS) dan kemampuannya mengkelat logam adalah yang tertinggi di dalam ekstrak metanol tumbuhan *in vivo*. Sementara itu, pecahan kloroform tumbuhan *in vivo* menunjukkan kemampuan memerangkap radikal 2,2-diphenyl-1-picrylhydrazyl (DPPH), kemampuan memerangkap radikal superoksida anion dan kemampuan memerangkap radikal hidrogen peroksida yang tertinggi berbanding ekstrak lain. Hubungan yang signifikan telah ditemui di antara aktiviti antioksidan ekstrak dan jumlah kandungan flavonoid dan fenolik.

Sitotoksisiti ekstrak tumbuhan telah diuji ke atas sel kanser manusia (sel karsinoma payudara manusia (MCF-7) dan sel karsinoma kolon manusia (HCT-116), menggunakan ujian MTT. Empat pelarut pengekstrakan yang berlainan telah digunakan untuk mengkaji kesan pelarut ke atas aktiviti sitotoksisiti ekstrak. Dua jenis sel kanser telah dirawat dengan ekstrak tumbuhan selama 24, 48 dan 72 jam. Kesemua ekstrak yang dikaji menunjukkan toksisiti ke atas sel kanser mengikut pertambahan masa, tetapi dalam potensi yang rendah berbanding doxorubicin (kawalan positif). Pecahan kloroform tumbuhan *in vivo* menunjukkan aktiviti toksisiti yang tertinggi terhadap sel MCF-7 (IC_{50} = 66.44 ± 2.05 µg/ml) tetapi ia tidak signifikan dengan aktiviti toksisiti pecahan kloroform tisu kalus (IC_{50} = 69.48 ± 2.66 µg/ml). Aktiviti toksisiti yang tertinggi terhadap sel HCT-116 telah ditunjukkan oleh pecahan kloroform tisu kalus (IC_{50} = 79.53 ± 2.00 µg/ml).

Kecekapan antimikrob ekstrak daripada *T. pratense* (tumbuhan *in vivo* dan *in vitro* termasuk kalus) telah dikaji menggunakan etanol dan metanol sebagai pelarut untuk pengekstrakan dan diuji ke atas empat patogen bakteria (dua gram negatif dan dua gram positif) dan tiga patogen fungi. Aktiviti antimikrob ekstrak methanol didapati menunjukkan zon perencatan yang tertinggi berbanding ekstrak etanol. Di antara kalus,

tumbuhan *in vivo* dan *in vitro*, ekstrak kalus menunjukkan aktiviti antimikrob yang lebih baik. Hal ini mendedahkan potensi baru penggunaan kalus *T. pretense.*

Untuk membandingkan parameter fotosintesis, kealiran stomata (g_s), kadar transpirasi (E) dan fotosintesis bersih (P_n) telah ditentukan ke atas tumbuh-tumbuhan yang ditanam dalam keadaan *in vitro* dan *in vivo*. Perbandingan data fotosintesis cahaya tepu mendedahkan bahawa kadar fotosintesis maksimum (P_{Nmax}) adalah 18.3 dan 11.3 μmol (CO_2) / m_2 / s di dalam tumbuhan *in vivo* dan *in vitro*, masing-masing. Respirasi (R_d) dan titik *compensation* (C_P) didapati 1.5 kali ganda dan dua kali ganda lebih tinggi di dalam tumbuhan *in vitro*, masing-masing. Sebaliknya, tumbuhan *in vitro* menunjukkan kadar transpirasi dan kealiran stomata yang lebih tinggi berbanding tumbuhan *in vivo*. Oleh itu, tahap pembezaan dari segi parameter fotosintesis wujud antara sampel tumbuhan *in vivo* dan *in vitro*. Hubungan secara langsung yang signifikan telah diperhatikan antara kadar fotosintesis bersih dan jumlah kandungan fenolik dan flavonoid daun *T. pratense*.

Kesan kepekatan optimum dan supra-optimum natrium klorida (NaCl) kepada pertumbuhan dan pertahanan antioksidan juga telah dikaji di dalam kultur *in vitro T. pratense*. Biji benih *T. pratense* telah dicambahkan dalam media Murashige dan Skoog (MS) yang mengandungi kepekatan NaCl (0, 50, 100, 150, 200 mM) yang berbeza. Panjang akar dan pucuk serta peratusan percambahan, kemampuan memerangkap radikal bebas (DPPH) dan superoxide dismutase (SOD) telah diperhatikan. Penurunan signifikan dalam percambahan dan pertumbuhan telah diperhatikan dalam biji benih yang didedahkan kepada 100, 150 dan 200 mM garam. Peratusan tertinggi percambahan ditemui dalam media MS yang mengandungi 50 mM NaCl, walaupun panjang akar dan pucuk yang tertinggi ditemui dalam media MS tanpa NaCl. Aktiviti antioksidan ekstrak metanol yang tertinggi berlaku dalam tumbuhan yang dikultur dalam media MS yang ditambah dengan 50 mM NaCl. Penurunan yang signifikan dalam kemampuan

memerangkap radikal bebas dan aktiviti superoksida dismutase telah ditemui dalam tumbuhan yang ditanam di dalam media yang mengandungi 100, 150 dan 200 mM garam.

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LIST OF SYMBOLS AND ABBREVIATIONS

µg	:	Microgram
µl	:	Microliter
µM	:	Micromolar
2,4-D	:	2,4-Dichlorophenoxyacetic Acid
ABTS	:	2,2'-azino-bis
$AlCl_3.6H_2O$	:	Aluminum Chloride Hexahydrate
ANOVA	:	Analysis of variance
BAP	:	Benzylaminopurine
C	:	Celsius
CFU	:	Colony-forming unit
CO_2	:	Carbon dioxide
CTE	:	Rutin equivalent
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
E	:	Transpiration rate
g/l	:	Gram per liter
GAE	:	Gallic acid equivalent
g_s	:	Stomatal conductance
h	:	Hours
HCl	:	Hydrochloric acid
HCT-116	:	Human colon carcinoma
IBA	:	Indolebutyric Acid
Kinetin	:	6-furfurylaminopurine

MCF-7	:	Human breast carcinoma
mg/l	:	Miligram per liter
MI	:	Mitotic index
ml	:	Mililiter
mm	:	Millimeter
mM	:	Millimolar
MS	:	Murashige and Skoog
MSO	:	Murashig and Skoog (without hormone)
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	:	Naphthalene Acetic Acid
NaCl	:	Sodium chloride
NaCl	:	Sodium chloride
$NaNO_2$	:	Sodium nitrite
NaOH	:	Sodium hydroxide
NBT	:	Nitro blue tetrazolium
nm	:	Nanometer
OD	:	Optical density
PGRs	:	Plant growth regulators
P_n	:	Net photosynthetic rate
PPFD	:	Photosynthesis photon flux density
R^2	:	Coefficient of determination
ROS	:	Reactive oxygen species
rpm	:	Rotation per minute
SD	:	Standard deveision
SE	:	Standard error
TDZ	:	Thiadiazuron

UV	:	Ultra violet
w/v	:	Weight per volume
AQY	:	Apparent quantum yield
R_d	:	Respiration
CP	:	Compensation point
P_{Nmax}	:	Maximum net photosynthetic
SOD	:	Superoxide dismutase

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CHAPTER 1: INTRODUCTION

1.1 Importance of Bioactivities Discovery from Plants

The plant kingdom represents an enormous reservoir of chemical compounds. There has been considerable amount of effort and research focused on finding novel bioactive compounds from natural sources such as tea, fruits, vegetables, herbs and spices (Kanazawa & Sakakibara, 2000). Most of the researches also suggested that the natural compounds found in fruits and vegetables may reduce the risk of degenerative ailments. Besides the interest in finding novel bioactive compounds from natural sources, there has been a worldwide move towards the use of traditional medicines due to concerns over the invasive, expensive and potentially toxic mainstream practices (Wyk, 2002; Ernst, 2002). Ethnopharmacological surveys have shed light on the fact that the therapeutic use of even 80% of 122 plant-derived drugs may have a link with their recommendations in traditional medicine (Fabricant & Farnsworth, 2001).

The efforts regarding understanding the natural products are always an intriguing goal for researchers over decades, especially on plants. With reference to past events, plants (fruits, vegetables, medicinal herbs, etc.) have given a decent wellspring of a wide variety of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other secondary metabolites, which are rich in valuable bioactivities, e.g., antioxidant, anti-inflammatory, antitumor, antimutagenic, anti-carcinogenic, antibacterial, or antiviral activities. In numerous oriental countries (China, Japan, etc), the traditional herbal medicines have been generally utilized for a large number of years. Herbal plants have turned into the primary object of chemists, biochemist, and pharmaceutics. Their examination assumes an essential part to discover and growing new medications, which are having ideally more viability and no side effects like most present day drugs.

Medicinal plants are natural resources which are often used in the treatment of various ailments. From ancient time, plants are rich source of effective and safe medicines. In recent years there has been more focus on plants with antimicrobial, antioxidant and anticancer activities.

Several studies have shown that aromatic and medicinal plants are sources of various nutrient and non-nutrient molecules, many of which show antioxidant, anticancer and antimicrobial properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus, it is important to characterize the different types of medicinal plants for their biological activities potential (Mothana & Lindequist, 2005; Bajpai et al., 2005; Wojdylo et al., 2007).

In this way, research on herbal plant significantly is considered for both to find active compounds and to locate the viable instrument of them to form into medications for treatment illnesses. Besides, the research additionally supplied general constituents and impacts that can energize the utilization of herbal plants as "nourishment" for escalating wellbeing and avert illnesses.

In developing countries like Malaysia, medicinal plants continue to be the main source of medication. Current developments in phytotechnology, phytochemistry and biotechnology have facilitated rapid progress in natural product research.

1.2 Plant Biotechnology

One of the most challenging problems in the development and use of medicinal plants to produce drugs is the non-economic isolation of their pharmaceutically important compounds due to their low production quantity (Kayser & Quax, 2007). Hence, several biotechnological techniques have been employed to enhance the production of bioactive compounds in plants such as tissue culture and genetic modification methods (Robins,

1994; Tripathi & Tripathi, 2003; Kayser & Quax, 2007; Karuppusamy, 2009; Chaudhury & Pal, 2010).

Plant biotechnology is the use of exploratory learning to the enhancement of plant characteristics. Improvements are in amount or quality. It is a specific process in which scientific techniques are used to develop molecular- and cellular-based technologies to improve plant productivity, quality and health; to improve the quality of plant products; or to prevent, reduce or eliminate constraints to plant productivity caused by diseases, pest organisms and ecological stresses. Almost all commercial improvements have been in productivity. Expanded profitability conserves land and water, brings down the expense of production, and can build the accessibility supply of food, feed, fiber, or pharmaceutical.

The field of plant biotechnology is involved in creating approaches to enhance the generation of plants so as to supply the world's requirements for food, fiber and fuel. Moreover, plants give us numerous pharmaceuticals and industrial compounds. As our populace develops, our needs additionally develop. To build the amount of crop production as well as to produce specific characteristics in plants, biotechnologists are using selective gene techniques. The two noteworthy routines of propagation are: plant tissue culture and genetic engineering.

Study in the zone of plant biotechnology will keep on concentrating on tackling major farming issues. Researchers will keep on moving in the direction of enhancing biotechnology tools for even more secure, more powerful utilization of biotechnology by all specialists. For instance, more effective models are being produced to assess genetically engineered plants and to diminish allergens in foods. Researchers will continue to monitor for potential environmental problems, such as insect pests becoming resistant to biotechnology.

1.3 Plant Tissue Culture and Micropropagation

The need for a sustainable food production is undeniably increasing. Hence, in order to fulfill the demand for food and to achieve a sustainable food production, plant breeding serves as a very vital tool. An example of plant breeding methods includes the use of tissue culture in plant regeneration and propagation. *In vitro* technique or tissue culture technique is an alternative propagation method which plays a very essential role in plant biotechnology. It can serve as a mean to improve the existing plant cultivars, to protect the cultivars from extinction and also to shorten the required time to generate the plants, as compared to conventional breeding (Akbar et al., 2003). Other than that, *in vitro* methods or micropropagation techniques can also be used to produce large amounts of uniform planting materials and to produce genetically engineered plant products and diseases-free plants (Maluszynski et al., 1995).

One example where the use of tissue culture has become increasingly in demand is in the oil palm industry. Malaysia with approximately 48% of palm oil production is one of the leading countries in the world to export palm oil, followed by Indonesia with 35% of palm oil production and other countries (Soh et al., 2003). This makes oil palm as one of the most valuable economic resources of the country. Due to such importance, many researches has been done to improve the oil palm cultivars and to obtain the highest yielding cultivars with enhanced oil production. One of the most sought-after technique is tissue culture, where high yielding cultivars were produced clonally (Soh et al., 2003).

Tissue culture techniques have already been integrated in biotechnology that permits the regeneration of plants either as clones or somaclones. The accessibility of a proficient protocol for *in vitro* regeneration is an essential to bridle any biotechnological approach for genetic improvement of crop plants.

The use of a plant tissue culture system to produce desirable medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenes, steroids, phenolics, flavonoids, and amino acids (Vanisree et al., 2004). Taxol, for example, is one of the most promising anticancer drugs, which was originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*). Taxol supply from this original source cannot meet the increasing demand for clinical use because of the scarcity and slow growth of *Taxus brevifolia*, and the costly synthetic process (Cragg et al., 1993). Tissue cultures of various Taxus species, therefore, have been studied extensively as an alternative route for production of Taxol and other useful taxane compounds (Wu & Lin, 2003; Kim et al., 2004; Naill & Roberts, 2005).

Term used to describe laboratory activities to produce plantlets from explants or callus of a parent plant is called micropropagation (Brown & Thorpe, 1995). In theory, all plants can be subjected to micropropagation to generate vast amounts of offspring plantlets. However, this is subjected to numerous limitations. Successful micropropagation protocols have been developed for different species, but its use in large scale production has become limited due to a variety of factors including the need of asepsis, labor cost for sub-culturing the micropropagated tissues between the tissue culture vessels and also problems with genetic variations in the resulting plantlets, verification, acclimatization and contamination problems (Brown & Thorpe, 1995).

The success of micropropagation depends to the few factors, such as the source of the explant and the type of the cultured medium. For example, tissues from shoot tips, shoots or roots of the seedling has been identified as excellent sources for regeneration of friable callus via tissue culture (Gamborg et al., 1976).

Another important influential factor in the success of micropropagation, is the type of the medium being used in the culture process. Media with the essential nutrients needed

for growth, is responsible in supplying the seedling and the growth of the cultured plants. For example, in the early years of micropropagation experiments, the white medium had been adopted in micropropagation researches. Since then many modifications had been done to improve the culture media in order to allow for a more efficient and successful growth of plant cells. This is because the white media was found to be insufficient in terms of its nitrogen and potassium content to maintain cell suspension culture and callus formation.

1.4 Importance of Forage Crops

Legume species provide high quality forages for animals with a positive effect on the environment due to reduction in the use of inorganic-N fertilizer due to their N2-fixation ability (Lüscher et al., 2014). However, the protein level in legumes is rather high and the natural digestion process of proteins in ruminant is quite inefficient. To cope with this problem, the use of grass–legume mixtures is interesting as they balance the energy, protein ratio, increase biomass production by transferring the symbiotically fixed N from legumes to grasses and can stimulate voluntary intake (Niderkorn et al., 2014).

Grass and legume plant species that are cultured for livestock feed are called forage crops. They are also very important for reclamation and land conservation. The vegetative portion of the plant, mostly stems and leaves, are used by livestock. Sheep, cattle and other ruminant livestock require the fiber in their diet for proper digestion. This fiber is naturally found in the forage crops. They also gain their required nutrients such as vitamins, minerals and protein from forage crops. Livestock can use the forage crops directly in the pastures, or the plants can conserve as hey or silage for winter feeding. Both annual and perennial plant species are used as forage crops, but perennials are much more commonly utilized.

One of the most important legume forage crops in the world is Alfalfa (*Medicago sativa* L.). It has excellent productivity, high quality, the ability to fix and utilize atmospheric nitrogen and drought tolerance. The superiority of alfalfa to the other forage crops is its higher production of protein per unit area. It can be grown alone or in combination with various grass species. It requires well-drained soil, a pH above 6.1, adequate fertility and proper harvest management. Alfalfa has some disadvantages like; poor persistence under grazing, low tolerance to acidic or variably drained soil and need to have fall rest period.

Sainfoin (*Onobrychis sativa*) is another important forage crop that is well adopted to poor lands where drought is common and cultural practices are minimal. It is native to Turkey, Iran and Europe. It can be used as an alternative to alfalfa. It contains condensed tannins which reduce its potential to produce bloat and improve protein digestion by grazing animals (McMahon et al., 2000). Sainfoin has a good nitrogen fixing ability, so can be grown in conjunction with other forage grasses to improve soil fertility (Lu et al., 2000). Sainfoin offers a superb forage for feeding animals and voluntary consumption of sainfoin by cattle and sheep is 20% higher than for the grass. Unlike many other legumes, it is non-bloating and is known to have anthelmintic properties, so reducing the problems associated with livestock worms. Livestock that are fed by sainfoin have very fast liveweight gains, so young stock can be finished sooner and with very good body grades (Hayot Carbonero et al., 2011). The flowers of sainfoin produce great amounts of nectar and are very attractive to honey bees and other pollinating insects (Ogle et al., 2007). Many investigations have been done to determine the sainfoin polyphenols which include tannins and flavonoids. Significant differences were found between sainfoin types and this will lead to further development of sainfoin plant breeding. Sainfoins are difficult to establish as pasture, are not persistent in grassland, and only yield one crop of hay or seeds per year, thus it is rarely used as a pure crop and is usually presented in pasture in

a grass-legume combination with red clover, white clover or other legumes. Unlike the alfalfa sainfoin is resistant to many pests, but in wet condition the sainfoin cannot survive for long time because of the root and crown rot disease (Morrill et al., 1998). Other reasons that often reduce the interest of using sainfoin are: high seeding rate, large seeds and higher price of seeds compared with other forage crops.

Another important forage crop is red clover (*Trifolium pratense* L.). It is a short-lived perennial plant that is more adapted to the soil with lower pH and wet condition, compared with the alfalfa and sainfoin, so it can be a good alternative to alfalfa in areas where alfalfa winterkill is a problem. Compared with many other legumes, red clover can grow faster, fix more nitrogen, produce more biomass and is more adapted to different soil types. It has lower seeding cost and easier establishment than alfalfa. Red clover is an excellent treatment for the soil, as its extensive root system permeates the top soil. It is greatly suitable for livestock whether at fresh leafy-growth stage or as hay or silage. It exhibited higher digestibility compared with alfalfa or sainfoin and the rate of decline in digestibility with maturation is slower. Because of the physical structure of red clover leaves, the breakdown of consumed forage is quicker and clearance of particles from the rumen is faster. In addition of livestock feeding, red clover have been known for many centuries as a valuable herb in traditional medicine of various cultures (Sabudak et al., 2008; Khan and Khatoon, 2008). Until recently, there are some studies on biological activities and beneficial effects of red clover, like antioxidant activity (Mu et al., 2009), anticancer properties (Liu et al., 2011), platelet activity (Simoncini et al., 2005), antiangiogenic action (Krenn & Paper, 2009), estrogenic effect (Yatkin & Daglioglu 2011).

1.5 Forage Crops in Malaysia

Malaysia with the population of 25 million people in the year 2006 spent around 12.2 billion Ringgits for the import of food. The imported items were consisted of vegetables, meat, fruit, fish dairy products and fish and animal foodstuffs. The amount of the imported products increased to 13.4 billion Ringgits in 2007. Livestock products were imported with the approximate value of 3.7 billion Ringgits including 70-75% for poultry, 20-25% for swine and 1-2% for ruminants, respectively.

Malaysia lacks natural grasslands. The main vegetation is evergreen equatorial rain forest. Where land is cleared, it is normally cultivated with plantation crops such as rubber, oil palm, cocoa and fruit crops.

One of the major limitations to efficient ruminant livestock production in Malaysia is the lack of adequate 1evels of high quality forage for feeding the ruminants. Nonetheless, the wide diversity of farming systems existing in Malaysia offers various potential ecological niches for the forage introduction and evaluation programs to identify adaptive forages and to encourage their adoption and use by the farmers for ruminant production. Some ad hoc research on pasture and fodder grasses was initiated in the early twenties by the Department of Agriculture to improve forage quality and quantity. At the same time, the government was promoting commercial enterprises in the beef and dairy production to increase livestock production to meet increasing meat demand and to raise the standard of living of the farmers through efficient production systems.

The Malaysian experience with the indigenous and introduced (exotic) forages has been variable. The indigenous species do not produce high dry matter yield and the exotic species are less persistent and prone to pest and disease damage. Poor seeding behaviour of the selected species often prevented commercialization (Chin et al., 1974). Although there is a need for a breeding program to upgrade the desirable characteristics of the

selected species, the present circumstances of forage development strategies in Malaysia do not permit or warrant such a research undertaking. Hence, introduction and evaluation of forage germplasm program has remained as the primary means of obtaining genetic resources for upgrading. Interest is now on collection of genetic materials of high productivity, acid soil tolerance, forage quality, shade tolerance, persistency to defoliation or grazing and tolerance to tropical pests and diseases.

1.6 Morphology of *Trifolium pratense*

Trifolium is a genus of about 300 species of plants in the leguminous pea family Fabaceae. The most widely cultivated clovers are *Trifolium repens* (white clover) and *Trifolium pratense* (red clover). As a member of the family Leguminosae or Fabaceae, *Trifolium pratense* L. (red clover) is a short-lived biennial plant which serves as food for livestock, but also as a health food for humans (Leung & Foster, 1996) (Table 1.1). Red clover is a valuable forage legume in temperate regions of the world. It is an herbaceous (non-woody), short-lived perennial plant. Stems develop from the crown and range in height from 18 to 36 inches at maturity. Leaves are arranged alternately on the stems. The leaves borne on the basal crown are long and petiolate, while the leaves borne on stems are often nearly sessile. The leaves are trifoliate or palmate-trifoliate, pubescent and alternate. Leaflets are oval or elliptic, 1-3 cm long x 0.5-1.5 cm broad. They are typically variegated with a white V-like mark. Flower heads, located at the tip of stems, usually have 75-125 individual pinkish-violet flowers (Figures 1.1 and 1.2). Red clover has a taproot system with many secondary branches.

Table 1.1: Taxonomic classification of *Trifolium pratense L.*

Taxonomic classification of *Trifolium pratense* L.	
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Genus	*Trifolium* L.
Species	*Trifolium pratense* L.
Common Name	Red Clover

Figure 1.1: Intact plant of *Trifolium pratense* L.
(Source: http://www.pfaf.org/user/Plant.aspx?LatinName=Trifolium+pratense)

Figure 1.2: Individual pinkish-violet flowers of *Trifolium pratense*
(Source: http://www.rolv.no/bilder/galleri/fjellplanter/trif_pra.htm)

1.7 Uses of *Trifolium pratense*

Trifolium pratense has been a popular, multi purpose crop since European immigrant farmers brought it to North America in the 1500s. It is widely grown as a fodder crop, and also the aboveground parts and blossoms are used in traditional medicine.

Trifolium pratense can be used as a cover crop that provides many benefits such as fixing nitrogen (N) to meet needs of the following crop, protecting soil from erosion, improving soil tilth, competing with weeds, as well as supplying forage needs. It is used primarily as a legume green manure killed ahead of corn or vegetable crops planted in early summer. Grown alone or mixed with grasses, it produces high yields and good quality forage in a wide range of soil types, pH, environmental and management conditions (Carrillo et al., 2004).

In recent years, the interest for red clover has increased, partly because it possesses unique properties that may affect animal performance and product quality. It has superior feeding value and higher ruminant performances (higher milk yield and growth rate) are often obtained when compared with grasses and Lucerne (Fraser et al., 2004; Vanhatalo et al., 2006).

Extracts of *T. pratense* have had a long history of medicinal uses. It was originally used as a medicinal herb by native indigenous people of North America for whooping cough, gout and cancer (Leung & Foster, 1996). Other traditional societies have used this plant in the treatment of asthma, bronchitis coughs, athlets's foot (Foster & Duke, 1990), also for eczema and psoriasis (Rijke et al., 2001). Extracts of *T. pratense* are commercially available as isoflavone enriched dietary supplements on the US and European markets for women suffering from menopausal complaints (Oleszek & Stochmal, 2002).

1.8 Problem Statement

The valuable characteristics of *T. pratense* (productivity, forage quality and bioactive compounds) are induced by its morphology and eco-physiology, which are correlated with the genetics of persistence, and with the environmental conditions.

It is axiomatic that to be persistent, a crop must be well adapted to its environment. *T. pratense* persistence is the result of an interaction between its adaptation and its stress load (Taylor & Quesenberry, 1996). Stress load may be defined as any factor, whether physiogenic or pathogenic, that affects the growth and development of the species, and consequently the maintenance of a closed canopy. Environmental stresses such as interspecific or/and intraspecific competition, winter hazards, management practices, and pathogens, are seen as reducing the vigor of the forage legume plants. Weakened plants are then susceptible to damage from other pathogens which increases root rot phenomenon that leads to shortened persistence through senescence occurrence and finally, to lose the forage stand.

Persistence, then, is determined by the species eco-physiological characteristics, the perennial ecotypes traits, the environmental influence and the grassland applied technologies. The most negative factors that diminish the clover persistence includes: improper harvesting performed before pre-bloom stage, late autumn harvesting, low temperatures and spring drought, and root rot phenomenon. It has been suggested that root rots in forage legumes may be an unavoidable disease (Rufelt, 1982).

In most places of the world the utilization of *T. pratense* by farmers is confined by the absence of persistence identified with high mortality of plants, determining a productive life of two to three years (Ortega et al., 2003). This likewise restrains the genetic progress of breeding projects because of the trouble in keeping up selected genotypes under field

or greenhouse conditions and also, due to the allogamous way of the species that makes it difficult to keep up elite clones by seed (Taylor & Quesenberry, 1996).

Considering these limitations, protocols for *in vitro* tissue culture of *T. pratense* have been developed to propagate, eliminate viruses, and maintain *T. pratense* clones. *In vitro* culture of plant offers the opportunity to select and regenerate plants with desirable characteristics. The technique has also been effectively utilized to induce tolerance which includes the use of some selective agents that permit the preferential survival and growth of desired phenotypes.

The application of *in vitro* selection techniques has facilitated the generation of disease resistant plants in other pathosystems (Pontaroli & Camadro, 2005).

1.9 Objectives of the Study

This study consists of two major parts. In the first part, micropropagation of *Trifolium pratense* using different plant hormones in tissue culture system was performed in order to mass propagate of this very important forage crop. In the second part of the study a comparison was made on some of the biological activities, cellular behavior and physiological activity of *in vivo* and *in vitro* grown *T. pratense* to determine whether there are any significant differences between plants grown *in vivo* and *in vitro* system.

The results are valuable for applications of *T. pratense* as a forage crop in the livestock industry and also for using this important traditional medicinal plant in pharmaceutical industries.

The aims of this study were:

- To establish an efficient *in vitro* micropropagation and callus formation system for *T. pratense*.

- To compare cellular behavior in root meristem cells of *in vivo* and *in vitro* grown *T. pratense.*
- To investigate and compare the antioxidant, antimicrobial and antitumor activities of *in vivo* and *in vitro* grown *T. pratense* and also the effect of extraction solvent on these biological activities.
- To compare the photosynthetic characteristics of in vivo and in vitro grown T. pretense and also to investigate the correlation between photosynthesis and total phenolic and flavonoid content of this plant.
- To investigate the effect of salt stress on seed germination of *T. pratense* using *in vitro* culture.

CHAPTER 2: LITERATURE REVIEW

2.1 Tissue Culture as a Tool in Plant Biotechnology

Often, the problem with commercially production of unique or uncommon plants is in availability or insufficient of plant materials required for traditional vegetative propagation and also to obtain sufficient quantities of uniform compounds. It is, however, possible to use a biotechnological approach in order to improve horticultural crop production (Chebet et al., 2003). Biotechnology can also be defined as the branch of molecular biology that examines the use of microorganisms to carry out specific industrial processes. Currently, biotechnology is recognized for its importance with respect to plant production, protection and improvement. One of the main biotechnological applications in plant biotechnology is tissue culture. Micropropagation by means of tissue culturing is commonly referred to as cell, tissue or organ culture *in vitro* (in glass) (Rout et al., 2006). Plant tissue culture is a form of biotechnology that is a powerful tool for plant breeders (Stewart, 2007). This technology utilizes plant cells, tissues or organs in a culture medium that contains plant growth regulators to produce plants in a controlled aseptic environment. *In vitro* culture is one of the main implements of plant biotechnology responsible for exploiting the totipotency character of plant cells (Rout et al., 2006). Additionally, *in vitro* propagation can be utilized to rapidly multiply cultivars with desirable traits and create healthy, disease-free plants without seasonal constraints (Pati et al., 2006). It is important to develop a micropropagation method or protocol in the case of plant production via micropropagation (Razdan et al., 2008). The development of an *in vitro* protocol is advantageous not only for maintainable utilization of a species, but also for germplasm conservation and genetic improvement (Chen et al., 2006). It is possible to store germplasm collections in cold storage *in vitro*, which keeps plants available for future study or distribution (Kovalchuk, 2009).

Tissue culture protocol development is challenging due to interaction of biotic and abiotic factors. Example of biotic factors warranting critical consideration includes the variability that exists in culture response with regards to the source of plant tissue or explant material (Skof et al., 2007). A second concern is fungal, bacterial and viral contamination that can occur *in vitro*, either from the explant materials or environmentally. Sources of microbial contamination in plant tissue culture are extremely difficult to determine (Kyte & Kleyn, 2001). Failure to control contamination will ultimately result in severe losses of the regenerated or *in vitro* grown plant cultures (Anjali et al., 2007).

Tissue culture involves the use of small pieces of plant tissues (explants) which are cultured in a nutrient medium under sterile condition. Using the appropriate growing conditions for each explant type, plant can be induced to rapidly produce new shoots, and, with the addition of suitable plant growth regulators (PGRs) new roots. The most important and complex biotic factor affecting tissue culture of plant is biochemical activity, specifically plant responses that result from the interaction of plant growth regulators. Plant responses elicited from phytohormones are dependent on type, mode of application, concentration, tissue sensitivity and species genotype (Goncalves et al., 2008). Research has shown that every species explicitly requires a particular concentration and combination of phytohormones in order to achieve optimal development (Ramirez-Malagon, 2008).

2.1.1 Benefits of Tissue Culture

In vitro culture of cells and tissues has a great value in providing powerful ways to study developmental processes in plants. Merits of tissue culture include: precise control over growth conditions, uniformity, increased levels of control and the ability to create recombinant proteins using acceptable manufacturing practices (Hellwig et al., 2004).

The production of plant recombinant proteins, also known as molecular farming, was first reported in 1989 by Hiatt et al. Recombinant proteins are encoded by recombinant Deoxyribonucleic acid (DNA) or generated from a recombinant gene. Products targeted for bioengineered plants include those for food, feed additives, human and animal health, industrial enzymes and technical reagents (Abranches et al., 2005). Furthermore, tissue culture techniques can be used to commercially produce and can save rare plants of medicinal value. Such propagation techniques could be necessary as a result of species physiological factors such as low productivity due to low seed germination rates (Chen et al., 2006). Similarly, seedless plants and varieties where seedlessness is commercially important can benefit from tissue culture techniques (Zhang et al., 2007).

Seed culture *in vitro* is a viable method for improving germination rates and assisting in breaking seed dormancy (Chen et al., 2006; Nikolic, 2006). Further, micropropagation methods can prove beneficial in rescuing species at risk of becoming extinct (Stephenson & Fahey, 2004). *In vitro* propagation is also beneficial for plants with seeds that are slow rooting or those with reduced multiplication rates (Chen et al., 2006).

Tissue culture systems offer better environmental containment than whole plants grown in the field so that regulatory requirements can be more easily met; production times are also shorter and, for proteins that are secreted from the cells, downstream processing and product purification are simpler and cheaper (Doran, 2006).

In the recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions. *In vitro* propagation also called micropropagation is infact the miniature version of conventional propagation, which is carried out under aseptic conditions. The advent of *in vitro* tissue culture technique has offered a new approach to the morphogenetic investigations. It allows a living system to be studied under controlled environmental conditions. This enables a

study of the complex biological phenomenon in parts. Moreover these partial processes are amenable to controlled investigations.

Plants raised through micropropagation are supposed to have the following characteristics:

i. Uniform quality
ii. Pathogen free
iii. Can be produced much more rapidly as new cultivars could become commercially available within 2 to 3 years from development rather than 5 to 10 years needed using conventional propagation.
iv. Produce uniformly superior seeds
v. Show improved vigor and quality
vi. year round production
vii. Clean of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture

2.1.2 Explant Materials

Selection of explant materials is the first and crucial aspect of micropropagation that requires three important considerations: 1) genetic and epigenetic characteristics of the source plant 2) pathogen control 3) physiological conditions of the plant prior to explant excision in order to optimize its ability to establish in a culture (Hartmann et al., 1990). Studies have revealed that somatic embryogenesis (formation of embryos from somatic cells without fusion of male and female genus) could be improved by choosing the most favorable female or male parent in specific cases (Park et al., 2006). The source of explant materials can be from seed, leaf, stem, flower or root tissue.

Generally, adventitious shoot meristems are genotypically independent and are preferred for *in vitro* examination due to their ease of handling compared to other sources of explants. Axillary shoot tip meristems are easier to grow than true apical meristems in culture due to the existing meristems (Sai et al., 2006). Large explants can increase chances of contamination, while small explants like meristems can sometimes show less growth (Staba & Seabrook, 1980). Explants are cleaned by distilled water and sterilized using mercuric chloride, ethyl alcohol and liquid bleach (Rout et al., 2000; Prakash & Van, 2007).

Regardless of whether the application of tissue culture is for research or market, it is important that the cultures be recognized *in vitro* free of biological contamination and be maintained as aseptic cultures during manipulation, growth, and storage. The risks from microbial contamination are spurious experimental results due to the effects of latent contaminants or losses of valuable experimental or commercial cultures. Microbial contamination of plant tissue cultures is due to the great nutrient availability in the Murashige and Skoog basal medium or variants of it. In recent years, it has been revealed that numerous plants, particularly perennials, are at least locally endophytically colonized intercellularly by bacteria. The latter, an intracellular pathogenic bacteria and viruses, may permit latently into culture and be spread horizontally and vertically in cultures. Growth of some potentially cultivable endophytes may be suppressed by the high salt and sugar content of the Murashige and Skoog basal medium and suboptimal temperatures for their growth in plant tissue growth rooms. The management of contamination in tissue culture involves three stages: disease screening of the stock plants with disease and endophyte elimination where detected; establishment and pathogen and contaminant screening of established initial cultures; observation, random sampling, and culture screening for micro-organism in multiplication and stored cultures. The increasing accessibility of both broad-spectrum and specific molecular diagnostics has resulted in

advances in multiple pathogen and latent contaminant detection. The hazard analysis critical control point management strategy for tissue culture laboratories is underpinned by staff training in aseptic technique and good laboratory practice.

2.1.3 Culture Media

Plant tissues and organs for *in vitro* micropropagation grow on artificial media which is supplied with nutrients necessary for cell growth. The success level of a plant tissue culture work is strongly influenced by the environment and formation of the culture medium. For healthy and vigorous growth, intact plants need large amounts of some inorganic elements (the so-called major plant nutrients and macronutrients) salts of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and small quantities of other elements (minor plant nutrients or trace elements) like iron (Fe), Sodium (Na). Chlorine (Cl), Manganese (Mn), Zinc (Zn), boron (B), Copper (Cu), molybdenum (Mo) and (possibly) nickel (Ni) (George, 1993).

Plant tissue culture media is therefore made up from some or all of the following components:

- Macronutrients (always used)
- Micronutrients (most of time is used; sometimes just iron, has been used)
- Vitamins (can be included/not included when purchase a ready media from suppliers)
- Amino acids and other nitrogen supplements
- Sugars
- A solidifying agent (Agar is the most common agent)

A wide range of media are available for plant tissue culture, but MS medium (Murashige & Skoog, 1962) is commonly used. Other media used are Linsmaier-Skoog

(LS) (Linsmaier & Skoog, 1965), Schenk and Hilderbrandt (SH) (Schenk & Hilderbrandt, 1972), woody plant medium (WPM) (Lioyd & McCown, 1980) and the Nitsch and Nitsch (NN) (Nitsch & Nitsch, 1969).

Prepared commercial MS media (Murashige and Skoog, 1962) with vitamins was used in this study due to the lack of some ingredients for making a manual media and also the cost of making the media in the lab. In addition, using already prepared MS media is almost simple and can save a lot of time and effort.

2.1.4 Plant Growth Regulators (PGRs)

Hormones also known as "plant growth regulators" are chemicals used in a small quantity to alter the growth of a plant or plant parts. They are natural chemicals produced within plant tissues, which control normal plant functions such as root growth, fruit set and drop, growth and other development processes. Good development of tissues and organs are influenced by the present of hormones in medium though a few monocotyledonous species find it optional (Mukhambetzhanov, 1997). In addition, if the explants already have existing endogenous hormones, therefore there is no need to supplement the medium with exogenous hormones (Hoe, 1992).

The use of plant growth regulators in agricultural production expand exponentially since they were first discovered and has been a major component of some agricultural commodity production (Frederick, 2006). It is acknowledged that without these regulators, *in vitro* culture is often impossible.

Some of the natural growth substances are prepared synthetically or through the fermentation processes and can be purchased from chemical suppliers. There are several recognized classes of plant growth substances:

- Auxins
- Cytokinins
- Gibberellins
- Ethylene
- Abscisic acid

Auxins and cytokinins are the most important for regulating growth and morphogenesis in plant tissue and organ cultures; in these classes, synthetic regulators have been discovered with a biological activity which equals or exceeds that of the equivalent growth substances (George, 1993).

IBA with the molecular formula $C_{12}H_{13}NO_2$ and NAA with the molecular formula $C_{12}H_{10}O_2$ are among the auxins and BAP with the formula $C_{12}H_{11}N_5$ and Kinetin with the formula $C_{10}H_9N_5O$ from Cytokinins are the most favored and commonly used hormones employed in various experiments for tissue culture and micropropagation (Keng & Stanley, 2007; Loc et al., 2005; Miachir et al., 2004; Georg, 1993).

Indole-3-butyric acid (IBA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) from Auxins and 6-Benzylaminopurine (BAP) from cytokinins were used for this study.

Auxins can promote the growth of plant tissues. Binding of auxin leads to lipid breakdown and acidification of the wall, increasing its extensibility and increasing the water potential of the cell; so water enters and cell expands. Auxins also affects the mRNA transcription and they cause the cell to produce biologically active cell oligosaccharides. Auxins are able to remove genetically the original physiology of whole plant tissues which had previously determined their differentiated state; therefore auxin causes the cell to divide (George, 1993). Auxins are generally used to stimulate callus production and cell growth in a culture medium or to initiate shoots, particularly roots,

and to induce somatic embryogenesis and stimulate growth from shoot apices and shoot tip cultures (George, 1993).

The second group, cytokinins, can stimulate protein synthesis. The cytokinins are used to stimulate cell division in the culture medium and induce shoot formation or auxiliary shoot proliferation (George, 1993).

2.2 *In Vitro* Propagation of Medicinal Plants

The use of medicinal plants in traditional health care system back to the prehistoric times and they are still the most important source of health care system for the majority of people around the world (Iwu, 1993; Leaman, 2006; Carvalho, 2004; Teklehaymanot & Giday, 2007; Heinrich, 2000; Uprety et al., 2010).

Recently, there has been restored enthusiasm in natural medicines that are obtained from plant parts or plant extracts. About 40% of the medicines currently used compounds in Western countries are now derived or at least partially derived from natural sources. The woodland harbor countless species, yet deforestation has been in charge of the quick loss of therapeutic plant wealth, such that numerous profitable medicinal plants are under the risk of ending. Pharmaceutical corporations depend largely upon materials obtained from naturally occurring stands that are being rapidly depleted. Plant tissue culture is an alternative method for commercial propagation (George & Sherrington, 1984) and is being used widely for the commercial propagation of a substantial number of plant species, including numerous medicinal plants.

Exploratory methodologies utilized for proliferation of therapeutic plants through tissue culture can be partitioned into three general classifications. The most widely recognized methodology is to disengage sorted out meristems like shoot tips or axillary buds and affect them to develop into complete plants. This arrangement of propagation is

regularly referred to as micropropagation. In the second method, adventitious shoots are started on leaf, root and stem segments or on callus derived from those organs. The third system of propagation includes induction of somatic embryogenesis in cell and callus cultures. This system is hypothetically most proficient as substantial quantities of somatic embryos can be gotten when the entire procedure is institutionalized. Biotechnology involving modern tissue culture, cell biology and molecular biology brings about the chance to grow new germplasms that are well adapted to evolving requests.

Micropropagation of several plant species, including many medicinal plants, has been described by many researchers during the last three decades (Murashige, 1977; Saurabh et al., 2005; Nishritha et al., 2008; Chitra et al., 2009; Mathew et al., 2014; Dakah et al., 2014).

In case of a practical and pharmaceutical perspective, propagation from existing meristems is not innovatively troublesome, and it yields plants that are genetically identical with donor plants. Micropropagation of medicinal plants has been achieved through rapid proliferation of shoot-tips and axillary buds in culture. Several issues are described to influence the success of *in vitro* propagation of different medicinal plants and, therefore, it is indiscreet to characterize any particular reason for the general micropropagation of medicinal plants (Rout et al., 2000).

The factors that affect micropropagation of medicinal and aromatic plants have been studied by Murashige (1977), Hu and Wang (1983), Bhagyalakshmi and Singh (1988), Rout (2000), Verma and Bansal (2013). The influence of plant regulators and their interactions on micropropagation of different plant species have been discussed in detail by Skirvin et al. (1990), Rout et al. (1989) and Su and Zhang (2014).

Shoot multiplication is viewed as the most significant stage of plant micropropagation. Shoot multiplication is achieved by exploiting the use of PGRs including auxins and cytokinins (Rout et al., 2006). PGRs alter various physiological processes that affect morphogenic responses. The concentration of PGRs required for a given response is cultivar and genotypically specific and depends on the unique cytokinin or auxin used (Bhatia et al., 2004). The effect of auxins and cytokinins on shoot multiplication of various medicinal plants were reported (Murashige & Skoog, 1962; Sharma and Singh, 1997; Handique & Bora, 1999; Sagare, 2000; Singh & Tiwari, 2010; Ramadevi et al., 2012; Ugraiah et al., 2013). Cytokinins levels were shown to be the most critical for multiplication of medicinal plants. Benjamin et al. (1987) reported that BAP at high concentration (1-5 ppm) stimulated the development of the axillary meristems and shoot tips of *Atropa belladonna*. Lal et al. (1988) reported a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0-5.0 mg/l. Singh and Tiwari (2010) indicated that the production of multiple shoots was higher in *Clitoria ternatea* L. on a medium having 1.5 mg/l BAP. Multiple shoot formation were reported from shoot tips (1-2cm) of field grown plants of *Paederia foetida* and *Centella asiatica* on MS medium supplemented with BAP (1.0 mg/l) within 7 days of culture (Singh et al., 1999). Addition of BAP (0.3 mg/l) and kinetin (0.2 mg/l) has been found to give a good response of shoot proliferation in *Withania somnifera* with a regeneration of 85 % (Kulkarni et al., 2007). In *Crataeva magna*, rapid multiplication was achieved on MS added with BAP (8.8 µm) (Beniamin *et al*., 2004). Namh et al., (2010) reported that the highest number of shoot formation from tissue culture of *Hypericum retusum* was obtained on MS medium supplemented with 0.5 mg/l BAP. Similarly, it was observed that cytokinin was required in optimal quantity for shoot proliferation in many genotypes but including of low concentration of auxins along with cytokinin triggered the rate of shoot proliferation. Faria and Illg (1995) reported that the addition of 10 µM BAP together with 5 µM IAA or 5 µM NAA induced a high rate

of shoot proliferation in *Zingiber spectabile*. MS medium with growth regulators such as BAP (0.5 mg/l) in conjunction with NAA (0.01 mg/l) has been reported to give optimum results in *Utleria salcifolia* (Gangaprasad et al., 2003). In *Peltophorum pterocarpum*, highest number of multiple shoots was observed on MS with kinetin (2.0 mg/l) + NAA (0.5 mg/l) (Uddin et al., 2005).

A fast rate of propagation relies on upon the subculturing of proliferating shoot cultures. In the case of prolonged cultures, the nutrients in the medium are gradually exhausted, and at the same time, the relative humidity in the vessels decreases leading to the drying of the culture medium. Subculturing also decreases the effects of competition of the developing shoots for nutrients. Upadhyay et al. (1989) reported a propagation profile for *Picrorhiza kurroa* and detected that the shoot multiplication rate regularly improved as the quantity of subcultures increased. Biswas et al. (2007) confirmed that the quantity of shoots per culture was increased with the quantity of subculturing in *Aristolochia tagala* Champ. Rout et al. (2000) reported that, a fast rate of propagation relies on upon the sub-culturing of proliferating shoots.

The induction of callus development and subsequent differentiation and organogenesis in medicinal plants is accomplished by the differential application of hormons and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. Several reports are available on the regeneration of various medicinal plants via callus culture. For instance callus cultures initiated from shoot base explants of *Curcuma aromatica Salisb* were maintained on Murashige and Skoog (MS) media supplemented with 2 mg dm^{-3} 2,4-D alone or with 0.5 mg dm^{-3} BAP and 0.5 mg dm^{-3} NAA. Approximately 8-10 plantlets were produced after 30-40 days of culture per 50 mg of callus inoculated

(Mohanty et al., 2008). Karami et al. (2009) reported the regeneration of shoots from callus of *Elaeagnus angustifolia* L. using appropriate concentration of BAP alone or BAP combined with NAA or IAA. Thomas and Maseena (2006) described *in vitro* regeneration of *Cardiospermum halicacabum* L. via callus culture. They reported that shoot regeneration form callus tissue was maximum on media supplemented with 8 µM kinetin and 0.5 µM IAA.

Saxena et al. (1997) reported plant regeneration via organogenesis from callus cultures derived from mature leaves, stems, petioles and roots of young seedlings of *Psoralea corylifolia*. In their study the calli differentiated into green nodular structures which developed into dark green shoot buds in the media supplemented with 2.5 mg/L BA and 1.0 mg/L NAA. Successful plant regeneration was reported from stem and leaf-derived callus of *Centella asiatica* on MS medium supplemented with 4.0 mg/L BA, 2.0 mg/L kinetin, 0.25 mg/L NAA, and 20 mg/L adenine sulfate. The regeneration frequency varied from 62.8% to 73.4% (Patra et al., 1998).

In vitro induction of roots from growing shoots is dependent on the interactions of internal and external factors. Factors include; species/cultivar, age and size of microshoots, media, inorganic salts, carbohydrates, activated charcoal, PGRs, culture vessel, light and temperature (Pati et al., 2006). Commercially, indole-3-acetic-acid (IAA) and Indole-3- butyric acid (IBA) are natural and synthetic auxins respectively that are used extensively to improve adventitious root development. However, IBA is the auxin of choice in micropropagation because IAA tends to break down during the autoclaving process. IBA is used extensively for rooting commercially and in agriculture due to its availability and reasonable cost. Anis et al. (2003) obtained 80% rooting from shoots cultured of mulberry (*Morus alba* L.) on the MS supplemented with IAA (1.0 mg/l). Rout et al. (1999) reported induction of rooting in microshoots of *Plumbago*

zeylanica on half-strength MS medium supplemented with 0.25 mg/L IBA with 2% sucrose. Prakash and Staden (2007), reported that *in vitro* regenerated shoots of *Hoslundia opposita* developed roots directly from the basal cut ends of shoots, on MS medium supplemented with 3.6 μM of IAA, after 30 days of incubation. Matu et al. (2006) reported that in micropropagation of *Maytenus senegalensis* the number of roots produced per shoot were significantly higher in the treatments containing IBA compared to IAA.

2.3 *In Vitro* Propagation of *Trifolium* Species

To increase the forage availability to the growing population of animals and decreasing the land resources research is required. Forage crop improvement requires a long-term and multidisciplinary approach, viz. plant breeding, genetics, agronomy, pathology, physiology, biotechnology and animal nutrition etc. Tissue and cell culture techniques are known as potentially valuable tools for the programs of crop improvement. The improvement of *Trifolium* species as important forage legumes in most part of the world has been largely confined to conventional breeding approaches. Using conventional methods have impeded improvement because of the some problems like: short life, associated with disease and barriers to interspecific gene transfer. So development of *in vitro* techniques could facilitate *Trifolium* breeding. The first establishment of *in vitro* culture of legumes back to the 1950's by Nickell (1955). Report on organogenesis in white clover (*Trifolium repens* L.) further indicates potential for the use of *in vitro* techniques in breeding research on *Trifolium* species (Pelletier & Pelletier, 1971). Beginning in the late 1970's different studies demonstrated whole plant regeneration from tissue cultures of various *Trifolium* species, however, most of these systems were of low regeneration frequency and primarily from un-adapted plant introduction material or wild *Trifolium* species (Phillips & Collins, 1979; Collins et al., 1982).

Cheyne and Dale (1980) tested different media either Blaydes (Blaydes, 1966) or Gamborg B5 basal media (Gamborg et al., 1976) with different hormones (IAA and BAP) for micropropagation of *Trifolium repens* and *Trifolium pratense*. Dale and Cheyne (1993) also used different media for micropropagation of red clover to eliminate clover diseases of seven cultivars and obtained good results using the B5 medium with identical PGR levels. On the other hand, Campbell and Tomes (1984) also achieved good results with the B5 medium and using the BAP (2.0 g/l) as a growth regulator.

Carrillo et al. (2004) investigated the effect of explant source, culture media and plant growth regulators on tissue culture of red clover. Their finding showed that stem meristems were presented better development and lower level of contamination compared with the crown meristems. They also found that the L2 medium supplemented with BAP shows better results compared with the B5 and MS media.

Uranbey et al. (2005) had developed an efficient micropropagation system for Persian clover (*Trifolium resupinatum* L.). They cultured different explants (node, hypocotyl and cotyledonary node) on MS medium supplemented with combination of BAP and IBA or BAP, Kn and IBA. In their finding direct multiple shoots developed within 6 weeks in all explants in most of the media tested. The best shoot multiplication capacity was obtained from cotyledonary node explants cultured on MS medium containing 7.1 µM BAP and 1 µM IBA. They also reported that high rooting of elongated shoots was achieved in half MS medium containing 8 µM IBA.

Nippon and Higuchi (1990), reported successful regeneration for *Trifolium amabile, T. heldreichianum, T. montanum, T. apertum, T. caucasicum, T. cherleri* and *T. alpestre* on Gamborg medium supplemented with different concentrations of 2,4-D, IAA and Kn.

Ding et al. (2003) have developed an efficient procedure for plant regeneration from cotyledonary explants for some of the *Trifolium* species including *T. repens, T. pratense, Tsubterraneum, T. michelianum* and *T. isthmocarpum*. They reported that plant regeneration frequencies, expressed as percentage of explants producing shoots, ranged from 65–97% for all tested cultivars of *Trifolium* species.

2.4 Cytological studies of Plants

Cytological characterization is crucial in the context of modern and traditional breeding. It is well known that different ploidy levels within a species give rise to different phenotypes and polyploidy often being associated with enhanced environmental success and in the case of crops agronomic superiority (Hegarty & Hiscock, 2008). Cytological analysis is usually performed to assess the mitotic process in experimental varieties and hybrids. Hydridization of the close genetic species produces regular chromosome hybrids while offspring of those more distantly related species have mitotic irregularities (Marfil et al., 2006). Despite existence of some few cytological studies of *Trifolium pratense*, little work rarely focused on detailed karyological criteria.

According to Karp (1997), basic cytological technique is very important and useful for the determination of accurate chromosome number and structure and should be in routine use for regenerated plants in tissue culture.

Meristematic cells of roots have long been employed as the standard tissue or cells for the cytological cell studies (Gimenez-Martin et al., 1966). The use of root in these studies are comparatively uncomplicated than using other meristematic tissues such as shoot tips, because they can be handled and produced easily, as root can be grown from seeds germinated in only water.

Cytological investigation of *Trifolium pratense* began in the 1970s with the main aim of developing interspecific hybrids and polyploids (Taylor, 2008). The karyotype of red clover was initially determined based on metaphase chromosome, and it was determined that the largest chromosome featured a large satellite (Taylor & Chen, 1988).

Cytogenetically, most species of *Trifolium*, an important temperate forage legume, have a diploid chromosome number of 14. Polyploidy is uncommon in *Trifolium*. Only 16% of the 248 species of *Trifolium* distributed throughout the world are polyploid. However, 70% of the known polyploids occur in the subgenus *Amoria*, which is considered to be one of the most primitive and unspecialized subgenera.

Trifolium pratense has an allogamous diploid genome (2n = 2x = 14, 1C = 440 Mb) (Sato et al., 2005), with a DNA content of 0.97 pg/2C,7 which is slightly larger than that of rice (Arumuganathan & Earle, 1991). Because it has a strong self-incompatible fertilization system, red clover has a highly polymorphic genome that has hampered genetic and genomic analysis.

2.5 Antioxidants

2.5.1 Importance of Antioxidant

Oxygen, the essential element for life (except for anaerobes), generates various types of reactive oxygen species (ROS) which can damage living tissues. The damage caused by the interaction of ROS with biocompounds increases the risk of many diseases. ROS can be classified as radicals (such as superoxide, hydroxyl, peroxyl and alkoxyl) and non-radicals (such as ozone, peroxynitrite, lipid peroxide and hydrogen peroxide) and can easily be formed in normal and pathological cell metabolisms (Trachootham et al., 2009). The imbalance between pro-oxidants and antioxidants lead to condition called oxidative stress. Based on high reactivity of free radicals, they can easily damage proteins, lipids and DNA molecules under oxidative stress conditions and are harmful to the intra/extra-

cellular systems of organisms. It has the potential to cause numerous kinds of diseases such as cancer, atherosclerosis, vasospasms, stroke, trauma, hyperoxia, Alzheimer, Parkinson and cardiovascular diseases (Halliwell & Gutteridge, 1985; Ames et al., 1993; Frei, 2012; Giasson et al., 2002).

Although the human natural antioxidant defense system is responsible for protection against the destructive action of ROS by production of natural antioxidants, any interruption in their function caused by environmental stresses or a disease can potentially increase the risk of oxidative damage in cells. Furthermore, an increase in the concentration of the oxidizing species can cause oxidative stress in the body and natural antioxidants may not adequately neutralize free radicals. Hence, to reduce the harmful effects of oxidative stress, consuming a diet high in antioxidants is recommended (Packer et al., 1999; Jeep et al., 2008).

There are several strong antioxidants which can be divided into lipid-soluble antioxidants, like vitamin E and butylated hydroxytoluene, and water-soluble antioxidants, like vitamin C and *gluthathione* (Donoghue & Donoghue, 1997; McLean et al., 2005).The above mentioned classification of antioxidants may not be a superior categorization as some antioxidants such as phenolics have various phytochemicals of which some are water-soluble and some are lipid-soluble compounds (Steven et al., 1996; Kong et al., 2008). Dividing the antioxidants into enzymatic and non-enzymatic groups is another way of classification of antioxidant compounds. Enzymatic antioxidants including superoxide dismutase, catalase, and glutathione peroxidase are produced endogenously in humans while the non-enzymatic antioxidants like tocopherols, carotenoids, ascorbic acid, flavonoids and tannins are mostly obtained from other sources such as plants (Naskar et al., 2010).

In addition to the above mentioned influence of oxidative reactions on human health, lipid and protein oxidations in foods during food processing and storage are also some of the main reasons for deterioration of flavor, aroma, colour and nutritive value (Stadtman & Levine, 2003; Baron et al., 2007). Usage of antioxidant compounds in foods as preservatives to avoid oxidative reactions has been practiced for many years (Artés et al., 2006).

Natural antioxidants have a diversity of biochemical activities, some of which include the inhibition of reactive oxygen species (ROS) generation, direct or indirect scavenging of free radicals and alteration of intracellular redox potential (Finkel & Holbrook, 2000). Many antioxidant substances have anticancer or anticarcinogenic properties (Dai & Mumper, 2010).

By controlling free radicals, antioxidants can make the difference between life and death, as well as influence how fast and how well we age. The more we understand about antioxidants and how they work, the more we will understand and appreciate the profound role they play in keeping us healthy and happy. Their role in the human body is nothing less than miraculous.

There is overwhelming scientific evidence demonstrating that those of us who eat a diet rich in antioxidants and take antioxidant supplements will live longer and healthier lives. Therefor in recent years, the use of natural antioxidants found in plants has attracted interest due to their presumed nutritional and therapeutic value. Antioxidant properties are an important mechanism of beneficial activity of plant-derived compounds and extracts.

2.5.2 Methods of antioxidant activity evaluation

There are various model systems to determine the antioxidant potential of different samples. However, due to the complex composition of antioxidant compounds, application of different methods of evaluation of the antioxidant capacity which are based on different features of the antioxidant activities is recommended (Wichtl, 2001). These assays can be classified into two group namely *in vivo* and *in vitro* models. While rats and mice are the usual test animals for *in vivo* evaluation of antioxidant activity techniques, *in vitro* models for evaluation of antioxidant activity of samples include several types of assays such as DPPH radical scavenging, super oxide anion radical scavenging, hydrogen peroxide scavenging, nitric oxide radical inhibition, reducing power, superoxide dismutase (SOD), oxygen radical absorbance, 2,2-azinobis(3-ethyl benzothiazoline-6-sulfonicacid) diamonium salt (ABTS), N, N-dimethyl-p-phenylene diamine dihydrochloride (DMPD), and chelating power. These are mostly spectrophotometeric assays. The parameters usually evaluated by *in vivo* models include protein oxidation, lipid peroxidation, reduced gluthinone content, superoxide dismutase, and catalse activities (Joharapurkar et al., 2003).

The antioxidant compounds can be recycled in the cell or are irreversibly damaged, but their oxidation products are less harmful or can be further converted to harmless substances (Halliwell & Gutteridge, 2007). At the cellular and organism level the antioxidant protection is provided by numerous enzymes and endogenous small molecular weight antioxidants such as ascorbic acid, uric acid glutathione, tocopherols and several others. Many compounds contain antioxidant activity in addition to their specialized physiological function, and their importance as antioxidants *in vivo* is sometimes ambiguous (Azzi et al., 2004).

Application of strong antioxidants as a positive control in all types of antioxidant activity assays is necessary. Ascorbic acid (vitamin C) is a strong antioxidant as it donates two electrons from the C-2 and C-3 double bond carbon atoms and is commonly used as a positive control in assays.

2.5.3 Antioxidant activity of plants

A variety of free radical scavenging antioxidants exist within the human body which many of them are derived from dietary sources like fruits, vegetables and teas. In recent decades, interest in chemopreventive plant natural products has grown rapidly. The etiology of several degenerative and aging-related diseases has been attributed to oxidative stress, and numerous studies have been undertaken to search for the most effective antioxidants (Aruoma, 2003; Soobrattee *et al*., 2005; Alvarado *et al*., 2006; Pérez-Jiménez *et al.*, 2008; Blasa *et al*., 2010; Wootton-Beard & Ryan, 2011). Vegetables and herbal infusions have been recognized as important sources of antioxidants (Alarcón *et al*., 2008, Almajano *et al.*, 2008 and Chan *et al*., 2010).

The adverse effects of oxidative stress on human health have become a serious issue. The World Health Organization (WHO) has estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components (Winston, 1999).

Some researchers suggest that two-thirds of the world's plant species have medicinal values; in particular, many medicinal plants have great antioxidant potential. Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases (Duduku *et al.*, 2010).

Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxylanisole (BHA) are currently used as food additives, and many plant species have similar antioxidant potentials as these synthetics. These species include *Diospyros abyssinica* (Maiga *et al*., 2006), *Pistacia lentiscus* (Abdelwahed *et al*., 2007), *Geranium sanguineum* L. (Sokmen *et al*., 2005), *Sargentodoxa cuneata* Rehd (Li *et al*., 2008), *Polyalthia cerasoides* (Roxb) (Ravikumar *et al*., 2008), *Crataeva nurvala* Buch-Ham. (Krishnanand, 2004), *Acacia auriculiformis* A. Cunn (Singh *et al*., 2007), *Teucrium polium* L. (Sharififar *et al*., 2009), *Dracocephalum moldavica* L. (Dastmalchi *et al*., 2007), *Urtica dioica* L. (Gülçin *et al*., 2004), *Ficus microcarpa* L. (Ao *et al*., 2008), *Bidens pilosa* (Deba *et al*., 2008). *Radiata Leea indica* (Boe *et al*., 2006), *Uncaria tomentosa* (Pilarski *et al*., 2006), *Salvia officinalis* L. (Bozin *et al*., 2007), *Momordica Charantia* L. (Horax *et al*., 2005), *Rheum ribes* L. (Öztürk *et al*., 2007), and *Pelargonium endlicherianum* (Tepe *et al*., 2006). The literature reveals that these natural antioxidants represent a potentially side effect-free alternative to synthetic antioxidants in the food processing industry and for use in preventive medicine (Chung, 1997).

2.6 Phenolic Compounds

At least one aromatic ring (C6) which bears one or more hydroxyl groups can indicate the phenolic compounds. Phenolics can be called as one of the major groups of plant products within of alkaloids and terpenoids (Ndakidemi & Dakora, 2003). Phenolics are usually classified into various groups based on their structures. In one of the most complete division it is classified into different groups of phytochemicals namely simple phenolics, phenolic acids and aldehydes, acetophenones and phenylacetic acids, cinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, xanthones, stilbenes, benzoquinones, anthraquinones, naphthaquinones, betacyanins, lignans, lignin, tannis, and phlobaphenes (Vermerris & Nichelson, 2007). Phenolics can also be divided into three groups based on the phenolic phytochemical structures, namely phenolic acids (such

as caffeic acid), flavonoids (such as anthocyanins) and non-flavonoid polyphenols (such as Ellagic acid) to make the above mentioned classification simple and easy to use (Basha et al., 2004).

Phenolics are of widely varied range of compounds with low molecular weight found in all higher plants (Makoi & Ndakidemi, 2007). They have been mostly classified as secondary metabolites as they are not directly involved in primary metabolism but involved in different plant processes such as biotic and abiotic stresses (Taylor, 1996; Michalak, 2006), fruit growth and maturation (Buta & Spaulding, 1997; Ding et al., 2001).

2.7 Antimicrobial

2.7.1 Importance of antimicrobial

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance (Okeke et al., 2005).

Antibiotic resistance is the major reason for the need to develop new antibiotics. Humans, animals, plants and even insects are all at risk to bacterial infections. The number of diseases caused by bacteria to humans, animal and plants is high and transmission is variable trough air, food, water, soil and contact. These pathogenic bacteria are from different genera, species and strains and therefore have different antibiotic resistances.

Natural antibacterial compounds can be produced by any bio-organism which carry antibacterial resistance gene/s. Discovering antibacterial compounds produced by animals (Conlon & Sonnevend, 2010), insects (Otvos, 2000), plants (Castro & Fontes,

2005), fungi (Qi et al., 2009) and bacteria (Eleftherianos, 2009) show that screening the antibacterial activity of natural products to find new antibacterial compounds is promising. These antibacterial compounds belong to different phytochemical families such as peptides (Boman, 2003), antibacterial phenolic compounds (Maddox & Laur, 2010), and organic acids (Raftari, 2009).

Bacteria has developed resistance to all different classes of antibiotics discovered to date (Rojas et al. 2003); this makes the present work more remarkable. This must be pointed out that, even with optimal antibiotic use, antibacterial resistances would not decline quickly and existing resistances are unlikely to vanish but we must avoid the emergence of new strains of resistant bacteria and limit the existing antibacterial resistance (Guillemot, 1999).

The emerging resistance of microbes to antifungal agents has serious implications in the management of infections. These antifungal compounds also act on targets found in mammalian cells which may result in toxicity or adverse drug interactions (Lucca & Walsh, 1999). Ketoconazole is one of the antifungal drugs used against both superficial and deep seated infections. However, its unpleasant side effects include nausea, abdominal pain, itching, toxicity, slow therapeutic response, and poor efficacy in immunocompromised patients (Pyun & Shin, 2005). Therefore, the discovery of novel antifungals is severely needed.

2.7.2 Methods of antimicrobial activity evaluation

Both *in vivo* and *in vitro* methods are applied to determine the antimicrobial activity of samples. The *in vivo* evaluation methods of antimicrobial compounds usually are carried out on rats and the duration of antimicrobial activity is determined (Lee et al., 2003). Several *in vitro* antimicrobial assays are developed but agar-well diffusion and disc diffusion methods are the most commonly used techniques to evaluate antimicrobial

activity of different kinds of samples (European Committee on Antimicrobial Susceptibility Testing, 2009). Inhibition zone diameters can be measured and antibacterial potential of samples can be stated to compare with a positive control that is usually a commercial antibiotic and antifungal.

2.7.3 Antimicrobial activity of plants

The idea that certain plants have healing potential and they contain what we would currently call antimicrobial compounds was well accepted before humans actually discovered the existence of microbes (Ríos & Recio, 2005). Since then, mankind have used plants to treat common infectious diseases and some of these traditional medicines are still being performed as antimicrobial treatments; for example, bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) are used to treat urinary tract infections, or lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) has a broad-spectrum of antimicrobial agents (Heinrich & Gibbons, 2004). It has been proven that the main compounds for antibacterial activities generally contain the essential oils of these plants rather than their extracts which bring a great and vast area of usage in the treatment for infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin. For example, in the case of *Melaleuca alternifolia*, the usage of the essential oil (tea tree oil) is a common therapeutic tool to treat acne and other infectious problems of the skin (Ríos & Recio, 2005).

2.8 Anticancer

2.8.1 Importance of anticancer

The use of natural products as anticancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine. Several drugs currently used in chemotherapy were isolated from plant species

or derived from a natural prototype. Most of the research performed today focuses on the development of new drugs to treat cancer. According to Cragg and Newman (2000), over 50 % of the drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them.

According to the Ferlay et al. (2015) there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide. According to the World Health Organization estimates for 2011, cancer now cause more deaths than all coronary heart disease.

Plant derived drugs are playing an important role in the up growth of cancer therapy (Conforti et al., 2008). Most of the active compounds in these extracts remain unidentified, and their presence is only detected by biological tests. Most of the identified compounds are products of plant secondary metabolism and belong to the classes of alkaloids, polyphenols, triterpenes, or steroid glycosides (Dzhambazov et al., 2002).

2.8.2 Methods of anticancer activity

To assess for preliminary anticancer activity in terms of cell viability, the MTT and MTS *in vitro* cytotoxicity assays are considered two of the most economic, reliable and convenient methods. This is based on their ease of use, accuracy and rapid indication of toxicity (Berg et al., 1994), as well as their sensitivity and specificity (Malich et al., 1997). Both assays are *in vitro* whole cell toxicity assays that employ colorimetric methods for determining the number of viable cells based on mitochondrial dehydrogenase activity measurement and differ only in the reagent employed. In the MTT assay, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is bioreduced by dehydrogenase inside living cells to form a coloured formazan dye, while in the MTS assay, a similar bioconversion takes places utilising 3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent (*Phenazine ethosulfate*).

Other methods like: Tryphan blue dye exclusion assay (Unnikrishnan & Ramadasan 1998), LDH (Lactic dehydrogenase) assay (Russo et al., 2006) and Sulforhodamine B assay (Voigt, 2005) also are used to determine the anticancer activity.

2.8.3 Anticancer activity of medicinal plants

The synthetic anticancer remedies are beyond the reach of common man because of cost factor. Herbal medicines have a vital role in the prevention and treatment of cancer and medicinal herbs are commonly available and comparatively economical. A great deal of pharmaceutical research done in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the herbal medicines used in the treatment of cancer. Some herbs protect the body from cancer by enhancing detoxification functions of the body. Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. Some herbs reduce toxic side effects of chemotherapy and radiotherapy.

Phytoconstituents derived from the herbs *Taxus species* (Malik et al., 2011), *Achyranthes bidentate* (Zhang et al., 2012), *Allium sativum* (Thomson & Ali, 2003), *Aloe vera* (Boudreau & Beland, 2006), *Angelica sinensis* (Cheng et al., 2004), *Astragals membranaceus* (Cho & Leung, 2007), *Glycine max* (Yanamandra et al., 2003), *Glycyrrhiza glabra* (Kherva, 2009), *Medicago sativa* (Rosenthal & Nkomo, 2000), *Morinda citrifolia* (Wang & Su, 2001), *Saussurea lappa* (Ko et al., 2005), *Taxus wallichiana* (Chattopadhyay et al., 2003), *Tinospora cordifolia* (Mathew & Kuttan, 1999), *Viscum album* (Zarkovic et al., 2001), *Withania somnifera* (Christina et al., 2004), *Zingiber officinale* (Habib et al., 2008) etc., have been used in various formulations to

help the body fighting cancer more effectively and reduce toxic side effects of chemotherapy and radiotherapy stages of cancer.

2.9 Biological Activities of *Trifolium* Species

Plants from the genus *Trifolium* have been known for many years as important forage crops and also valuable herbs in folk medicine. Nowadays, the therapeutic use of *Trifolium* plants is still based mainly on traditional medicine recommendations, but the number of scientific data on the biological activity of clovers and their possible therapeutic effects has been growing. The majority of the studies on the biological properties of clovers concerns *Trifolium pratense* and is focused on its phytoestrogenic action, being a result of isoflavone content. However, it should be emphasized that the beneficial effects of red clover as well as other clovers may be dependent on the action of various biologically active substances occurring in these herbs, not only isoflavones. There are significant gaps in the ethnopharmacological knowledge of medicinal relevance to *Trifolium* genus. Contrary to the existing evidence of therapeutic properties of *T. pratense*, obtained from *in vivo* and clinical examinations, the curative applications of other *Trifolium* species are supported mainly by basic investigations or traditional medicine.

Clovers have been used by Oriental and European cultures for the treatment of eczema and psoriasis. In Turkish traditional medicine, *Trifolium repens*, *Trifolium arvense* and *Trifolium pratense* have been administered as expectorant, antiseptic, analgesic, sedative and tonic mixtures (Sabudak et al., 2008). *Trifolium repens* and *Trifolium pratense* are popular herbs in Pakistan, useful in the treatment of sore throat, fever, pneumonia, meningitis and feverish feeling (Khan & Khatoon, 2008). Native Americans have used *Trifolium pratense* to cure external skin problems, lung illnesses, as well as some disorders of nervous and reproductive system (Sabudak & Guler, 2009). Barros et al.

(2010) report that in Portugal, *Trifolium angustifolium* decoctions are a folk medicine for stomachache and diarrhea. *Trifolium repens* is a deworming remedy in the traditional medicine of the Naga tribes of India. This activity of white clover has been confirmed by *in vivo* study on animals (Tangpu et al., 2004).

The chemical profile of clovers is partly recognized. It is known that besides isoflavones, *Trifolium* plants synthesize a wide range of phenolic and polyphenolic compounds such as flavonoids, saponins, clovamides (caffeic acid esters), phenolic acids and other substances (Oleszek & Stochmal, 2002; Oleszek et al., 2007).

The antioxidative activity of some *Trifolium* species may be also a result of the abundance of flavonoids and other phenolic compounds, such as catechins, saponins, clovamides and phenolic acids, present in clovers. . Sabudak et al. (2009) have found the antioxidative activity *in vitro* of hexane extracts, obtained from *T. balansae, T. stellatum, T. nigrescens, T. constantinopolitanum* and *T. resupinatum*. Moreover, these effects correlated with the content of unsaturated fatty acid in the tested extracts. Kolodziejczyk et al., (2011) reported *in vitro* antioxidant effects of the clovamide rich fraction, obtained from aerial parts of *Trifolium pallidum,* in the protection of blood platelets and plasma against the nitrative and oxidative damage, caused by peroxynitric. Mu et al. (2009), reported *in vivo* antioxidant activity and the estrogenic effect of formononetin from *Trifolium pratense*. Contrary to the mentioned reports suggesting the high anti-oxidative activity of *Trifolium* plants, the ethnobotanical study of Barros et al. (2010) demonstrated relatively weak antioxidant properties of *T. angustifolium in vitro,* most likely being a result of a low content of phenolics and flavonoids.

A study by khan et al. (2012) demonstrated the antibacterial activity of *Trifolium alexandrinum* leaves extract against seven Gram-positive and eleven Gram-negative hospital isolated human pathogenic bacteria strains. According to this study all the

extracts had the antimicrobial activity against at least six types of microorganisms. They also found that the polar (methanol or ethyl acetate) extracts displayed considerably stronger antibacterial activity. Flythe and Kagan, (2010) reported that the phenolic compounds from *Trifolium pratense* possessed antimicrobial activity against hyper ammonia-producing bacteria (*Clostridium sticklandii*). Turker et al. (2009) reported that the alcoholic extracts of *Trifolium pannonicum* showed a broad antibacterial spectrum against four different fish pathogens: *Aeromonas hydrophila, Yersinia ruckeri, Streptococcus agalactia* and *Lactococcus garvieae*.

Trifolium pratense is said to retard progress of cancerous tumors before ulceration has taken place (Phatak, 1977). The isoflavone constituents in red clover have estrogenic properties. They are thought to have positive effects on menopausal disorders such as osteoporosis, cardiac risk factors or breast cancer (Pagliaaci et al., 1994; Fugh-berman & Kronenberg, 2001; Beck et al., 2005). Genistein isolated from *Trifolium* species is a molecule of great interest as a potentially chemotherapeutic agent or as a lead compound in anticancer drug design (Polkowski et al., 2004). Lellau and Liebezeit (2003), reported tumor growth inhibiting activities of the *Trifolium fragiferum* and *Trifolium repens* extracts. Moderate cytotoxic activity (LC_{50} between 30 and 50 µg/ml) by *Trifolium campestre* and *Trifolium repens* against the brine shrimp were reported by Khalighi-Sigaroodi et al. (2012).

2.10 Photosynthetic and Leaf Gas Exchange of Plant

Photosynthesis is a process used by plants and other organisms to convert light energy, normally from the Sun, into chemical energy that can be later released to fuel the organisms' activities. This chemical energy is stored in carbohydrate molecules, such as sugars, which are synthesized from carbon dioxide and water. Photosynthesis maintains atmospheric oxygen levels and supplies all of the organic compounds and most of the

energy necessary for life on Earth. Although photosynthesis is performed differently by different species, the process always begins when energy from light is absorbed by proteins called reaction centers that contain green chlorophyll pigments. In plants, these proteins are held inside organelles called chloroplasts, which are most abundant in leaf cells. Although all cells in the green parts of a plant have chloroplasts, most of the energy in higher plants is captured in the leaves.

Photosynthesis measurement systems are not designed to directly measure the amount of light absorbed by the leaf. But analysis of chlorophyll-fluorescence, P700- and P515-absorbance and gas exchange measurements reveal detailed information about e.g. the photosystems, quantum efficiency and the CO_2 assimilation rates. With some instruments even wavelength-dependency of the photosynthetic efficiency can be analyzed.

Many physiological parameters such as light regime, plant nutrition, water stress, leaf age and environmental temperature are known to be effective on photosynthetic CO_2 assimilation by leaves. For example higher temperature cause heat stress in plants and under this conditions plant growth and productivities are less (Porter & Semenov, 2005). In some cases, the decrease in crop productivity under warmer conditions is related to effects on the phenology and reproductive stages. The rate of photosynthesis declines as plant water stress increases (Flexas & Medrano, 2002), and as occurred with high temperature, this decline may be a consequence of limitations in CO_2 diffusion and/or photosynthetic metabolism.

Studies have shown that high altitude plants have higher photosynthetic efficiency than the low altitude ones (Körner & Diemer, 1994; Körner et al., 1988). The leaves of high altitude plants are capable of positive net photosynthetic rate (PN) at lower CO_2 concentrations and lower temperatures than low altitude plants (Friend & Woodward

1990). At high altitude plants, leaves achieve maximal photosynthetic rate (P_{Nmax}) at higher irradiance than those at low altitude (Friend & Woodward, 1990).

Photosynthesis process has been identified as one of the most important factors that limit crop production. It is also known as one of the environmental factors that affects secondary metabolism of plants.

The net photosynthetic rates vary considerable with plant species, temperature, Photosynthesis Photon Flux Density (PPFD) and other conditions. Within a large group of herbaceous, the leaf photosynthetic rates were more or less the same but they were lesser than 15 μmol/m^2/s when measured under full sunlight, normal air and optimum temperatures. The variation in the net photosynthesis and the related gas exchange parameters have been suggested as the determinants of plant productivity and the early selection criteria to enhance plant efficiency (Wang et al., 1995). The irradiance level is a significant ecological factor for photo-autotrophic plants. Inappropriate light level can create stress on plants by restricting photosynthesis and consequently net carbon gaining and plant growth. Hence, the mathematical description derived from light-response curves is valuable because it includes variables with a comprehensible physiological meaning.

Heraut-Bron et al. (2000) assessed the effects of red:far-red (R:FR) ratio on net CO_2 assimilation, photosynthetic activity and morphology of young white clover leaves. They found that a leaf that developed in lower irradiance had a lower net CO_2 assimilation rate and a greater leaf area and petiole length than leaves that developed in higher irradiance. In addition, they did not find any significant effect of FR light on photosynthesis of white clover.

2.11 Plant Salt Tolerance

Salinity is known as one of the main environmental constraints due to its high value of impact and wide distribution that is affecting the arable lands and therefore yields of most crops all over the world. Salinity of soil is due to high evapo-transpiration rate and an inappropriate poor-quality water irrigation, which, then, affects almost every aspect of plant physiology at both the whole-plant and cellular levels (Munns & Tester, 2008).

The main responsible factors for increasing soil salinity are using saline irrigation water and application of fertilizer (Epstein et al., 1980). The levels of salinity in the soil vary depending on the soil depth and season (Daniells et al., 2001) and also great potential for interactions with other environmental factors (Perez-Tornero et al., 2009). For this reason studies about salt stress are preferably conducted in soil-less culture with nutrient solutions of known salt concentration (Vijayan et al., 2003). Therefore, one of the good tools for salinity studies is *in vitro* culture of plants using plant tissues or cells.

There have been many investigations on plant seed tolerance against salinity stress. Karimi et al. (2004) reported that endurance of salinity in germination level has special importance because it can cause absence some proteins and enzymes. Shannon (1984) stated that in many of plants, the most sensitivity levels to salinity in period of plant life are the stages of budding and flowering. It is known that legumes are generally more sensitive to salinity (Rogers et al., 1997).

The production of reactive oxygen species (ROS) is one of the biochemical changes occurring in plants subjected to salt stress (Ashraf and Harris, 2004; Panda & Upadhyay, 2004; Jehan et al., 2012). To alleviate the disadvantageous effects of ROS, plants have evolved specific antioxidative mechanisms to scavenge ROS. Hence, antioxidant enzymes seem to be most important determinants of plant tolerance to salt stress (Vranova et al., 2002; Ashraf et al., 2008). Many studies reported that NaCl tolerance will affected

on antioxidant response in different plant species such as *Malus domestica* (Wang et al., 2013), Soybean (Amirjani, 2010), *Sesuvium portulacastrum* (Lokhande et al., 2011), pea (Miljuš-Djukić et al., 2013).

Maas (1977), reported that *T. pratense* has a low salt tolerance. Researches by Asci (2011) and also Winter and Lauchli, (1982), showed a lower survival potential for *T. pratense* at high concentration of NaCl treatment.

CHAPTER 3: MATERIALS AND METHODS

3.1 *In Vitro* Regeneration and Callus Induction of *Trifolium pratense*

3.1.1 *In vivo* plant samples

Seeds of *Trifolium pratense* L. were purchased from Stock Seed Farms (Murdock, US) and were planted in polybags filled with the ratio of 1:1:1 of coco peat, sand and compost. Then, the organic fertilizer was added to each polybag and kept in the growth room (temperature of 25 ± 1 °C, 16 hours photoperiod, 40 µmol/m^2/s of photon flux density, 60% relative humidity) (Figures 3.1 and 3.2).

Figure 3.1: *In vivo* culture of *T. pratense* L. after 5 days

Figure 3.2: *In vivo* plants of *T. pratense* after 6 weeks

3.1.2 *In vitro* plant samples

3.1.2.1 Use of aseptic techniques

The use of aseptic techniques is very crucial in this part of expriment. Aseptic techniques were used when culturing the seeds or explants onto the culture media. This is to prevent contamination from occurring. For instance, whilst preparing the culture media, after the media has been prepared, the conical flask were sealed with an aluminum foil and would be autoclaved at 121° C for 20 minutes to sterilize the media.

All tissue culture experiments were conducted in the Laminar Flow Chamber and before any tissue culture experiments were done, the UV light was switched on for about 15 to 20 minutes to get rid of the adhering microorganism in the laminar flow chamber. After that the inner surface of the laminar were wiped clean with 70% alcohol to reduce the risks of contamination due to the bacteria and fungi.

Every equipment and lab ware such as the outer surfaces of the culture flasks containing the culture media, scalpels, petri dishes and etc, were wiped clean with 70% alcohol before being brought into the laminar flow.

3.1.2.2 Preparation of culture media

For *in vitro* seed germination MS media without the addition of any hormones were used. The MS media from Murashige and Skoog (including vitamins) with the weight of 4.4g was used for media preparation. Media was prepared for one liter (L) of water. For making a liter of MS medium, 800 ml distilled water was poured in a beaker and placed on the stirrer (using magnetic beads for stirring) and stirred while adding 4.4 g MS powder (Sigma with vitamins) and 30 g sucrose. The pH of the media solution was then measured and adjusted to 5.7 by adding 1M NaOH or 1M HCL. Following that, for solidifying the media, 2.8 gram phytogel agar was added while turned on the heating button in order to melt the agar. Then, the media solution was topped up to 1L with distilled water and the conical flask would be wrapped with an aluminum foil and autoclaved at 121° C for 20 minutes.

On the other hand, for tissue culture of explants, MS media supplemented with hormones were used. The same protocol as described in the previous paragraph was used, but with an addition of desired hormones combination (with the desired concentrations) before measuring the pH.

3.1.2.3 *In Vitro* seed germination

Before the seeds were cultured in MS media, they were first washed and sterilized by using an appropriate sterilization protocol. During sterilization, the seeds were washed with a series of Clorox dilutions, followed by washing with distilled water. This was done to effectively remove impurities and contaminants that adhere to the surface of the seeds. Some of the purchased seeds were surface sterilized by first placing under running tap

water for 30 minutes, rinsed once using sterile distilled water and dunked in 50 % Clorox for 2 minutes. The seeds were then washed 5 times using sterile distilled water and soaked in 10 % Clorox for one minute. The seeds were brought into the laminar flow and rinsed 5 times using sterile distilled water and dipped in 70 % ethanol for 1 minute. Lastly the seeds were rinsed 5 times using sterile distilled water and blotted on sterilized filter paper. Immediately after the sterilization process, the seeds were cultured on MS (Murashige and Skoog) medium without adding any hormones (MSO) and incubated in the culture room at 25 ± 1° C with 16 hours light and 8 hours dark (40 µmol/m^2/s of photon flux density and 60% relative humidity) (Figure 3.3). Since the seeds were very small in size, 6 seeds were cultured in each conical flask containing the MS basal medium. It was observed that the seeds started to germinate after 5 to 10 days and became complete seedling after 3-4 weeks. The plants were subcultured after 6 weeks, to allow them to grow better. The nodal parts of these aseptic seedlings were used as the explant for subsequent micropropagation experiments of *Trifolium pratense* L.

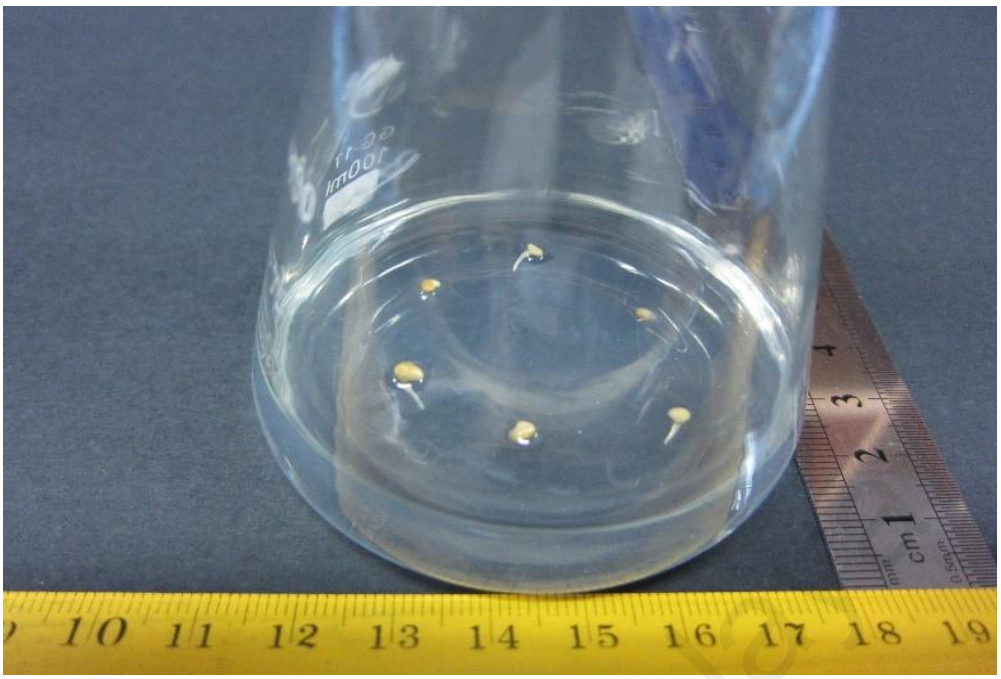

Figure 3.3: *In vitro* seed germination of *Trifolium pratense* L. on MS basal medium after 1 day

3.1.2.4 Sterilization of explants

Stringent sterilization procedure is critical as it minimizes the risk of contamination and therefor ensures efficient production of aseptic seedlings. However, in this part of experiment, the explants used in the culture process were already sterile, as they were excised from aseptic seedlings obtained from seed culture.

3.1.2.5 Effects of hormones on explants

Different hormones will yield different results or responses in plant regeneration. The nodal explants from four-week old plants were collected. Each nodal cutting was approximately the same size. Three cuttings were placed vertically into each flask containing 40 ml of medium (Figure 3.4). Each explant was spaced equal distances apart in the flasks. This study was designed to identify a medium with the optimal combination of PGRs to support shoot multiplication, root formation and callus induction. The study was conducted with 29 treatments consist of control, MS medium with different

combination of BAP and IBA and different combination of BAP and 2,4-D were used. The hormones combination used in this part of study are shown below:

1. MS media + no hormone
2. MS media + 0.5 mg/L BAP
3. MS media + 1.0 mg/L BAP
4. MS media + 1.5 mg/L BAP
5. MS media + 2.0 mg/L BAP
6. MS media + 0.5 mg/L BAP + 0.25 mg/L 2,4-D
7. MS media + 0.5 mg/L BAP + 0.5 mg/L 2,4-D
8. MS media + 0.5 mg/L BAP + 0.75 mg/L 2,4-D
9. MS media + 1.0 mg/L BAP + 0.25 mg/L 2,4-D
10. MS media + 1.0 mg/L BAP + 0.5 mg/L 2,4-D
11. MS media + 1.0 mg/L BAP + 0.75 mg/L 2,4-D
12. MS media + 1.5 mg/L BAP + 0.25 mg/L 2,4-D
13. MS media + 1.5 mg/L BAP + 0.5 mg/L 2,4-D
14. MS media + 1.5 mg/L BAP + 0.75 mg/L 2,4-D
15. MS media + 2.0 mg/L BAP + 0.25 mg/L 2,4-D
16. MS media + 2.0 mg/L BAP + 0.5 mg/L 2,4-D
17. MS media + 2.0 mg/L BAP + 0.75 mg/L 2,4-D
18. MS media + 0.5 mg/L BAP + 0.25 mg/L IBA
19. MS media + 0.5 mg/L BAP + 0.5 mg/L IBA
20. MS media + 0.5 mg/L BAP + 0.75 mg/L IBA
21. MS media + 1.0 mg/L BAP + 0.25 mg/L IBA
22. MS media + 1.0 mg/L BAP + 0.5 mg/L IBA
23. MS media + 1.0 mg/L BAP + 0.75 mg/L IBA
24. MS media + 1.5 mg/L BAP + 0.25 mg/L IBA

25. MS media + 1.5 mg/L BAP + 0.5 mg/L IBA
26. MS media + 1.5 mg/L BAP + 0.75 mg/L IBA
27. MS media + 2.0 mg/L BAP + 0.25 mg/L IBA
28. MS media + 2.0 mg/L BAP + 0.5 mg/L IBA
29. MS media + 2.0 mg/L BAP + 0.75 mg/L IBA

Each of the 29 treatments was replicated 30 times (15 conical flasks for each treatment, each flask containing 2 explants). Flasks were placed on a shelf in the growth chamber under light condition (1000 Lux) and at a temperature of 25 ± 1 ºC. Cultures were observed regularly and the contaminated flasks were replaced with fresh cultures. After a period of 6 weeks, the media and explants were screened and the responses recorded.

Figure 3.4: Culture of nodal explants of *Trifolium pratense* L. on MS medium after 5 days.

3.1.3 Acclimatization of *Trifolium pratense* plantlets

The *in vitro* plantlets were taken out from their respective containers carefully and all adhering MS media stuck onto the plantlets were washed off using sterile distilled water. The plantlets were then transferred onto the three different types of soils (black soil, red soil and combination of black and red soil in ratio of 1:1) in vases covered with plastic bags and acclimatized in culture room for one month. The plantlets were then transferred to a green house and their ability to fully adapt to the natural conditions was monitored.

3.1.4 Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was used to analyze the data using IBM SPSS Statistics version 19. The means were compared with Duncan's Multiple Comparison Test (DMCT) and $p < 0.05$ was considered to indicate statistical significance.

3.2 Cellular Behavior of *Trifolium pratense*

3.2.1 Determination of standard primary root growth

Growth measurement is obtained through measurements of average values of the parameters tested, from a group of roots that are as homogenous as possible (Pilet, 1991). Determining the standard root age and root length allows subsequent experiments to be conducted using root segments that are uniform throughout the roots population and can be considered as good and valid sample representative of the population. This is important as the selection of the roots would not only depend on the age of the seedlings after planting, but also with consideration of their physiological age and conditions depending on the growth pattern of the roots (Jensen, 1955).

In order to measure the standard growth of the primary root length for the *in vivo* plants, the sterilized seeds were cultured in a petri dish containing sterilized wet cotton wool. In this part 15 petri dishes with 10 seeds in each one were used. The cotton wools

in each petri dish were wetted twice everyday by using sterile distilled water and the cultures were maintained in 16 hours light and 8 hours dark, at 25 ± 1° C (Figure 3.5).

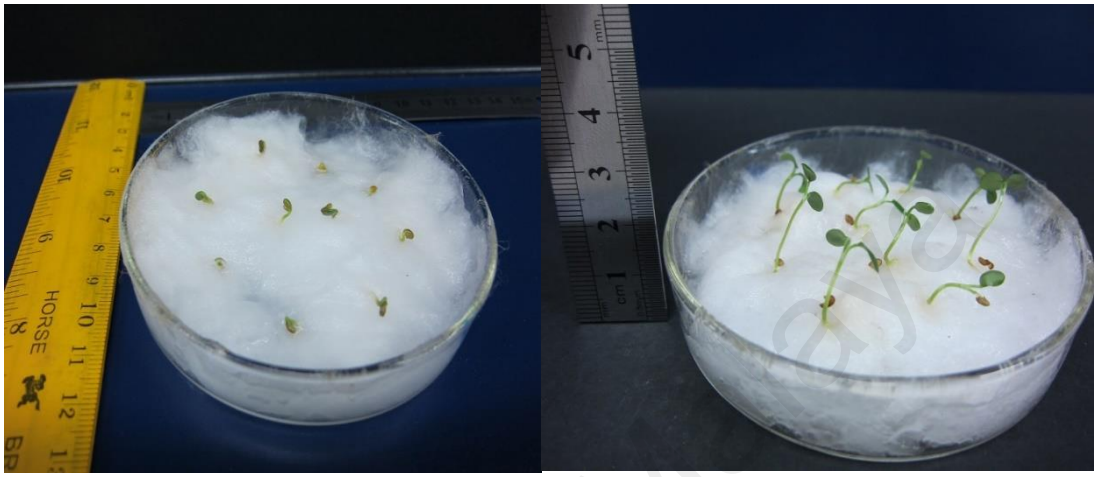

Figure 3.5: The seeds of *Trifolium pratense* L, germinated in petri dishes containing sterilized wet cotton wool

Standard growth of *in vitro* primary root length was also measured as a comparison. For this purpose, the sterilized seeds were cultured in petri dishes containing sterilized MSO media by using aseptic techniques in the laminar flow. Similar to the protocol used in measuring the standard growth for the *in vivo* plants, 15 petri dishes with 10 seeds in each one were used. The petri dishes were wrapped with parafilm to prevent contamination. The cultures were kept in the same condition with the *in vivo* samples.

The length of primary root of the seedlings was measured using a thread and a ruler, at the same time every day, until majority of primary roots developed secondary roots. The length was averaged and was plotted into a graph of the average length of the primary roots (Y-axis) against time (X-axis). Then, a linear regression analysis was conducted to find the relationship between both factors.

3.2.2 Cytological studies of primary roots of *Trifolium pratense*

In order to study cellular activities of *in vivo* and *in vitro* seedlings of *Trifolium pratense,* parameters such as chromosome count, mitotic index (MI) and mean cell and nuclear areas were determined.

For *in vitro* cytological studies, sterilized seed were cultured again in full strength MS medium without hormones and incubated in the culture room at 25 ± 1 ˚C with 16 h light and 8 h dark for 4 weeks. The nodal parts of the 4 weeks-old plants were used as explants and cultured on MS medium supplemented with combination of 1.5 mg/l BAP and 0.5 mg/l IBA, for the micropropagation of *T. pretense* (based on the results determined in Chapter 4).. The cultures were maintained in the culture room at 25 ± 1 ˚C with 16 h light and 8 h dark. After two weeks of culture (the needed time to achieve the standard root length), root with the standard length of 33 mm were harvested for the cytological analysis. For the cytological studies of *in vivo* grown *T. pratense*, the seeds were cultured in the black and red soil with the ratio of 1:1 and transferred to the growth room with 16 hours light and 8 hours dark, at 25 ± 1° C. After two weeks of culture the primary roots with the standard length of 33 mm were harvested for cytological studies.

3.2.3 Slide preparation and image analysis

Root tip meristems obtained from seedlings were pre-treated in 8-hydroxyl-quinalin (2 mM) at 4° C for 5 hours, fixed farmer's fluid (methanol 3 : 1 acetic acid) and carnoys solution (1:glacial acetic acid, 3:chloroform, 6:ethanol) at three different times of 15 min, 60 min and overnight. The samples were then hydrolyzed in 5N HCL for 40 min at room temperature (cold method) or in 1N HCL for 10 and 30 minutes at 60° C (hot method). After washing with distilled water, the root tips were soaked in Feulgen reagent for 3 hours. The root segments were then rinsed in several changes of distilled water for 5 to 10 minutes and any excess water was blotted using a filter paper. The apical meristem of

the root segments were subdivided and squashed, followed by addition of a small drop of 45% acetic acid on clean slides. The slides were then heated gently by passing over a spirit lamp a few times and the coverslips were levered off using a razor blade. The slides were then viewed using Axioskop Zeiss microscope attached to AxioCam MRc video camera and were then analyzed using AxioVision 4.7 software.

3.2.4 Measurement of Mitotic Index

Mitotic index (MI) is the sum of cells that are undergoing division or mitosis, such as cells in prophase, metaphase, anaphase and telophase that are expressed in terms of percentage of all cells counted. The mitotic index was measured by scoring these cells from at least 1000 cells in a series of random transect across the permanent slide. The data obtained in three replicates from three slides. The average MI values for three slides were calculated.

3.2.5 Chromosome count

The number of chromosomes was counted from 15 metaphase plates with suitable chromosome spread. This was done in three replicates, from which the average values were calculated.

3.2.6 Mean cell and nuclear areas

The mean cell and nuclear areas were measured from 150 prophase cells from 3 slides. The ratio of nuclear to cell area was also determined from each sample.

3.3 Biological Activities

3.3.1 Extract preparation

The aerial parts of *in vivo* and *in vitro* grown plants and also the callus obtained from *in vitro* cultures were collected and dried in the dark. The dried samples were powdered in an electronic blender before being used for solvent extraction. For the preparation of

extract, 25 g of fine powder was extracted with 100 ml of 95% methanol at room temperature for 48 hours. The extracts were filtrated through Whatman No. 1 and combined and this was followed by a concentration using rotary evaporator under pressure that was reduced at 45 ˚C. The filtrate obtained was suspended in distilled water (25 ml) and n-hexane (50 ml) was added to it, where the mixture was shaken well and the layers were enabled to be separated for 6 h. After separation of n-hexane layer, again 50 ml of n-hexane was added to get the n-hexane fraction. In a similar manner, the protocol was repeated with the rest of the solvents (ethyl acetate and chloroform). Each fraction obtained was dried using a rotary evaporator (Kil et al., 2009). The dry extract obtained with each solvent was weighed out and the percentage yield was expressed in terms of the air dried weight of plant materials. Samples were stored in an airtight container at – 20 ˚C until it was time to conduct further analysis.

3.3.2 Total phenolic content

The total phenolic content of the obtained extracts was spectrometrically analyzed in adherence to the Folin-Ciocalteu method (Dewanto et al., 2002). In short, 100 µl of each extract (dissolved in methanol) or gallic acid standard solution was mixed with 2 ml of 2% (w/v) sodium carbonate solution. The mixture was then incubated for 5 minutes, and afterwards 100 µl of Folin-Ciocalteu reagent was added. After being left for 30 min at room temperature for color development, absorbance was measured at 750 nm using a spectrophotometer. Results are expressed as mg gallic acid equivalents (GAE) per gram dry-matter of sample.

3.3.3 Total flavonoid content

Total flavonoid content was ascertained based on the method by Zhishen et al., (1999). In brief, 50 mg of each fraction was dissolved in 10 ml of 80% aqueous methanol and filtered through Whatman filter paper No. 42 (125 mm). In a 10 ml test tube, 300 µl of

each extract, 3.4 ml of 30% methanol, 150 µl of 0.5 M $NaNO_2$ and 150 µl of 0.3 M $AlCl_3.6H_2O$ were added and mixed, followed by 5-min incubation and the addition of 1 ml of NaOH (1 M). Absorbance was measured at 510 nm with a spectrophotometer. The standard curve for total flavonoids was made using rutin standard solution (0–100 mg/l) using the same aforementioned procedure. The total flavonoid content was shown as milligram of rutin equivalents (CTE) per gram dry matter of sample.

3.3.4 Antioxidant assays

Each sample was dissolved in 95% methanol at a concentration of 1 mg/ml and then diluted in order to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparative purposes in all assays.

3.3.4.1 DPPH radical scavenging activity assay

The DPPH radical scavenging assay was conducted following the method of Zhu *et al*., (2006). In brief, 2 ml of DPPH solution (0.1 mM, in methanol) was blended with 2 ml of the samples at varying concentrations (50, 100, 150, 200, 250 and 300 µg/ml). As the next stage, the reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank. Ascorbic acid and rutin standard as positive controls were prepared in a similar manner, as for the test group except for the antioxidant solution's replacement. The inhibition of the DPPH radical by the sample was calculated based on the formula below:

DPPH scavenging activity (%)

= [(absorbance of control – absorbance of sample) / (absorbance of control)] x 100

3.3.4.2 Superoxide anion radical scavenging assay

The assay for superoxide anion radical scavenging activity leaned on a riboflavin-light-NBT system (Beauchamp & Fridovich, 1971). The reaction mixture had 0.5 ml of

phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml phenazinemethosulphate (PMS) (20 mM), and 0.1 ml nitro blue tetrazolium (NBT) (0.5 mM), before 1 ml sample solution was added at varying concentrations (50, 100, 150, 200, 250 and 300 µg/ml). Reaction began as the reaction mixture was illuminated with different concentrations of the extracts using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The percentage of inhibition of superoxide anion generation was calculated based on the following formula:

Scavenging activity (%) = (1 – absorbance of sample / absorbance of control) x 100

3.3.4.3 ABTS radical scavenging assay

The ABTS radical scavenging assay was performed adhering to the method of Re *et al*., (1999) with slight modification. The ABTS radical was generated through the oxidation of ABTS with potassium persulfate. In brief, the ABTS solution (7 mM) had reacted with potassium persulfate (2.45 mM) solution and was stored in the dark for 12-16 h to produce a dark coloured solution containing ABTS radical cation. Before being used in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.70 (± 0.02) at 745 nm, with the temperature control fixed as 30 ˚C. Free radical scavenging activity was evaluated by mixing 3 ml of ABTS working standard with 300 µl of test sample (50, 100, 150, 200, 300, 400, 500 µg/ml) in a microcuvette. The decrease in the absorbance was measured at the exact time of one min after mixing the solution, then until it reached 6 min. The final absorbance was noted then. The inhibition percentage was calculated based on the following formula:

Scavenging effect (%)

= [(absorbance of control – absorbance of sample) / (absorbance of control)] x 100

3.3.4.4 Hydrogen peroxide scavenging activity

The capability of scavenging hydrogen peroxide by the extract was determined based on the method of Ruch et al., (1989). A hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 ml) of the extracted sample (different concentration of 50, 100, 150, 200, 250, 300 µg/ml) were transferred into the test tubes and their volumes were made up to 0.4 ml with 50 mM phosphate buffer (pH 7.4). After adding 0.6 ml hydrogen peroxide solution, tubes were vortexed and the absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. The abilities to scavenge the hydrogen peroxide were calculated based on the following equation:

Hydrogen peroxide scavenging activity = (1 – absorbance of sample / absorbance of control) × 100

3.3.4.5 Reducing power assay

The reducing powers of the samples were determined following the method of Atmani et al., (2009). Two millilitres (2 mm) of each extract solution (300 µg/ml) were mixed with 2 ml of phosphate buffered saline (0.2 M, pH 6.6) and 2 ml of potassium ferrocyanate (10 mg/ml). The incubation for this mixture was set at 50 ˚C for 20 min. In the next stage, 2 ml of trichloroacetic acid (100 mg/l) was added to the mixture. In a test tube, a volume of 2 ml from each of the above mixtures was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) ferric chloride. The absorbance was measured at 700 nm after reaction was started for 10 minutes. The increased absorbance of the reaction mixture suggested that the reducing power was high.

3.3.4.6 Chelating power

The ability of the extract to chelate iron (II) was estimated based on the method of Dinis et al., (1994) with minimal modification. Various sample solution (50 – 300 µg/ml) were prepared with dissolving the extracts in the methanol. An aliquot of each sample

(200 µl) was mixed with 100 µl of $FeCl_2.2H_2O$ (2 mM) and 900 µl of methanol. After 5 min incubation, an initial reaction was fuelled by the addition of 400 µl of ferrozine (5 mM). After 10 min incubation, the absorbance at 562 nm was recorded. The percentage of the chelating activity was calculated based on the following equation:

Chelating activity (%) = [(absorbance of control – absorbance of sample) / (absorbance of control)] × 100

3.3.5 Determination of anticancer activity

3.3.5.1 Cell line and culture

HCT-116 (human colon carcinoma) and MCF-7 (human breast carcinoma) cell cultures were purchased from the American Tissue Culture Collection (ATCC) and were cultured in Roswell Park Memorial Institute medium (1640, pH 7.4), with addition of 10% fetal bovine serum (FBS), antibiotic (penicillin, 100 U/ml) and streptomycin sulphate (100 µg/ml). For obtained the cell attachment the cell lines were incubated at 37 °C in 5% CO_2 overnight.

3.3.5.2 MTT assay

The anticancer activity of extracts was distinctive according to the method of Zhao *et al*., (2007) using MTT (3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide) assay. In this essay the reduction of MTT to blue formazan product by mitochondrial dehydrogenase, which reflects the normal function of mitochondrial and hence the cell viability, will be detected. Briefly two human cancer cells (MCF-7 and HCT-116) were plated at 2×10^4 cells per well in 96-well microtiter plates with 100 µl RPMI growth medium and 24 h incubation at 37 °C, with 5% CO_2 in a humidified atmosphere, during which period a partial monolayer was formed. Later, the media was replaced with the fresh growth media containing different concentration (25, 50, 75, 100, 125, 150 µg/ml) of tested plant extract. After incubation at 37 °C (24, 48 or 72 hours) with 5% CO_2, the

growth medium was replaced with the MTT reagent (0.1 mg/ml). The MTT reagent was removed after incubation at 37 °C for 4 hours and 100 µl of dimethylsulphoxide was added to each well before shaking for 15 minutes. The absorbance of the solution was measured at 570 nm, using as ELISA reader. The growth media without adding plant extract was used as the negative control. Doxorubicin as a conventional anticancer drug was used as the positive control in this test. Cellular growth inhibition by the tested samples was computed as the inhibition activity percentage and expressed as the IC_{50} value.

3.3.6 Statistical analysis

All assays were carried out in triplicates and their results were expressed as mean ± standard deviation. The EC_{50} (half maximal effective concentration) of various fractions for different antioxidant assays were analyzed using ANOVA test with least significant difference (LSD) $P < 0.05$ as a level of significant. Experimental results were examined further for Pearson correlation coefficient of phenolic and flavonoids with different antioxidants assays and its significance tested using the student's t-test ($p < 0.05$).

3.3.7 Antimicrobial activities

3.3.7.1 Extract preparation

The extract for antimicrobial activities test were prepared according to the method described in section 3.3.1, but only methanol and ethanol solvent were used for extraction. The plant extracts (100 mg/ml) were dissolved in Dimethyl sulfoxide (DMSO) and kept at 4°C until required for the experiments.

3.3.7.2 Antibacterial activity assay

The antibacterial potentials of ethanol and methanol extracts of red clover were studied using the paper disc diffusion method of Kil et al., (2009). Two gram-negative pathogenic bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two gram-

positive pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were obtained from the Microbiology Division of Institute of Biological Sciences, University of Malaya and then grown in nutrient broth medium to yield a final concentration of 10^7 colony forming unit (CFU)/ml. The test bacteria (0.1 ml) were streaked on Mueller Hinton medium plates using sterile cotton swab. Sterilized filter paper discs were soaked in ethanol or methanol extract (100 mg/ml) and were then placed in the center of test bacteria plates. Each disk must be pressed down with forceps to ensure complete contact with the agar surface. The plates were incubated for 24 h at 37°C and the diameters of the inhibition zones were measured. Tetracycline disc (30 μg) and DMSO were used as the positive and negative controls, respectively. All measurements were performed in triplicate and mean values ± SD were recorded.

3.3.7.3 Antifungal activity assay

Antifungal activity was measured using paper disc diffusion method of Erturk (2006). Twenty mL of Sabouraud Dextrose Agar (SDA) was poured into each 15 cm petri dish. The fungal strains used in this study were *Aspergillus niger*, *Candida albicans* and *Fusarium verticillioides* which were grown in Sabouraud Dextrose Broth at 27 ˚C for 48 hours. Growth was adjusted to OD (600 nm) of 0.1 by dilution with Sabouraud Dextrose Broth. 0.1 mL of suspension containing approximately 10^8 fungal/mL were placed over agar in petri dishes and dispersed using sterile cotton swab. Then sterilized filter paper discs (6 mm diameter) were soaked in ethanol or methanol extracts of each sample (100 mg/ml) and were then placed in the center of test fungal plates. One hundred units of Nystatin were used as a positive control and DMSO as a negative control. Inhibition zones were determined in mm after incubation at 27 ˚C for 72 hours. All experiments were done in triplicate.

3.4 Leaf Gas Exchange and Photosynthetic Light Response Curve

In the fully expanded leaves with highest flavonoid and phenolic contents, the stomatal conductance (gs), transpiration rate (E) and net photosynthetic rate (Pn) were determined by means of a portable infrared photosynthesis system LICOR 6400, at standard cuvette condition including 1000 µmol/m^2/s PPFD, 400 µmol/mol CO_2 reference, 60% relative humidity at 25 - 30 ºC leaf temperature. The measurements were carried out between 9 to 11 am. For accurate measurement with this portable instrument two zeroing process were needed; the first one was for the built in flow meter and second one was for the infra-red gas analyzer (Haniff, 2006).

Photosynthetic light response curve is produced when the photosynthesis rates are plotted against light intensity. Carbon dioxide level was calibrated at 400 µmol/mol (60% humidity, flow rate of 300 mL/min and temperature of 25 – 30° C), and the irradiance level (Photosynthesis Photon Flux Density, PPFD) were varied keeping the following order: 1200, 1000, 800, 600, 400, 250, 200, 150, 100, 50 and 0 µmol/m2/s. photosynthesis rate at the light saturation point was considered maximum net photosynthetic rate (P_{Nmax}). At light saturation point, enhancing the light does not cause an increase in photosynthesis. The photochemical efficiency (α: ATP formation) was estimated measuring the slope of the linear phase of the same light response curve. In other words, the slope of the linear phase of the light curve was an estimation of photosynthetic efficiency. The y intercept of the linear part of the light response curve indicated O_2 exchange extrapolated to darkness. Since the y-intercept is less than 0, this showed a net uptake of O_2. Oxygen uptake in leaves was the consequence of two processes; one was cellular respiration, whereas the other was photorespiration. The light intensity value on the X-axis through which the line passes is known as the light compensation point.

The CP rate (X) was calculated using the following formula:

$X = [-b \pm (b^2 - 4ac)^{1/2}] / 2a$ (derived from $y = ax^2 + bx + c$ when $y = 0$)

Where, c = constant parameter in $y = ax^2 + bx + c$, and b is AQY

3.5 Effect of Salinity Stress on Seed Germination of *Trifolium pratense*

Trifolium pratense seeds were purchased from Stock Seed Farms (Murdock, US). The sterilization of seeds was done as explained in section (3.1.2.3). Control seeds were germinated on a hormone-free basal medium (MS) containing 3% sucrose and 0.7% phytogel agar without adding NaCl in culture flasks. The treatment seeds were maintained on the same MS medium with addition of 50, 100, 150 and 200 mM of NaCl in culture flasks. All seeds were germinated under a 16-h light and 8-h dark photoperiod and a constant temperate of 25 ± 1 ˚C in a growth chamber. Germination percentage as well as root and shoot length (mm) were recorded for each treatment after 6 weeks of culture, then the grown plants were dried for the analyzing of antioxidant activity of extract.

CHAPTER 4: RESULTS

4.1 Identification of Shoot Regeneration and Root Formation Media

The direct production of plant organs such as shoots or roots from an explant or from callus, is known as organogenesis and plant regeneration. The success of plant regeneration depends on the use of plant hormones, as they can produce new organs from the cultured explants by promoting the ability of plant cells to divide and differentiate.

The used explants in this part of study were obtained from one month aseptic seedlings initiating from seeds cultured on MS basal medium without any hormones.

This study depicts successful direct regeneration and callus formation of *Trifolium pratense* L. through tissue culture system. The nodal explants were shown to regenerate roots and shoots directly when they were cultured on MS medium supplemented with different concentrations of BAP, IBA and 2,4-D. Direct regeneration was successfully achieved when nodal explants managed to produce shoots and roots after few days of culture and complete plantlets were developed after 2 weeks of culture. Most of the combinations of BAP and IBA used in this study depicted this finding, as the production of shoots and roots were shown in most of these combinations (Table 4.1).

Table 4.1: The effects of different concentrations and combinations of BAP and IBA on the nodal explants of *Trifolium pratense* cultured on MS medium after 4 weeks. The cultures were maintained at 25 ± 1 ˚ C with 16 hours light and 8 hours dark.

Growth regulators (mg/l)		Observations	% explant producing shoots	No. of shoots per explant	% explant producing callus	No. of roots per explant
BAP	**IBA**					
0.5	**0.0**	Necrotic	_	_	_	_
	0.25	Callus	_	_	$26.14 \pm 2.25_{b}$	_
	0.5	Callus	_	_	$21.36 \pm 3.13_{b}$	_
	0.75	Roots	_	_	_	$2.5 \pm 0.14_{ab}$
1.0	**0.0**	Shoots	$62.44 \pm 3.17_{b}$	$3.1 \pm 0.26_{c}$	_	_
	0.25	Shoots	$69.33 \pm 5.57_{b}$	$4.3 \pm 0.21_{bc}$	_	_
	0.5	Shoots and Roots	$87.00 \pm 4.63_{ab}$	$4.3 \pm 0.68_{bc}$	_	$2.6 \pm 0.11_{ab}$
	0.75	Shoots and roots	$90.00 \pm 3.33_{a}$	$4.13 \pm 0.27_{bc}$	_	$2.2 \pm 0.24_{b}$
1.5	**0.0**	Shoots	$91.5 \pm 5.57_{a}$	$3.70 \pm 0.19_{c}$	_	_
	0.25	Shoots	$90.00 \pm 4.72_{a}$	$4.10 \pm 0.14_{bc}$	_	_
	0.5	Shoots and roots	$100.00 \pm 0.0_{a}$	$6.05 \pm 0.28_{a}$	_	$3.1 \pm 0.17_{ab}$
	0.75	Shoots and roots	$100.00 \pm 0.0_{a}$	$4.65 \pm 0.17_{b}$	_	$3.3 \pm 0.21_{a}$
2.0	**0.0**	Necrotic	_	_	_	_
	0.25	Callus	_	_	$64.48 \pm 3.32_{ab}$	_
	0.5	Callus	_	_	$79.60 \pm 3.28_{a}$	_
	0.75	Callus	_	_	$75.67 \pm 4.53_{a}$	_

For each treatment, the means within the column followed by different superscript letters were significantly different at $p < 0.05$.

Generally, the response of the explants begun as early as 6 days when cultured on different culture media. As shown in Table 4.1, the combination of 1.5 mg/l BAP and 0.5 mg/l IBA yielded the highest mean number of shoots per explant (6.05 ± 0.28), whereby 100% of the explants produced shoots. This was observed that among the two different combinations of hormones (BAP + IBA or BAP + 2,4-D), the more efficient direct regeneration was successfully achieved through the use of BAP and IBA.

In the combination of BAP and 2,4-D, the highest mean number of shoots (3.05 ± 0.33) was found in the media containing 1.5 mg/l BAP and 0.25 mg/l 2,4-D, whereby 77.28 ± 4.66 % of the explants produced shoots (Table 4.2).

Table 4.2: The effects of different concentrations and combinations of BAP and 2,4-D on the nodal explants of *Trifolium pratense* cultured on MS medium after 4 weeks. The cultures were maintained at 25 ± 1 ˚ C with 16 hours light and 8 hours dark.

Growth regulators (mg/l)		Observations	% explant producing shoots	No. of shoots per explant	% explant producing callus	No. of roots per explant
BAP	2,4-D					
0.5	0.0	Necrotic	_	_	_	_
	0.25	Callus	_	_	$63.11 \pm 5.44_{c}$	_
	0.5	Callus	_	_	$72.12 \pm 3.13_{c}$	_
	0.75	Callus	_	_	$69.80 \pm 4.33_{c}$	_
1.0	0.0	Shoots	$62.44 \pm 3.17_{b}$	$3.1 \pm 0.26_{bc}$	_	_
	0.25	Shoots	$71.56 \pm 4.31_{b}$	$2.74 \pm 0.19_{c}$	_	_
	0.5	Callus	_	_	$85.43 \pm 4.6_{bc}$	_
	0.75	Callus	_	_	$85.18 \pm 3.21_{bc}$	_
1.5	0.0	Shoots	$91.5 \pm 5.57_{a}$	$3.70 \pm 0.19_{a}$	_	_
	0.25	Shoots	$77.28 \pm 4.66_{b}$	$3.05 \pm 0.33_{bc}$	_	_
	0.5	Callus	_	_	$100.00 \pm 0.0_{a}$	_
	0.75	Callus	_	_	$90.22 \pm 5.33_{b}$	_
2.0	0.0	Necrotic	_	_	_	_
	0.25	Callus	_	_	$90.51 \pm 4.72_{b}$	_
	0.5	Callus	_	_	$88.64 \pm 5.37_{b}$	_
	0.75	Necrotic	_	_	_	_

For each treatment, the means within the column followed by different superscript letters were significantly different at $p < 0.05$.

Production of multiple shoots from nodal explants of *Trifolium pratense* are shown in Figure 4.1 and 4.2, while regeneration of whole plantlet from nodal explant is shown in Figure 4.3.

Figure 4.1: Development of multiple shoots from nodal explants of *T. pratense* cultured on MS medium supplemented with 1.5 mg/l BAP and 0.25 mg/l 2,4-D.

Figure 4.2: Development of multiple shoots from nodal explants of *T. pratense* cultured on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA

Figure 4.3: Complete plantlets regeneration from nodal explants of *T. pratense* cultured on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA

The results also indicated that most of the media containing BAP (1 mg/l – 1.5 mg/l) and IBA (0.5 mg/l – 0.75 mg/l) hormones were able to induce rooting. The highest mean number of roots per explant (3.3 ± 0.21), were found in the media containing 1.5 mg/l BAP and 0.75 mg/l IBA (Table 4.1), while the lowest root formation were observed when the nodal explants were cultured in the media supplemented with 1.0 mg/l BAP and 0.75 mg/l IBA. As shown in Table 4.2, the explants cultured in the media supplemented with the combination of BAP and 2,4-D failed to form any roots.

Green coloured callus was obtained when the nodal explants were cultured in the MS medium supplemented with different concentrations of hormones in this research (Figure 4.4). However, the combinations of BAP and 2,4-D were found to be more effective on callus formation of nodal explants of red clover (Table 4.2). The highest percentage of callus formation (100 %) was found in the media supplemented with 1.5 mg/l BAP and 0.5 mg/l 2,4-D.

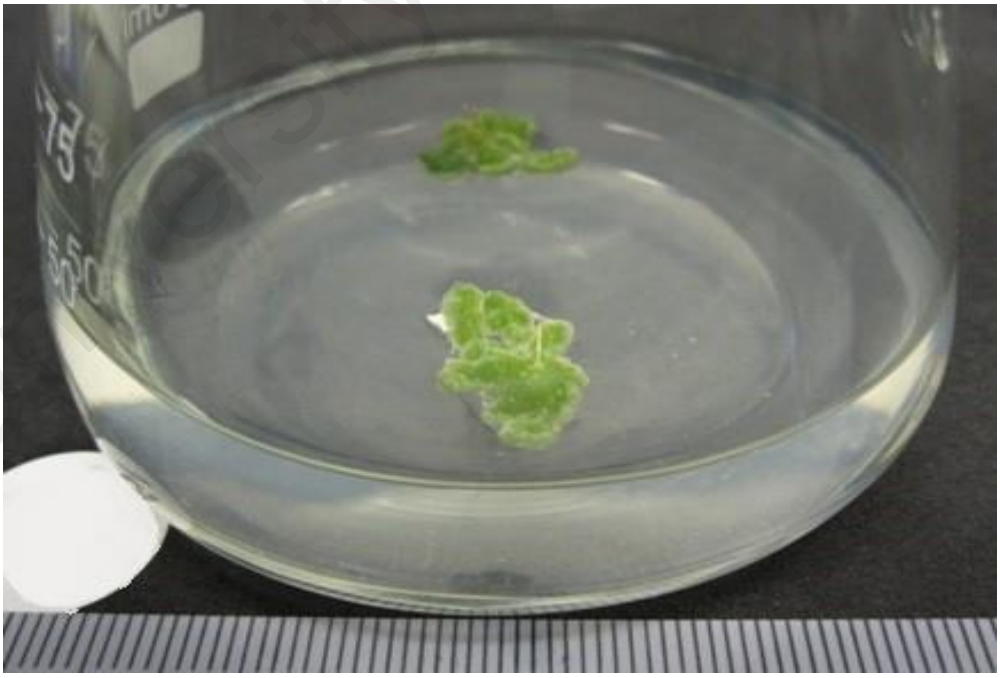

Figure 4.4: Callus derived from nodal explants of *T. pratense*, cultured on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l 2,4-D.

4.2 Acclimatization of Plantlets

The plantlets obtained from *in vitro* direct regeneration were cultured in three different types of soil (black soil, red soil and combination of black and red soil 1:1 ratio) and their growth was monitored in comparison to the plantlets derived from aseptic seedlings.

As shown in Table 4.3, the survival rates of the acclimatized plantlets depend to the type of soil used. The highest survival rate (93.71 ± 4.64) was observed in the acclimatized plantlets grown on the combination of black and red soil (1:1 ratio). Figures 4.5 and 4.6 show the acclimatization of *T. pratense* plantlets in culture room.

Table 4.3: Survival rates of the acclimatized plantlets grown on different types of soil.

Type of the soil	Survival rate %
Black soil	$80.46 \pm 5.32_b$
Red soil	$67.14 \pm 4.33_c$
Black soil + Red soil (1:1)	$93.71 \pm 4.64_a$

Mean values with different letters within a column are significantly different at $p < 0.05$

Figure 4.5: Two-month-old *T. pratense* L. plantlet after two weeks of acclimatization in culture room

Figure 4.6: Three-month-old *T. pratense* L. plantlet after six weeks of acclimatization in culture room

4.3 Standard Growth of Primary Roots of *T. pratense*

Results of primary root growth under two different growth conditions (on cotton and on MS media) were evaluated. The growth of primary roots of *Trifolium pratense* was quite slow, as it took 10 days for the secondary roots to start appearing for *in vivo* (on cotton) cultured plants and 12 days for the *in vitro* cultured plants.

The primary roots started to grow on second day of culture and the mean root length increased rapidly until 5 days after culture but after day 6 the increase of root length was found very slowly until the emergence of secondary roots on day 10.

The mean root length of the samples versus time was imported into spreadsheet and the data was plotted in a graph. The graph depicting the linear regression line showing the relationship between mean root lengths versus time as shown in Figures 4.7 and 4.8

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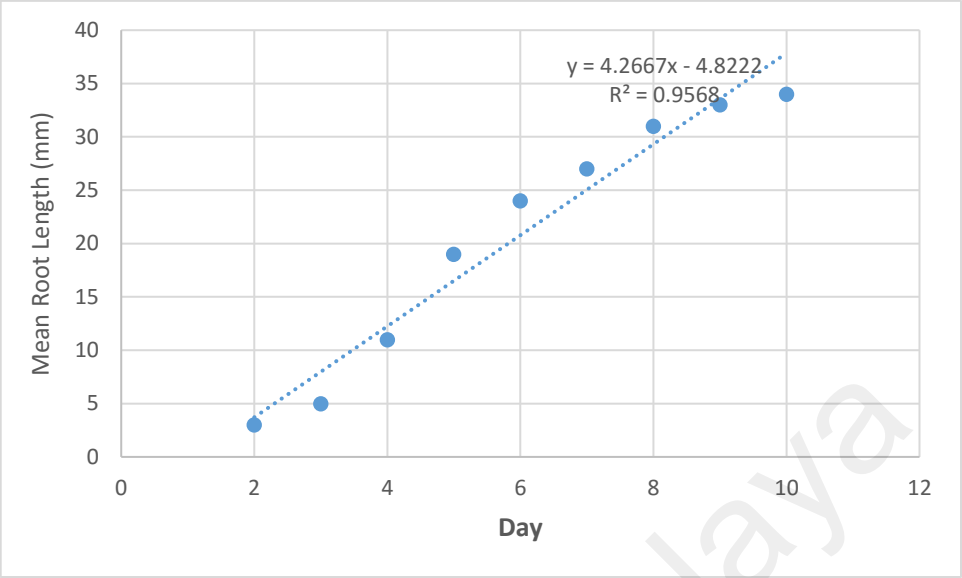

Figure 4.7: The growth of primary roots of *T. pratense*, germinated on moist cotton wool.

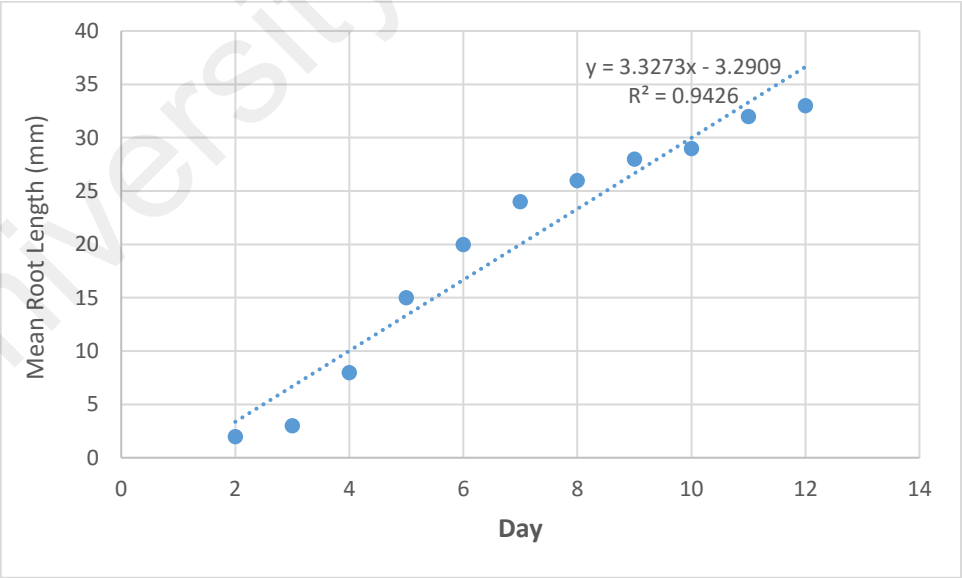

Figure 4.8: The growth of primary roots of *T. pratense* germinated on MS media.

As shown in Figures 4.5 and 4.6, the relationship between mean primary root length (mm) and time (day) showed a linear regression, with the equation below:

In cotton west: $Y = 4.2667X - 4.8222$ $R^2 = 0.9568$

In MS medium: $Y = 3.3273X - 3.2909$ $R^2 = 0.9426$

The high amount of R^2 for root length for germinated seeds in MS media and cotton west show that the data are closed to the fitted regression line.

4.4 Cytological Studies

The cellular behavior studies of some legume plants are difficult for observation and analysis because of their small chromosomes. In this thesis, the carnoy's solution (24h) with hot hydrolysis (30 min) and Feulgen's reagent was chosen for carrying out the cytological studies of *Trifolium pratense*. In order to study cellular activities of *in vivo* and *in vitro* plantlets of *T. pratense,* parameters such as chromosome count, mitotic index (MI), cell and nuclear areas were investigated.

Using Axioskop Zeiss microscope equipped with AxioCam MRc video camera, all four stages of mitosis (prophase, metaphase, anaphase and telophase) were observed from the samples and the images of the mitosis phases were successfully captured (Figure 4.9).

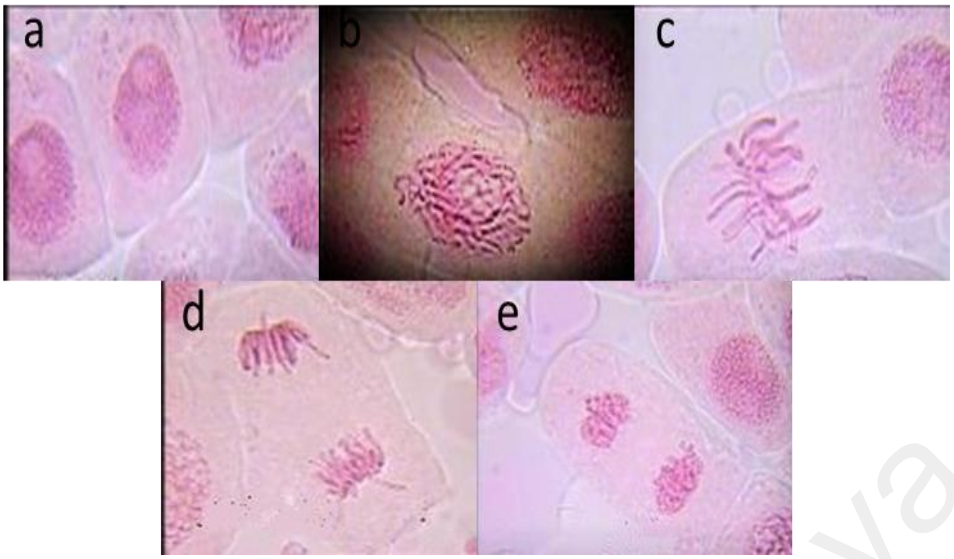

Figure 4.9: Stages of mitosis observed from squashed preparation of *in vivo T. pratense* root tip meristem cells (a: interphase, b: prophase, c: metaphase, d: anaphase and e: telophase)

4.4.1 Mitotic Index (MI)

In order to calculate the mitotic index, at least 1000 cells were observed in a series of random transect across the permanent slides. The percentage of cells undergoing mitosis among all observed cells including interphase cells was calculated. The comparison of the mitotic index of root meristem cells from *in vivo* and *in vitro* grown *T. pratense* are summarized in Table 4.4.

Table 4.4: Distribution of mitotic cells in root meristem of *in vivo* and *in vitro* grown *T. pratense* (Mean ± SD)

Growth Condition	Interphase %	Prophase %	Metaphase %	Anaphase %	Telophase %	Mitotic Index %
In Vivo	65.18 ± 2.48	24.21 ± 0.87	8.30 ± 0.33	0.72 ± 0.11	1.28 ± 0.17	35.12 ± 1.89 $_a$
In Vitro	59.55 ± 2.78	27.56 ± 1.33	10.23 ± 0.64	0.94 ± 0.17	1.72 ± 0.22	41.89 ± 1.34 $_a$

Mean values with different letters whitin a column are significantly different at $p < 0.05$

As shown in Table 4.4 the Mitotic Index of root meristem cells of *in vitro* grown *T. pratense* was not significantly different with the *in vivo* grown *T. pratense*. *In vivo* grown plants showed lower amount of Mitotic Index in comparison with the *in vitro* grown plants. So, it can be said that the addition of plant hormones may influenced the Mitotic Index of *T. pratense*, although this increase is not significant.

4.4.2 Chromosome counts

Three slides of 15 metaphase plates were used for the determination of mean number of chromosomes. The mean number of chromosomes of *in vivo* and *in vitro* grown *T. pratense* were shown in Table 4.5.

Table 4.5: Mean number of chromosomes of *in vivo* and *in vitro* grown *T. pratense* (mean ± SD).

Growth condition	Mean number of chromosomes
In vivo	14 ± 0.29
In vitro	14 ± 0.15

As observed from Table 4.5 the number of chromosomes of *in vivo* and *in vitro* grown *T. pratense* remained similar. So, it can be suggested that the tissue culture conditions had no influence on chromosome number in the present research.

4.4.3 Mean cell and nuclear areas

The mean cell and nuclear areas and also the ratio of nuclear areas to cell areas were calculated from 3 replicates of 150 prophase cells (Table 4.6).

Table 4.6: The mean cell and nuclear areas and their ratios in root meristem cells of *in vivo* and *in vitro* grown *T. pratense.*

Growth condition	Mean area (μm^2)		
	Nuclear (N)	Cell (C)	Ratio (N/C)
In vivo	34571 ± 68.49	168682 ± 121.34	0.20 ± 0.03 $_b$
In vitro	17251 ± 85.12	58902 ± 101.46	0.29 ± 0.02 $_a$

Mean values with different letters whitin a column are significantly different at $p < 0.05$

From Table 4.6 it was observed that the ratio of nuclear area to the cell area for the *in vitro* grown *T. pratense* is significantly higher than that of the *in vivo* grown *T. pratense.*

4.5 Total Phenolic, Total Flavonoids and Extraction Yield

The percentage yields of the methanol extract and different fractions of *in vivo* and *in vitro* grown plants as well as the callus tissue of red clover are shown in Table 4.7. The extraction yield of these samples was in a range of 2.35 ± 0.5% to 13.89 ± 0.54%. The highest extraction yield (13.89 ± 0.54 %) was obtained from the methanol extract of *in vivo* grown plants, while the lowest extraction yield (2.35 ± 0.5 %) was from the ethyl acetate fraction of the *in vitro* grown samples.

The total phenolic contents of the extracts, expressed as gallic acid equivalents, varied from 46.88 ± 1.07 mg GAE/g for the methanol extract of *in vivo* grown plants to 16.9 ± 1.19 mg GAE/g for the ethyl acetate extract of *in vitro* grown plants (Table 4.7). Results also showed that there was scaling increase in total phenolic content among the tested samples. In comparison, the total phenolic content of the *in vivo* grown red clover extract was found to be significantly higher than that of *in vitro* grown plants and callus tissue.

The content of total flavonoids expressed as mg of rutin equivalents per gram of dry sample, that ranged from 6.11 ± 0.79 to 26.61 ± 0.92, amounts of which were comparable with results verified in the literatures for other extracts produced (Ao et al., 2008; Manian et al., 2008). The methanol extract of *in vivo* grown red clover significantly contained ($p < 0.05$) higher flavonoids concentration as compared to other samples involved. The lowest flavonoid value in this study was recorded in ethyl acetate fraction of *in vitro* grown plants (6.11 ± 0.79 mg CTE / g dry sample).

Table 4.7: Total phenolic, total flavonoid and extraction yield of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants (aerial parts) and also callus tissue of *Trifolium pratense*

Plant extract	Total Phenolic (mg Gallic acid / g dry sample)	Total flavonoid (mg rutin equivalent / g dry sample)	Extraction Yield (%)
In Vivo			
Methanol extract	46.88 ± 1.07 ^{a}	26.61 ± 0.92 ^{a}	13.89 ± 0.54 ^{a}
n-Hexane fraction	30.52 ± 0.72 ^{cd}	16.06 ± 1.58 ^{d}	10.79 ± 0.59 ^{c}
Ethyl acetate fraction	27.57 ± 0.49 ^{e}	11.71 ± 1.43 ^{fg}	6.39 ± 1.12 ^{e}
Chloroform fraction	40.17 ± 0.88 ^{b}	19.56 ± 1.11 ^{b}	12.23 ± 0.54 ^{b}
In Vitro			
Methanol extract	31.94 ± 1.68 ^{c}	13.03 ± 0.79 ^{ef}	10.84 ± 0.72 ^{c}
n-Hexane fraction	20.06 ± 0.41 ^{g}	8.23 ± 0.96 ^{h}	5.20 ± 0.54 ^{f}
Ethyl acetate fraction	16.90 ± 1.19 ^{h}	6.11 ± 0.79 ^{i}	2.35 ± 0.5 ^{g}
Chloroform fraction	25.43 ± 0.87 ^{f}	10.39 ± 0.79 ^{g}	7.18 ± 0.82 ^{e}
Callus			
Methanol extract	40.82 ± 1.5 ^{b}	17.84 ± 0.53 ^{c}	11.36 ± 0.55 ^{bc}
n-Hexane fraction	31.77 ± 1.14 ^{c}	12.70 ± 0.94 ^{ef}	6.13 ± 0.34 ^{ef}
Ethyl acetate fraction	19.23 ± 0.66 ^{g}	7.21 ± 0.74 ^{hi}	2.98 ± 0.66 ^{g}
Chloroform fraction	29.25 ±1.62 ^{de}	14.02 ± 1.08 ^{e}	8.70 ± 0.52 ^{d}

Each value is represented as mean ± SD (n = 3).
Means in the same column not sharing the same letter are significantly different at (Duncan) P < 0.05.

4.6 Antioxidant Activity

4.6.1 DPPH radical scavenging activity

The antioxidant activity of samples was assessed in terms of radical scavenging activity which is related to the inhibition in the initiation step of free radical processes. In this manner, the efficient concentration (EC_{50}) was calculated as the concentration of the sample necessary to cause 50% inhibition, which was obtained by interpolation from linear regression analysis. So the lower EC_{50} shows the higher DPPH radical activity.

Figure 4.10 highlights the DPPH radical scavenging ability of *in vivo*, *in vitro* and callus samples of red clover with different extraction solvents. The chloroform fraction of *in vivo* grown plants showed the lowest reading of EC_{50} of DPPH radical scavenging (81.04 ± 2.33 µg/ml), while the highest EC_{50} belonged to the ethyl acetate fraction of *in vitro* grown plants (>>300 µg/ml) (Table 4.8). There is significant difference between the DPPH activity of *in vivo*, *in vitro* and callus tissues of red clover.

Table 4.8: DPPH radical scavenging activity of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants and callus tissues of *T. pratense* L.

Plant Extract	EC_{50} of DPPH radical scavenging activity (µg/ml)		
	In Vivo	*In Vitro*	Callus
Methanol extract	94.25 ± 1.15 $_{c}$ *	205.47 ± 2.90 $_{b}$ ***	128.42 ± 2.40 $_{c}$ **
n-Hexane fraction	131.42 ± 3.20 $_{d}$ **	291.95 ± 2.98 $_{c}$ ***	120.74 ± 3.45 $_{b}$ *
Ethyl acetate fraction	> 300 $_{e}$ *	>> 300 $_{d}$ ***	> 300 $_{e}$ **
Chloroform fraction	81.04 ± 2.33 $_{b}$ *	208.86 ± 2.20 $_{b}$ ***	155.17 ± 2.80 $_{d}$ **
Ascorbic acid	21.17 ± 0.76 $_{a}$	21.17 ± 0.76 $_{a}$	21.17 ± 0.76 $_{a}$

Each value in the table is represented as mean ± SD (n = 3).
Means not sharing the same symbols (, **, and ***) are significantly different (Duncan) at P < 0.05 in the same row*
Means not sharing the same letters are significantly different (Duncan) at P < 0.05 in the same column.

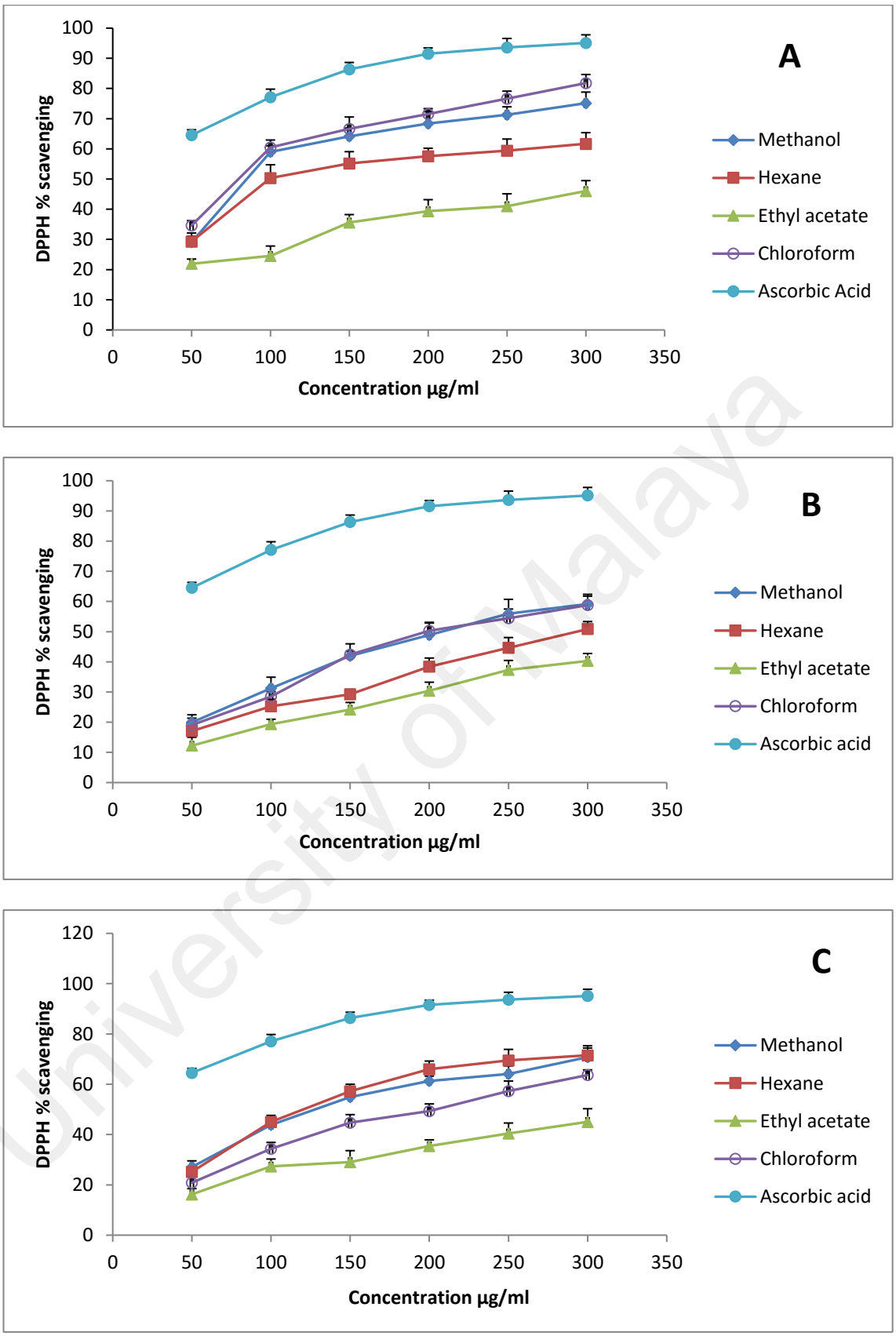

Figure 4.10: DPPH radical scavenging activity of different extracts from the methanol extract of *Trifolium pratense* by different solvent at different concentration. A: *In vivo* grown plants, B: *In vitro* grown plants, C: Callus tissue. Each value represents as mean ± SD (n = 3).

4.6.2 Superoxide Radical Scavenging Activity

The superoxide radical scavenging effect of these varying fractions of methanol extract of *in vivo* and *in vitro* grown as well as the callus tissue of red clover was drawn in comparison with the same doses of ascorbic acid in a range of 50 to 300 µg/ml as shown in Figure 4.11. The results had suggested that the lowest EC_{50} value (70.69 ± 1.76 µg/ml) belongs to the chloroform fraction of *in vivo* samples, whereas the highest (260.01 ± 1.46 µg/ml) belongs to the ethyl acetate fraction of *in vitro* samples (Table 4.9).

Table 4.9: Superoxide anion activity of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plant and callus tissue of *T. pratense* L.

Plant Extract	EC_{50} of superoxide anion activity (µg/ml)		
	In Vivo	In Vitro	Callus
Methanol extract	74.68 ± 1.59 $_{c}$ *	179.77 ± 1.60 $_{c}$ **	74.36 ± 1.42 $_{b}$ *
n-Hexane fraction	139.83 ± 2.40 $_{d}$ *	246.59 ± 2.09 $_{d}$ ***	190.06 ± 2.56 $_{d}$ **
Ethyl acetate fraction	201.55 ± 1.94 $_{e}$ *	260.01 ± 1.46 $_{e}$ ***	228.41 ± 1.52 $_{e}$ **
Chloroform fraction	70.69 ± 1.76 $_{b}$ *	155.91 ± 1.73 $_{b}$ ***	104.32 ± 1.45 $_{c}$ **
Ascorbic acid	23.44 ± 0.84 $_{a}$	23.44 ± 0.84 $_{a}$	23.44 ± 0.84 $_{a}$

Each value in the table is represented as mean ± SD (n = 3).
Means which are not sharing the same symbols (, **, and ***) are significantly different (Duncan) at P < 0.05 in the same row.*
Means which are not sharing the same letters are significantly different (Duncan) at P < 0.05 in the same column.

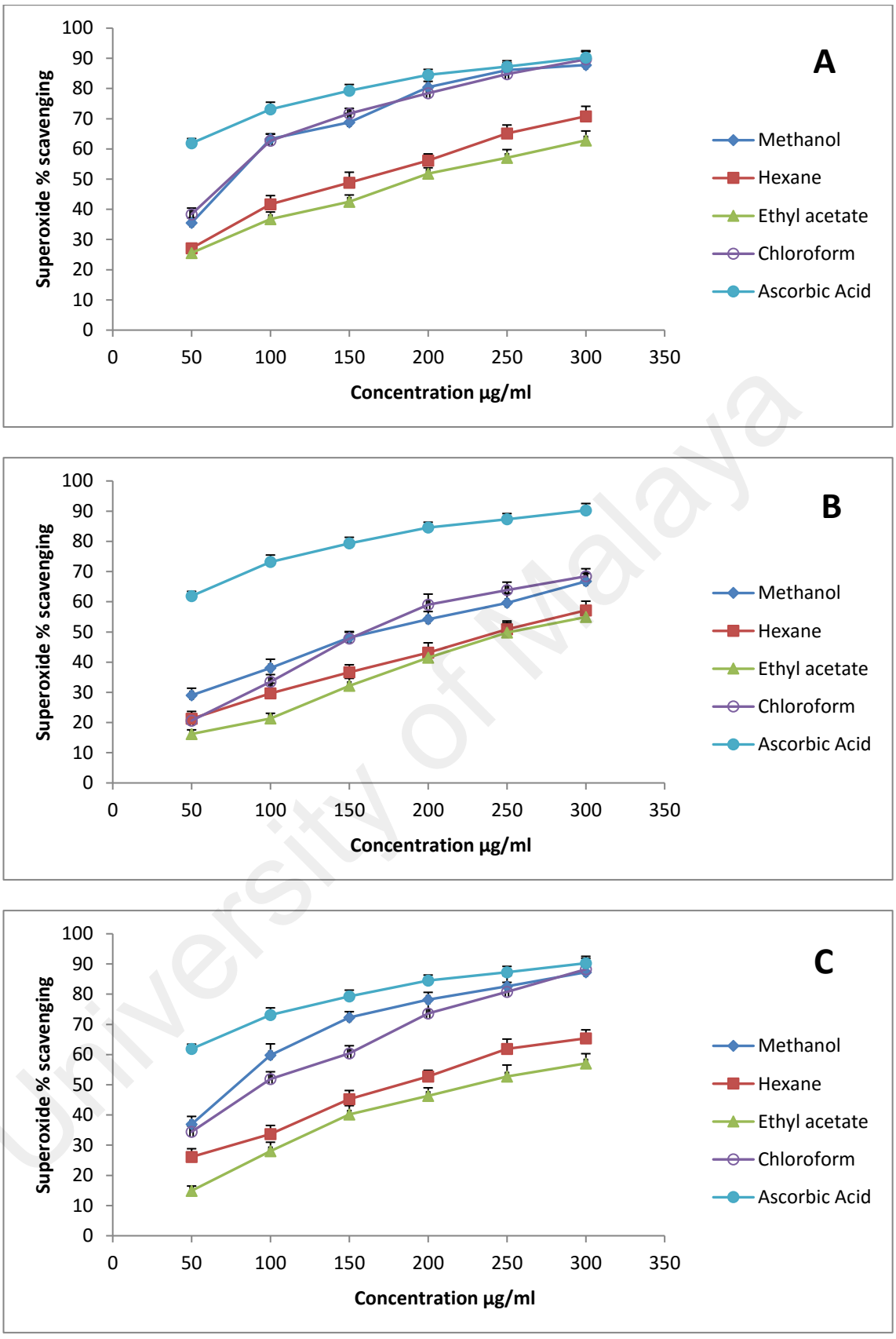

Figure 4.11: Superoxide radical scavenging activity of different extracts from the methanol extract of *T. pratense* L. by different solvent at different concentration. A: *In vivo* grown plants, B: *In vitro* grown plants, C: Callus tissue. Each value represents as mean ± SD (n = 3).

4.6.3 ABTS radical scavenging activity

The samples' ABTS radical scavenging capability can be ranked as such: *in vivo* > callus > *in vitro* samples. The methanol extract of *in vivo* grown samples had demonstrated the highest radical scavenging activity when it reacted with the ABTS radicals. By contrast, the ethyl acetate fraction of *in vivo*, *in vitro* and callus samples and also the n-hexane fraction of *in vitro* samples did not illustrate any leveling effect at the highest concentration, but it was a fact that their radical scavenging effects were much less ($p < 0.05$) than that of the other extracts tested (Table 4.10). Significant differences among the EC_{50} values of all the fractions and ascorbic acid had also been noted ($p < 0.05$).

Table 4.10: ABTS radical scavenging of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plant and callus tissue of *T. pratense* L.

Plant Extract	EC_{50} of ABTS (µg/ml)		
	In Vivo	*In Vitro*	Callus
Methanol extract	111.84 ± 1.46 $_{b}$ *	351.46 ± 2.02 $_{c}$ ***	164.32 ± 1.61 $_{b}$ **
n-Hexane fraction	210.76 ± 1.71 $_{d}$ *	> 500 $_{d}$ ***	225.63 ± 2.05 $_{d}$ **
Ethyl acetate fraction	> 500 $_{e}$ *	>> 500 $_{e}$ ***	> 500 $_{e}$ **
Chloroform fraction	122.34 ± 2.29 $_{c}$ *	297.68 ± 1.68 $_{b}$ ***	172.18 ± 1.55 $_{c}$ **
Ascorbic acid	34.67 ± 0.53 $_{a}$	34.67 ± 0.53 $_{a}$	34.67 ± 0.53 $_{a}$

Each value in the table is represented as mean ± SD (n = 3).
Means not sharing the same symbols (, **, and ***) are significantly different (Duncan) at P < 0.05 in the same row.*
Means not sharing the same letters are significantly different (Duncan) at P < 0.05 in the same column.

4.6.4 Hydrogen peroxide

ExtractS from *in vivo*, *in vitro* and callus tissue of red clover were capable of scavenging hydrogen peroxide in a concentration dependent manner (50–300 µg/ml). As compared with the EC_{50} values, the hydrogen peroxide scavenging activity of chloroform fraction of *in vivo* grown red clover (88.35 ± 1.27 µg/ml) was more effective ($P < 0.05$) than that of other samples. The lowest hydrogen peroxide scavenging activities were found in the n-hexane and ethyl acetate fractions of *in vitro* grown plants with the EC_{50} values of more than 300 µg/ml (Table 4.11).

Table 4.11: Hydrogen peroxide scavenging of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plant and callus tissue of *Trifolium pratense*.

Plant Extract	EC_{50} of Hydrogen peroxide (µg/ml)		
	In Vivo	*In Vitro*	Callus
Methanol extract	103.44 ± 1.47 $_{c}$ *	219.86 ± 1.57 $_{b}$ ***	141.74 ± 1.93 $_{c}$ **
n-Hexane fraction	270.56 ± 1.72 $_{e}$ *	>> 300 $_{e}$ ***	287.63 ± 1.59 $_{e}$ **
Ethyl acetate fraction	138.16 ± 1.93 $_{d}$ **	>300 $_{d}$ ***	132.26 ± 2.03 $_{b}$ *
Chloroform fraction	88.35 ± 1.27 $_{b}$ *	231.49 ± 2.02 $_{c}$ ***	146.31 ± 1.67 $_{d}$ **
Ascorbic acid	26.84 ± 0.83 $_{a}$	26.84 ± 0.83 $_{a}$	26.84 ± 0.83 $_{a}$

Each value in the table is represented as mean ± SD (n = 3).
Means not sharing the same symbols (, **, and ***) are significantly different (Duncan) at P < 0.05 in the same row.*
Means not sharing the same letters are significantly different (Duncan) at P < 0.05 in the same column.

4.6.5 Chelating activity

The EC_{50} values of iron chelating activity for different extracts of *in vivo* and *in vitro* grown as well as callus tissues of red clover were shown in Table 4.12. The highest chelating power shown by the methanol extract of *in vivo* grown plants with the EC_{50} value of 49.11 ± 0.97 µg/ml, whilst, the lowest was found in ethyl acetate fraction of *in vitro* grown samples with the EC_{50} value of 183.44 ± 2.48 µg/ml.

Table 4.12: Chelating activity of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plant and callus tissue of *Trifolium pratense*.

Plant Extract	EC_{50} of Chelating power (µg/ml)		
	In Vivo	*In Vitro*	Callus
Methanol extract	49.11 ± 0.97 $_{b}$ *	142.87 ± 1.71 $_{c}$ ***	72.56 ± 1.16 $_{d}$ **
n-Hexane fraction	52.33 ± 1.97 $_{c}$ *	131.84 ± 2.05 $_{b}$ ***	67.52 ± 2.15 $_{c}$ **
Ethyl acetate fraction	105.67 ± 1.56 $_{e}$ *	183.44 ± 2.48 $_{e}$ ***	118.23 ± 2.02 $_{e}$ **
Chloroform fraction	86.32 ± 1.89 $_{d}$ **	161.75 ± 1.36 $_{d}$ ***	60.17 ± 1.42 $_{b}$ *
Catechin	22.76 ± 0.37 $_{a}$	22.76 ± 0.37 $_{a}$	22.76 ± 0.37 $_{a}$

Each value in the table is represented as mean ± SD (n = 3).
Means not sharing the same symbols (, **, and ***) are significantly different (Duncan) at P < 0.05 in the same row.*
Means not sharing the same letters are significantly different (Duncan) at P < 0.05 in the same column.

4.6.6 Reducing power activity

Table 4.13 shows the reducing power absorbance of different extracts of *in vivo* and *in vitro* grown plants as well as callus tissue of red clover. Among the examined extracts the methanol extract of *in vivo* samples showed the highest reducing power with the absorbance of 1.05 ± 0.07 at 700 nm, so this extract could act as electron donors and also could convert free radicals to more stable products. Although, in comparison with the

positive control (ascorbic acid) it is significantly lower. The lowest reducing power (0.38 ± 0.01) belongs to the ethyl acetate fraction of *in vitro* grown plants.

Table 4.13: Reducing power of methanol extract and soluble fraction (300 µg/ml) of *in vivo* and *in vitro* grown plant and callus tissue of *Trifolium pratense*.

Plant Extract	Reducing power absorbance at 700 nm		
	In Vivo	*In Vitro*	Callus
Methanol extract	1.05 ± 0.07 $_{b}$ *	0.72 ± 0.03 $_{b,}$ ***	0.82 ± 0.03 $_{c}$ **
n-Hexane fraction	0.88 ± 0.04 $_{c}$ *	0.52 ± 0.04 $_{c,}$ ***	0.65 ± 0.02 $_{d}$ **
Ethyl acetate fraction	0.59 ± 0.03 $_{d}$ *	0.38 ± 0.01 $_{d}$ ***	0.52 ± 0.03 $_{e}$ **
Chloroform fraction	0.95 ± 0.03 $_{c}$ *	0.71 ± 0.04 $_{b}$ **	0.91 ± 0.03 $_{b}$ *
Ascorbic acid	2.45 ± 0.03 $_{a}$	2.45 ± 0.03 $_{a}$	2.45 ± 0.03 $_{a}$

Each value in the table is represented as mean ± SD (n = 3).
Means not sharing the same symbols (, **, and ***) are significantly different (Duncan) at P < 0.05 in the same row.*
Means not sharing the same letters are significantly different (Duncan) at P < 0.05 in the same column.

4.7 Relationship between Antioxidant Activities and Total Phenolic and Flavonoid Contents

Through the correlation analysis for phytochemical contents with EC_{50} values of radical scavenging activity and antioxidant ability of the extract of red clover and its various soluble fractions, the phenolic and flavonoid contents had exhibited excellent association with DPPH, superoxide, ABTS radical scavenging activities and reducing power of *in vivo*, *in vitro* and callus samples (Tables 4.14, 4.15 and 4.16).

Table 4.14: Correlation between the antioxidant activity and total phenolic and flavonoid of the extract of *in vivo* grown *Trifolium pratense*

Assays	Correlation R^2	
	Total flavonoid	**Total phenolic**
DPPH radical scavenging activity	0.759**	0.745**
Superoxide radical scavenging activity	0.856**	0.903**
ABTS radical scavenging ability	0.814**	0.801**
Hydrogen peroxide radical scavenging activity	0.414	0.598*
Chelating power	0.670*	0.500
Reducing power	0.884**	0.861**

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.15: Correlation between the antioxidant activity and total phenolic and flavonoid of the extract of *in vitro* grown *Trifolium pratense*

Assays	Correlation R^2	
	Total flavonoid	**Total phenolic**
DPPH radical scavenging activity	0.893**	0.876**
Superoxide radical scavenging activity	0.793**	0.806**
ABTS radical scavenging ability	0.825**	0.827**
Hydrogen peroxide radical scavenging activity	0.784**	0.833**
Chelating power	0.502	0.441
Reducing power	0.862**	0.862**

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4.16: Correlation between the antioxidant activity and total phenolic and flavonoid of the extract of callus tissue of *Trifolium pratense*

Assays	Correlation R^2	
	Total flavonoid	Total phenolic
DPPH radical scavenging activity	0.849**	0.822**
Superoxide radical scavenging activity	0.912**	0.917**
ABTS radical scavenging ability	0.903**	0.876**
Hydrogen peroxide radical scavenging activity	0.023	0.020
Chelating power	0.775**	0.734**
Reducing power	0.777**	0.753**

**. Correlation is significant at the 0.01 level (2-tailed).

4.8 Anticancer Activity

In the present study, the cytotoxic effects of different extracts of *in vivo* and *in vitro* grown plants as well as callus tissue of *Trifolium pratense* on two human cancer cell lines (MCF-7 and HCT-116) were evaluated using the MTT assay. Cell lines were incubated with different concentrations of sample extracts. After 24, 48 and 72 hours, the cell inhibition was determined and IC_{50} was calculated for all the plant extracts (Tables 4.17 and 4.18).

Treatment of MCF-7 and HCT-116 cell lines with different extracts of red clover resulted in a time dependent increase in the anticancer activity and the greatest anticancer activity was observed when the cells were exposed to the plant extract for 72 h.

All the examined extracts inhibited growth of MCF-7 and HCT-116 cell lines in a concentration dependent manner. This is displayed in Figure 4.12 for the two most active extracts: chloroform fraction of *in vivo* plants and callus tissues.

The chloroform fraction of *in vivo* plants showed a reading of the lowest IC_{50} (66.44 ± 2.05 µg/ml) of inhibition against the MCF-7 cell line, although it is not significantly different with the IC_{50} value of chloroform fraction of callus tissues (69.48 ± 2.66 µg/ml). The lowest IC_{50} (79.53 ± 2.00 µg/ml) of inhibition against the HCT-116 cell line was shown by the chloroform fraction of callus tissue. Although the cytotoxic activity of the fractions were less ($p < 0.05$) than those of the doxorubicin (positive control), the study had revealed that *in vivo* grown plants and callus tissues of red clover have inhibition activity against MCF-7 and HCT-116 cell lines, more than *in vitro* grown red clover.

Table 4.17: Cytotoxic activity of the different extracts of *in vivo*, *in vitro* and callus tissues of *T. pratense* L. against human breast carcinoma (MCF-7). (n = 3)

Extract	IC_{50} µg/ml		
	24 hours	48 hours	72 hours
In Vivo			
Methanol extract	104.25 ± 2.17 ^{c}	86.97 ± 2.05 ^{d}	83.62 ± 1.58 ^{c}
n-Hexane fraction	147.89 ± 1.86 ^{f}	126.12 ± 2.22 ^{h}	117.57 ± 2.42 ^{g}
Ethyl acetate fraction	207.24 ± 2.73 ^{h}	176.45 ± 1.92 ^{l}	169.96 ± 2.55 ^{k}
Chloroform fraction	93.9 ± 2.32 ^{b}	75.26 ± 1.68 ^{b}	66.44 ± 2.05 ^{b}
In Vitro			
Methanol extract	131.01 ± 1.39 ^{e}	115.85 ± 1.87 ^{g}	110.62 ± 2.49 ^{f}
n-Hexane fraction	222.29 ± 5.13 ^{j}	163.98 ± 2.49 ^{k}	155.50 ± 0.76 ^{j}
Ethyl acetate fraction	250.42 ± 4.2 ^{k}	181.5 ± 1.11^{m}	178.89 ± 2.03 ^{l}
Chloroform fraction	144.68 ± 1.87 ^{f}	110.72 ± 2.33 ^{f}	101.11 ± 2.26 ^{e}
Callus			
Methanol extract	118.11 ± 2.18 ^{d}	97.47 ± 1.53 ^{e}	88.73 ± 1.85 ^{d}
n-Hexane fraction	188.64 ± 1.87 ^{g}	145.3 ± 2.22 ^{i}	131.30 ± 2.06 ^{i}
Ethyl acetate fraction	213.47 ± 2.19 ^{i}	158.54 ± 2.36 ^{j}	124.64 ± 1.73 ^{h}
Chloroform fraction	100.71 ±1.8 ^{b}	79.35 ± 1.88 ^{c}	69.48 ± 2.66 ^{b}
Doxorubicin*	22.49 ± 1.33 ^{a}	19.25 ± 0.87 ^{a}	17.54 ± 0.69 ^{a}

IC_{50} value represents the concentration of the tested samples to kill 50% of the cancer cells.
Each value in the table is represented as mean ± SD (n = 3).
*Positive control
For each treatment, the means within the column followed by different superscript letters were significantly different at $p < 0.05$.

Table 4.18: Cytotoxic activity of the different extracts of *in vivo*, *in vitro* and callus tissues of *T. pratense* L. against human colon carcinoma (HCT-116). (n = 3)

Extract	IC_{50} µg/ml		
	24 hours	48 hours	72 hours
In Vivo			
Methanol extract	142.68 ± 1.56 ^{d}	115.99 ± 1.47 ^{e}	100.75 ± 2.33 ^{e}
n-Hexane fraction	200.82 ± 3.26 ^{g}	166.13 ± 1.83 ^{i}	138.60 ± 1.71 ^{h}
Ethyl acetate fraction	262.02 ± 2.22 ^{j}	215.40 ± 2.49 ^{l}	180.28 ± 2.54 ^{k}
Chloroform fraction	118.64 ± 1.63 ^{c}	96.95 ± 1.53 ^{c}	87.80 ± 2.34 ^{c}
In Vitro			
Methanol extract	185.43 ± 2.84 ^{f}	144.58 ± 2.15 ^{g}	128.67 ± 2.42 ^{g}
n-Hexane fraction	293.44 ± 4.60 ^{l}	214.82 ± 2.01 ^{l}	185.53 ± 2.48 ^{l}
Ethyl acetate fraction	276.16 ± 2.26 ^{k}	185.00 ± 2.26 ^{j}	161.55 ± 2.92 ^{j}
Chloroform fraction	155.59 ± 1.93 ^{e}	126.76 ± 1.47 ^{f}	113.24 ± 1.90 ^{f}
Callus			
Methanol extract	145.05 ± 2.24 ^{d}	107.96 ± 2.05 ^{d}	96.02 ± 2.7 ^{d}
n-Hexane fraction	217.10 ± 2.29 ^{h}	148.78 ± 2.37 ^{h}	127.73 ± 2.26 ^{g}
Ethyl acetate fraction	254.57 ± 2.73 ^{i}	192.84 ± 2.77 ^{k}	157.36 ± 2.43 ^{i}
Chloroform fraction	109.54 ± 2.44 ^{b}	85.55 ± 2.95 ^{b}	79.53 ± 2.00 ^{b}
Doxorubicin *	31.24 ± 1.65 ^{a}	26.32 ± 0.55 ^{a}	25.18 ± 1.31 ^{a}

IC_{50} value represents the concentration of the tested samples to kill 50% of the cancer cells.
Each value in the table is represented as mean ± SD (n = 3).
*Positive control
For each treatment, the means within the column followed by different superscript letters were significantly different at $p < 0.05$.

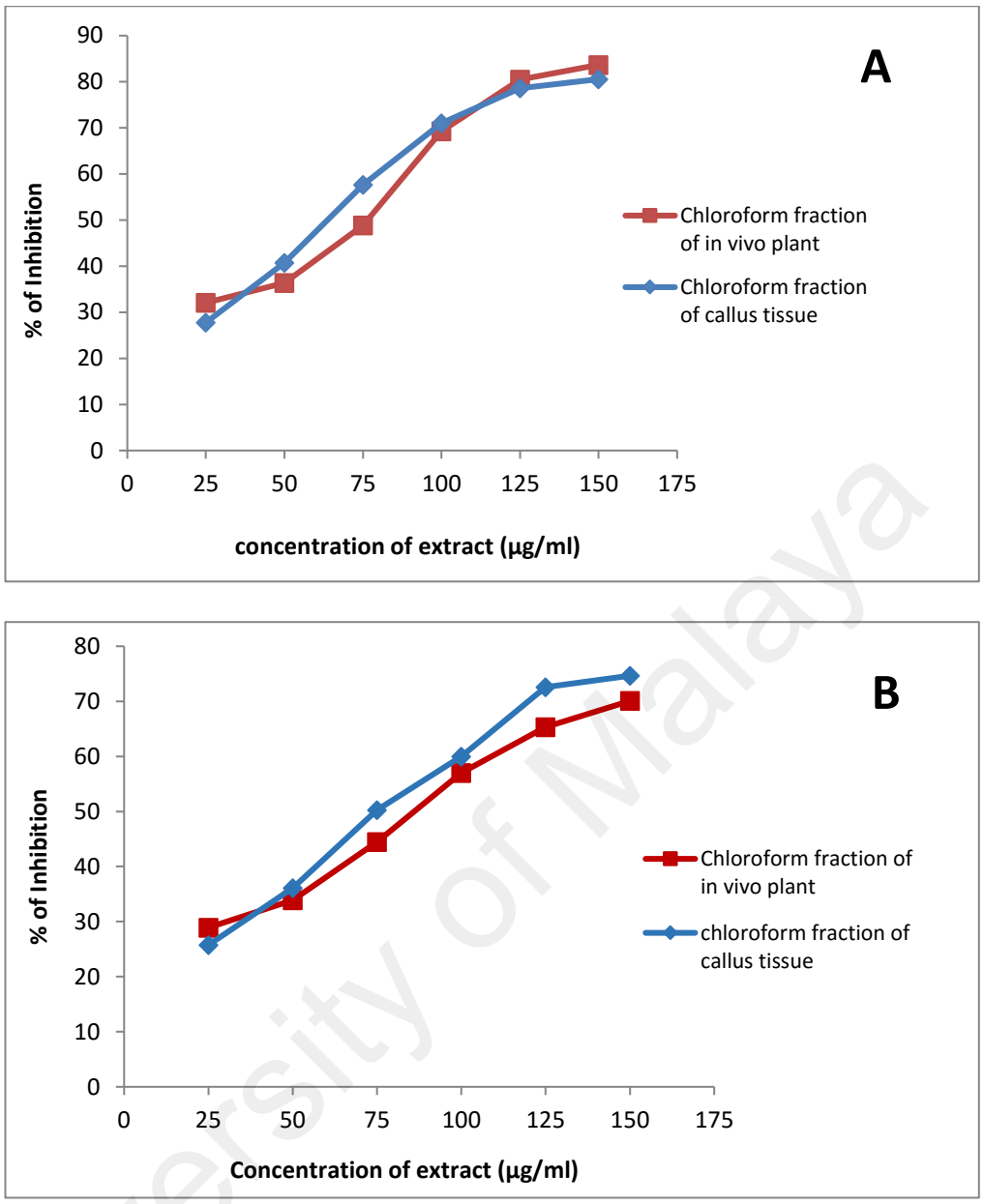

Figure 4.12: Cytotoxic activity of chloroform fraction of *in vivo* grown plants and callus tissues of *T. pratense* L. against (A): human breast cancer cell line (MCF-7) and (B): human colon cancer cell line (HCT-116) after 72 hours.

4.9 Antimicrobial Activity

In the present investigation, the inhibitory effect of ethanolic and methanolic extracts of *in vivo, in vitro* and callus from *Trifolium pratense* were evaluated against both fungicidal and bacterial strains. The antibacterial and antifungal activities were determined using paper disc diffusion method and summarized in Tables 4.19 and 4.20.

The antimicrobial potential of the experimental plants were evaluated according to their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with the activity of the standards. The results revealed that all callus, *in vivo* and *in vitro* grown plants extracts are potent antimicrobial against three of the examined bacteria strains (*Staphylococcus aureus, Bacillus cereus* and *Escherichia coli*) and two of the examined fungal strains (*Aspergillus niger* and *Candida albicans*). The callus extract showed antimicrobial activities against all the examined microorganisms. Among the two different solvents used for extraction, methanol extract showed higher degree of inhibition followed by ethanol extract. For all the investigated microorganisms, the callus extract showed maximum antibacterial and antifungal activities. In ethanolic extract, maximum inhibition zone diameter among all the tested microorganisms was obtained in *E. coli* with diameter of 16 ± 1.76 mm. Similarly, methanol extract showed maximum inhibition zone with diameter of 16 ± 1.66 mm in *B. cereus* and 16 ± 1.44 mm in *C. albicans*.

Table 4.19: Antimicrobial activity (inhibition zone) of ethanolic extract of callus, *in vitro* and *in vivo* grown *Trifolium pratense*

Sample	Inhibition Zone (mm)						
	Bacteria strains				Fungal strains		
	E.c	***P.a***	***S.a***	***B.c***	***A.n***	***C.a***	***F.v***
In Vivo	10 ± 1.3	-	8 ± 0.76	11 ± 1.57	7 ± 0.66	9 ± 1.57	-
In Vitro	11 ± 1.63	-	7 ± 1.41	14 ± 0.88	6 ± 1.4	10 ± 1.63	-
Callus	16 ± 1.76	-	11 ± 1.22	15 ± 1.54	10 ± 1.33	13 ± 0.57	4 ± 0.36
Tetracycline	24 ± 1.6	11 ± 1.33	20 ± 0.57	21 ± 0.88	NT*	NT	NT
Nystatin	NT	NT	NT	NT	20 ± 1.5	27 ± 2.6	16 ± 1.57

E.c (Escherichia coli); P.a(Pseudomonas aeruginosa); S.a (Staphylococcus aureus); B.c(Bacillus cereus); A.n(Aspergillus niger); C.a(Candida albicans); F.v(Fusarium verticillioides)
*NT (not tested)

Table 4.20: Antimicrobial activity (inhibition zone) of methanolic extract of callus, *in vitro* and *in vivo* grown *Trifolium pratense*

Sample	Inhibition Zone (mm)						
	Bacteria strains				Fungal strains		
	E.c	***P.a***	***S.a***	***B.c***	***A.n***	***C.a***	***F.v***
In Vivo	11 ± 1.57	-	6 ± 1.41	13 ± 1.3	8 ± 1.33	7 ± 1.88	-
In Vitro	13 ± 1.88	-	9 ± 0.57	13 ± 1.57	9 ± 0.88	12 ± 0.33	-
Callus	15 ± 0.66	7 ± 0.88	14 ± 1.33	16 ± 1.66	13 ± 0.66	16 ± 1.44	7 ± 1.33
Tetracycline	24 ± 1.6	11 ± 1.33	20 ± 0.57	21 ± 0.88	NT*	NT	NT
Nystatin	NT	NT	NT	NT	20 ± 1.5	27 ± 2.6	16 ± 1.57

E.c (Escherichia coli); P.a (Pseudomonas aeruginosa); S.a (Staphylococcus aureus); B.c(Bacillus cereus); A.n (Aspergillus niger); C.a(Candida albicans); F.v (Fusarium verticillioides)
*NT (not tested)

4.10 Photosynthesis and Leaf Gas Exchange of *T. pratense* L.

The measurements of stomatal conductance (g_s), transpiration rate (E) and net photosynthetic (P_n) were done using the LICOR 6400 portable photosynthesis at the standard cuvette conditions (i.e PPFD1000 µmol/m2/sec, carbon dioxide reference 400 µmol/mol, relative humidity 50 – 60 %) and leaf temperature standardized at 30 °C. All measurements were obtained 6 and 12 weeks after planting from fully expanded leaves. Results obtained showed that, significant differences were found among the samples in correlation with the leaf gas exchange in which the highest P_n (15.51 ± 0.34) were identified in 6-weeks old *in vivo* grown plants, while, the highest g_s (164.49 ± 4.95) and E (6.35 ± 0.14) were identified in 6-weeks old *in vitro* grown plants (Figure 4.13). The mean analysis results of dual correlation of traits are shown in Table 4.21. The net photosynthesis was found to be positively correlated with secondary metabolites (total phenolic, TP; total flavonoids, TF) at the significant level of 0.01.

In the natural environment, photon flux density (PPFD) of leaves varied greatly over time (Knapp & Smith 1990). Therefore, photosynthetic utilization of fluctuating PFD is important in estimating leaf photosynthetic capacity and water-use efficiency (Tang 1997). The maximum net photosynthetic (P_{Nmax}), apparent quantum yield (AQY), compensation point (CP) and respiration (R_d) were calculated from the light response curve shown in Figure 4.14 (regression were used to fit the data). Significant differences found in the accessions of the initial gradient in the light response curve, the light compensation point, the rate of dark respiration and the maximum rate of the photosynthetic, achieved from fitting the light response curves.

The light response curves attributes were determined for both *in vivo* and *in vitro* samples. Saturation PPFD of *in vitro* grown plants was 720 µmol/m^2/s, while in *in vivo* grown plants was 960 µmol/m^2/s. A comparison was made for the observed data for the

light-saturated photosynthetic among the treatments revealed significantly greater rates for P_{Nmax} (18.36 µmol/m^2/s) and AQY (0.0389 µmol/m2/s) in *in vivo* plants (Figure 4.15). Moreover, *in vitro* plants were observed to contain the highest rate of R_d (0.828 µmol/m2/s) and CP (29.48 µmol/m^2/s). The form of light–response curves had a logarithmic part with the maximum gradient (maximum quantum efficiency) at the start of curves, i.e., when the irradiation was reaching zero. The ‘‘S’’-shaped curves, which signify quantum efficiency under low light intensities tends to zero, were identified in a limited number of investigations.

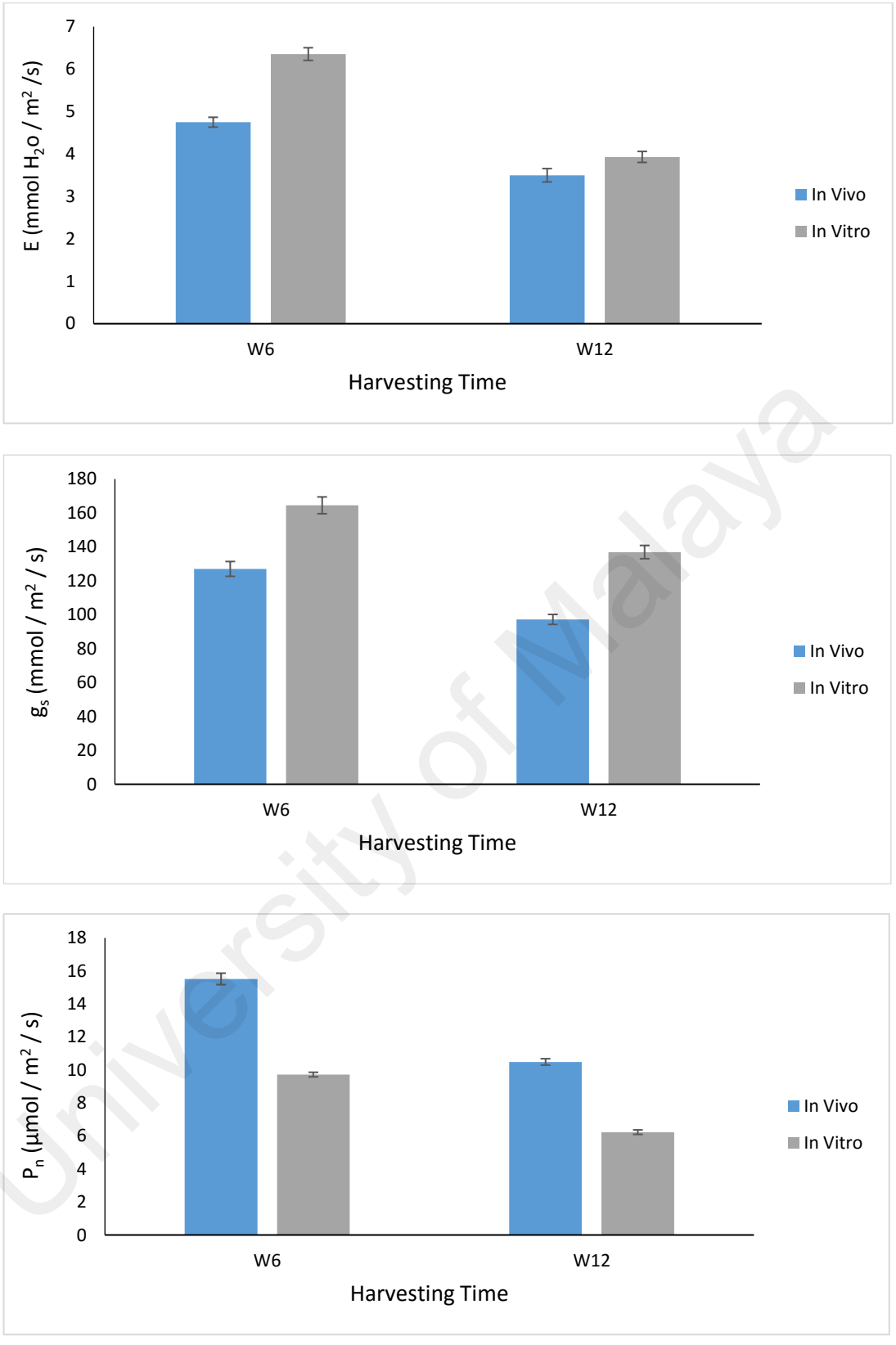

Figure 4.13: Net photosynthesis (P_n), Stomatal conductance (g_s) and Transpiration rate (E) of *in vivo* and *in vitro* grown *Trifolium pratense*, six and twelve weeks after planting. Each experiment repeated 5 times and data presented as the mean ± standard error.

Table 4.21: Correlation analysis between secondary metabolites and leaf gas exchange parameters of *T. pratense* in mean traits of *in vivo* and *in vitro*

	P_N	g_s	E	TF
TP	0.994**	-0.326	0.059	0.994**
TF	0.975*	-0.429	-0.041	
E	0.154	0.872		
g_s	-0.222			

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

TP = Total phenolic, TF = Total flavonoid, E = Transpiration rate, g_s = Stomatal conductance

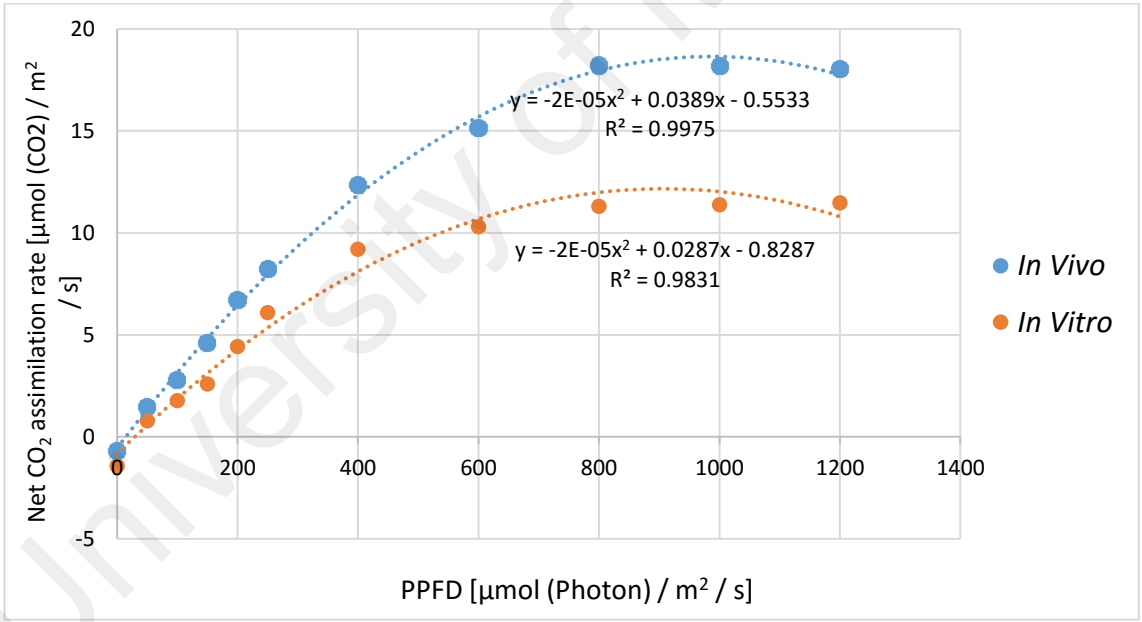

Figure 4.14: Light response curve in leaves from *in vivo* and *in vitro* grown plants of *T. pratense*. (n = 5, at each PPFD)

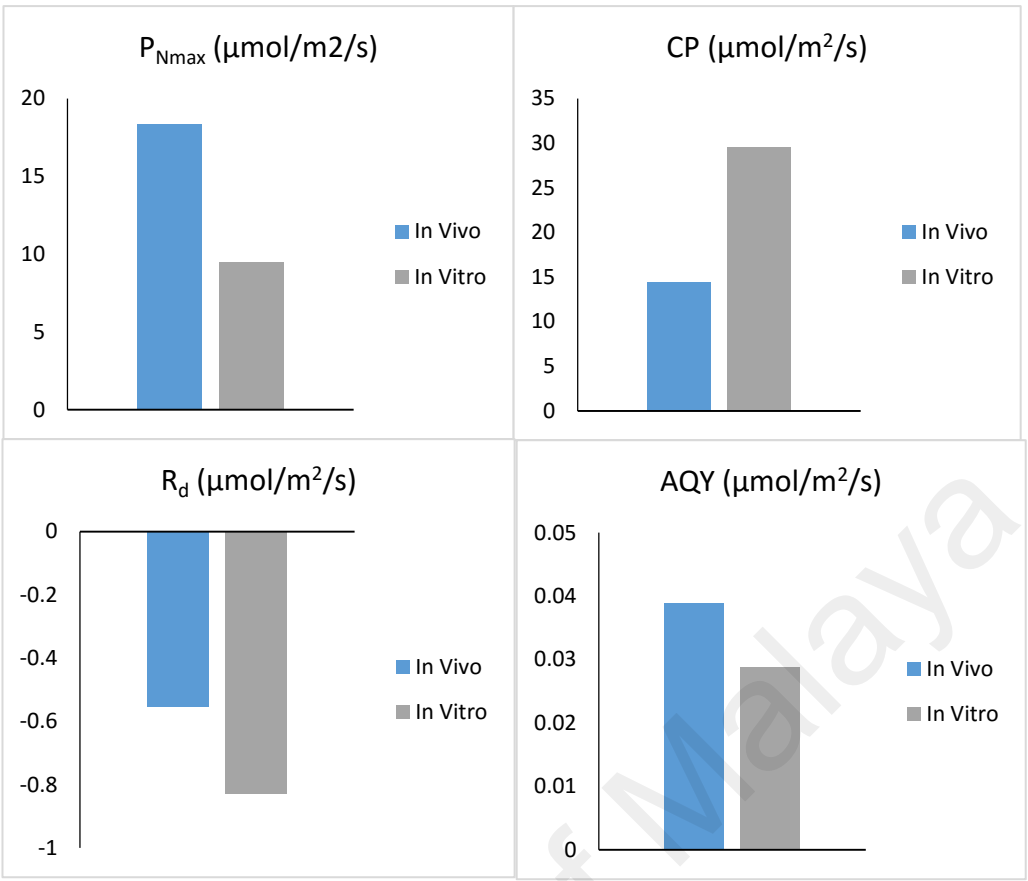

Figure 4.15: Maximum net assimilation rate (P_{Nmax}), apparent quantum yield (AQY), compensation point (CP) and respiration rate (R_d) in *in vivo* and *in vitro* grown *Trifolium pratense*, six weeks after planting

4.11 Effect of Salt Stress on Germination and Growth of *T. pratense*

The seed germination and growth of *T. pratense* was not significantly affected by the 50 mM NaCl treatment, although they were inhibited with the increase of NaCl concentration to 100, 150 and 200 mM. It was found that in the presence of 50 mM NaCl in MS medium the seed germination was a bit higher while the shoot and root length were slightly lower in the same condition. The germination percentage in red clover seeds treated with 100, 150 and 200 mM of NaCl were reduced by 46%, 22.4% and 10.6%, respectively (Figure 4.16).

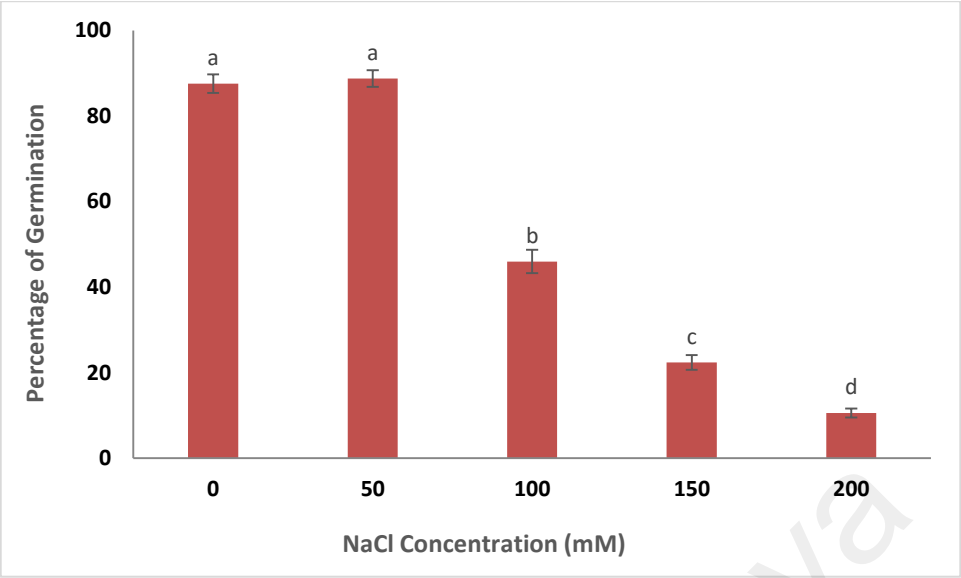

Figure 4.16: Germination percentage of Red clover seeds under different saline treatment. Each treatment repeated 5 times and data presented as the mean ± standard error. Values which are not sharing a common letter are significantly different at $p \leq 0.05$

At the high concentration of NaCl (100, 150 and 200 mM), the length of shoots and roots were inhibited significantly, although to different extent. The most severe growth restriction of shoots and roots (8.97% and 8.76% of the control, respectively) were found in the treatments with 200 mM of NaCl (Figures 4.17 and 4.18).

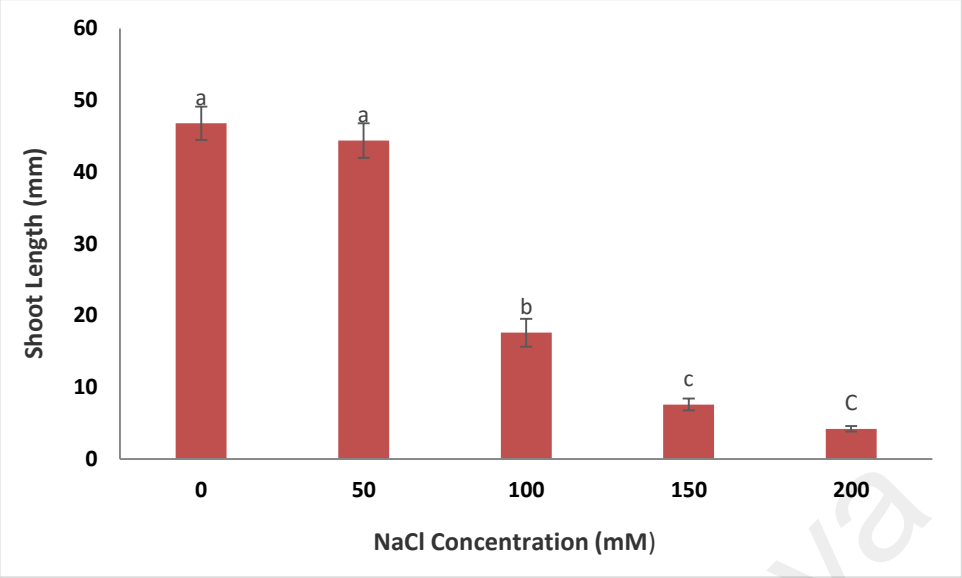

Figure 4.17: Shoot length of germinated *T. pratense* seeds under different saline treatments after 6 weeks of culture. Each data is the mean of 5 replicates of 25 explants per treatment (mean ± standard error). Values which are not sharing a common letter are significantly different at $p \leq 0.05$.

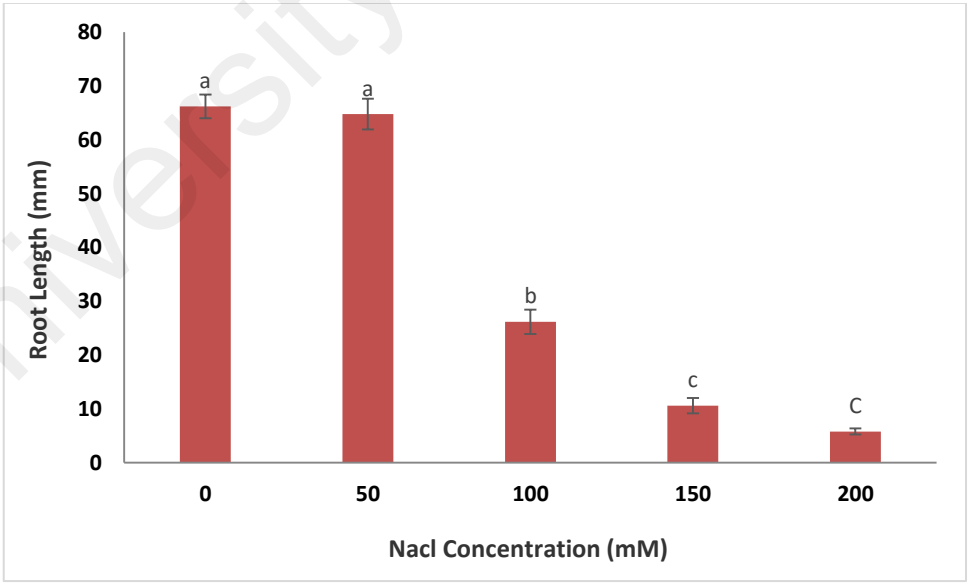

Figure 4.18: Root length of germinated *T. pratense* seeds under different saline treatments after 6 weeks of culture. Each data is the mean of 5 replicates of 25 explants per treatment (mean ± standard error). Values which are not sharing a common letter are significantly different at $p \leq 0.05$.

4.12 Effect of Salt Stress on Antioxidant Capacity Assay

Evaluation of antioxidant activity was done by examining the effect of extract samples on superoxide dismutase (SOD) and DPPH radical scavenging activities in all treatments. Treatments with 50 mM of NaCl showed slightly higher scavenging activity of DPPH with IC_{50} value of 59.58 ± 1.54 that was reduced when compared with the IC_{50} value of control (65.45 ± 1.42), while the scavenging activity was strongly diminished at 100, 150 and 200 mM of NaCl (IC_{50} values augmented to 1.77-, 2.64- and 3.32-fold the control respectively) (Table 4.22).

The activity of superoxide dismutase was inhibited with salt stress in all examined concentrations of NaCl except treatment with 50 mM NaCl. The concentration of extracts required for 50% scavenging superoxide dismutase at the presence of 100, 150 and 200 mM of NaCl were strongly increased (130.28 ± 7.38, 226.46 ± 6.54 and 324.04 ± 5.73 µg/ml, respectively) (Table 4.22).

Table 4.22: Antioxidant activity (IC_{50} values) of *in vitro* grown *T. pratense* under different concentrations of saline treatment after 6 weeks of culture. Values (means of 5 replicates ± SD) of each parameter followed by same letter are not significantly different at $p \leq 0.05$.

NaCl concentration (mM)	DPPH (IC_{50} µg/ml)	SOD (IC_{50} µg/ml)
0	65.45 ± 1.42 [a]	104.1 ± 8.48 [b]
50	59.58 ± 1.54 [a]	79.66 ± 5.36 [a]
100	116.38 ± 3.1 [b]	130.28 ± 7.38 [b]
150	173.44 ± 3.53 [c]	226.46 ± 6.54 [c]
200	217.48 ± 3.38 [d]	324.04 ± 5.73 [d]

CHAPTER 5: DISCUSSION

Trifolium pratense is one of the most important forage crop from temperate regions. It is highly productive, with nitrogen-fixing ability, protein- and mineral-rich species adapted to a wide range of soils and environmental conditions. Its erect growth habit makes it suitable for hay and silage making. High nutritive value and voluntary intake characteristics lead to good animal performance. It is a valuable break crop in arable farming and organic farming on account of N- fixation ability. Its high nutritive value allied to its high intake characteristics combine to give improved individual animal performance from different classes of animals compared with grass forage. As a human food the young leaves are harvested before the plants come into the flower, and are used in salads, soups and etc. It is safe and effective herb with a long history of medicinal usages.

The potential of tissue culture techniques for the production of several secondary metabolites has been known for many years. Evidently tissue cultures stimulate the production or induce the biosynthesis of novel compounds not found in the mature plant. The development of callus culture could provide an alternative supply of phytochemicals such as novel phytoestrogens to be used in medicine. Phytoestrogens in tissue cultures may prove to be medically beneficial for diseases such as cancers, cardiovascular diseases, and osteoporosis (Vanishes et al., 2004).

Tissue culture is a technique to enhance the application of biotechnological approaches to crop plants. Micropropagation and callus initiation of *T. pratense* were studied on Ms medium supplemented with concentrations of hormones (auxin and cytokinin). The tissue culture techniques established in this study can be utilized to achieve mass propagation of *Trifolium pratense*. The present study is divided into several parts that investigate the *in vitro* micropropagation, induction of callus, acclimatization of *in vitro* regenerated

plantlets, comparison of cellular behavior, biological activities and photosynthetic of *in vivo* and *in vitro* grown *Trifolium pratense*. The effects of different concentrations of salt on *in vitro* grown of this plant was also studied to find out the differences between growth and antioxidant of the plants grown under salt stress and grown under normal condition.

For micropropagation of *T. pratense,* various hormones (BAP, IBA and 2,4-D) of different concentrations and combinations were tested to find the most suitable hormone to achieve optimum regeneration *in vitro*. All experiments conducted in this part used MS (Murashige & Skoog, 1962) media. To date, the most widely used culture media is the MS media, as it is suitable for tissue culture of almost all plant species. MS media contains a wide range of important mineral salts, vitamins and a carbon source (for example: sucrose) and is distinctive from the rest of the other culture media as it also contains a high amount of potassium, nitrate and ammonium, comparative to the other commercial nutrient media.

Production of shoots and roots are very essential to ensure the success of plant regeneration. The use of auxins in tissue culture can promote root formation, while the use of cytokinins can promote shoot production. In this study, combinations of different cytokinin and auxin hormones, as well as individual cytokinin were tested. The hormones were added to the media and their effects on the explants were observed.

In this study direct regeneration of shoots or adventitious roots was observed when the nodal explants were cultured in MS medium supplemented with different hormones. This was observed when BAP alone or in combination with IBA or 2,4-D were used (Tables 4.1 and 4.2). However different results were obtained. Nodal explants managed to produce shoots after a few days of culture and managed to produce complete plants after 3 weeks of culture.

The optimum shoot production was observed when the nodal explants were cultured on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA, with 6.05 ± 0.28 shoots per explant (Table 4.1). The production of whole plant was observed when the MS media supplemented with the combination of BAP and IBA.

As demonstrated in Table 4.2, it was shown that the nodal explants responded readily to most of the combinations of BAP and 2,4-D to produce callus. Although in the presence of lower concentration of 2,4-D shoot formation was found in some of the treatments. Formation of roots was not observed in any of the treatments with combination of BAP and 2,4-D. These observation indicate that although the use of 2,4-D in combination with BAP did promote shoot production, it is not a very efficient hormone in regeneration this plant species directly, but is a very good hormone for callus induction.

Campbell and Tomes (1984) reported that for the multiplication of red clover the cytokinin BAP was most effective for shoot multiplication at 2.0 mg/l with maximum shoot production by four weeks. However, in the present study it was found that the maximum shoot formation of red clover occured in the presence of a lower concentration of BAP (1.5 mg/l).

The study by Uranbey et al. (2005) on micropropagation of Persian clover (*Trifolium resupinatum* L.) also is in agreement with the finding of this thesis that the best shoot multiplication capacity was obtained from cotyledonary node explants on MS medium containing 7.1 µM BAP and 1 µM IBA.

Cheyne and Dale (1980) tested different media either Blaydes (Blaydes, 1966) or Gamborg B5 basal media (Gamborg et al., 1976) with different hormones (IAA and BAP) for micropropagation of *Trifolium repens* and *Trifolium pratense*. They found a good plantlets regeneration for both the *T. repens* (63-82 %) and *T. pratense* (53-76 %) in the

Gamborg B5 medium. Dale and Cheyne (1993) also reported that B5 medium, as the best media for micropropagation of *T. pratense.*

Induction and growth of callus tissues depends on medium composition and concentrations of hormones (Brisson et al., 1988). The conventional approach to establishing or improving callus cultures has been to test a range of media and explants (Jauhar, 2006). Modifications involve qualitative and quantitative change in micronutrient elements, sources of carbon and growth regulators responsible for particular hormonal balance in the growth system.

Theoretically, any parts of the plants of any species can be cultured to induce the production of callus, via manipulation of plant hormones. However, the success of callus production largely depends on the plant species itself, whether they are dicotyledons or monocotyledons, the type of explants and the use of plant hormones.

The hormone 2,4-D had been shown to be very effective in callus induction (Kolganova et al., 1992). Gu et al. (2006), established a protocol for callus induction of *Trifolium pratense* using hypocotyl and cytoledon as explants. Their results showed that the optimum callus induction was observed in the MS basal medium supplemented with 2 mg/l 2,4-D and 0.5 mg/l BAP. It is similar with the finding of this thesis, that the presence of 2,4-D and BAP were needed for higher percentage of callus induction of *T. pratense*. Callus obtained from this species were soft, friable and greenish-white. Earlier studies by Katarina et al., 1998 indicated that callus cultures differ in anatomy, cell size and friability and growth potential with every species. The development of callus culture could provide an alternative source and supply of phytochemicals such as novel phytoestrogens to be used in medicine.

The resulting *in vitro Trifolium pratense* plantlets were then subsequently acclimatized to ensure further growth and development when they are fully transferred to the natural environment, as described in chapter 4. Plantlets through direct regeneration via micropropagation were acclimatized in three different types of soil (black soil, red soil and combination of black and red soil at the ratio of 1:1) and their survival rate were monitored (Table 4.3).

The highest survival rate of acclimatized plantlets was found when the plantlets cultured in the combination of black and red soil. As cultivation of *Trifolium pratense*, require well drainage sandy soil that is slightly acidic (Rice et al., 1977). This is an anticipated, as plantlets generated as a result of subsequent tissue culture experiments are generally smaller than the *in vivo* grown plants at the same age.

There are many researches that reported different growth potting mixes show different results for acclimatization of different plant species. For example Khan et al. (2007) reported that *in vitro* plantlets of *Saintpaulia ionantha*, yielded 95% survival rate when they acclimatized on 100% sand. While the survival rates of the *in vitro* grown plants of the same species acclimatized on 1:1 mixture of sand : farmyard manure and 1:1 mixture of sand : coconut husk were found 80% and 70%, respectively.

The cytological studies were carried out in order to compare the cellular behavior between *in vivo* and *in vitro* grown meristem cells of *T. pratense*, to detect any early occurrence of somaclonal variation at the cellular level. Cellular parameters such as chromosome counts, Mitotic Index (MI), cell and nuclear areas and also the ratios of nuclear area to the cell areas were investigated from meristem cells of *in vivo* and *in vitro* grown plants root.

For this purpose, initially the standard growth of primary roots of *in vivo* and *in vitro* grown *T. pratense* were determined in order to obtain mean samples of the population for the subsequent cytological studies. The secondary roots of *T. pratense* started to appear after 10 days for the *in vivo* grown plants and after 12 days for the *in vitro* grown plants. Therefore the root samples aged 11-days-old for *in vitro* grown plants and 9-days-old for the *in vivo* grown plants with the mean root length of 33 mm (based on the standard growth curve) were used for the preparation of permanent slides.

The Mitotic Index (MI) of *in vitro* regenerated *T. pratense* is not significantly different with the MI value of the *in vivo* grown plants, although the MI value of the *in vivo* grown plants was slightly lower than that of the *in vitro* regenerated plantlets (Table 4.4). The use of plant growth hormones had been reported to affect the Mitotic Indeces of different species. For example, Das et al. (1997) reported that the MI values of parenchyma cells of tobacco pith increased after 6 days of culture when supplemented with IAA and kinetin.

Also it was found that the chromosome number of *in vitro* and *in vivo* grown plants of *T. pratense* were similar ($2n = 14$) (Table 4.5), therefore the tissue culture condition and using of plant hormones did not have any influence on the chromosome count of *T. pratense* in this study.

The chromosome number for at least 184 species of *Trifolium* were observed that more than 80% of the examined species were found to have $2n = 16$ chromosome. Although, aneuploidy ($2n = 10$, 12 or 14) is known for 31 *Trifolium* species, that 11 of them were found to have both aneuploidy and diploid ($2n = 16$) counts (Ellison et al., 2006).

However, the present finding is in agreement with those of Yeh et al. (1986), Semerenko, (1989) and Wang and Holl, (1988), in which they also reported that $2n = 14$ for the chromosome number of *T. pratense*. Wang and Holl (1988) had revealed that that

the chromosome number of regenerated *T. pratense* from tissue culture studies was the same with the chromosome number of the *in vivo* grown plants which is in agreement with the finding of this thesis. Raha and Roy (2003) also reported similar observations, where by the same number of chromosomes (22) was observed in *in vitro* grown *Holarrhena antidysenterica* Wall. However, *in vitro* grown plants of *Populus sp*. have been reported to show a different chromosome number (Rani et al., 1995).

The mean cell and nuclear areas and also the ratio of nuclear areas to cell areas for root meristem cells of *in vivo* and *in vitro* grown *T. pratense* were also investigated. From the results, it was found that the mean cell and nuclear areas of *in vivo* grown samples were significantly higher than that of the *in vitro* grown samples (Table 4.6). These decrease in the mean cell and nuclear areas of *in vitro* samples could be due to the stress in tissue culture system and also could be due to the organogenesis.

The ratio of mean nuclear areas to the mean cell areas of *in vitro* grown samples were found to be higher than that of the *in vivo* grown plants.

According to previous studies, a wide range of polyphenolic compounds such as different flavonoids, clovamides and other phenolic compounds were found in the phytochemical profile of *Trifolium* plants (Oleszek et al., 2007; Sabudak & Guler, 2009), but the content of these substances are different in the individual species substantially. Most of the studies on the biological properties of clovers concerning red clover and is focused on its phytoestrogenic action, being a result of isoflavone content. However, it should be emphasized that the beneficial effects of red clover maybe dependent on the action of various biologically active substances occurring in this herb.

The antioxidant activity of some *Trifolium* species may be a result of the abundance of flavonoids and other phenolic compounds, present in clovers. A study by

Kolodziejczyk et al. (2011) on different concentration (12.5-100 µg/ml) of *Trifolium pallidum*, revealed antioxidant action of all examined concentrations against oxidative stress-induced damage, in comparison to the samples exposed to oxidative stress in the absence of the extract. Sabudak et al. (2009) have found the antioxidative activity of hexane extracts, obtained from *Trifolium balansae, Trifolium stellatum* L., *Trifolium nigrescens, Trifolium constantinopo* and *Trifolium resupinatum* L.

Contrary to the mentioned reports suggesting the high anti-oxidative activity of *Trifolium* plants, the ethnobotanical study of Barros et al. (2010) demonstrated relatively weak antioxidant properties of *Trifolium angustifolium* L., most likely being a result of a low content of phenolics and flavonoids.

Most of the previous studies on biological activities of red clover used only in nursery cultivated plants to prepare the extract. There is relatively little available information about the phenolic content and biological activity of *in vitro* grown and callus tissues of *T. pratense.*

Thus, in the present study, the total phenolic, total flavonoids and antioxidant activities of the extracts prepared from the aerial parts of *in vivo* and *in vitro* grown red clover as well as callus tissue of this plant were assessed. Different solvents for preparation of the extract and also different assay to assess the antioxidant activity were used.

Good extraction methods provd to be a crucial step for getting extracts with acceptable yields and strong antioxidant activity (Moure et al., 2001). According to the results obtained in this study methanol extraction of *in vivo* grown plants had illustrated the highest amount of extraction yield (13.89 ± 0.54 %), and conversely, the extraction yield of *in vitro* grown plants with ethyl acetate was prominently lower (2.35 ± 0.5 %) ($P < 0.05$) when compared to that of the other samples (Table 4.7).

As plant secondary metabolites, the phenolics or polyphenols are very important judging from the virtue of their antioxidant activities by chelating redox-active metalions, inactivating lipid free radical chains and avoiding the hydroperoxide conversions into reactive oxyradicals.

In all *in vivo* and *in vitro* plants and also callus tissue, the methanolic extract had illustrated the highest total content of phenolic, whereas the content obtained with ethyl acetate were much lower ($P < 0.05$) (Table 4.7), which is similar to the reports compiled by Sahreen et al. (2010) and Ao et al. (2008).

There are some studies that compared total phenolic and flavonoids between *in vivo* and *in vitro* grown plants, e.g. *Crataegus monogyna* (Rakotoarison et al., 1997), *Salvia officinalis* (Grzegorczyk et al., 2007), and *Ephedra* (Parsaeimehr et al., 2010). Most of these studies reported lower amounts of total phenolic in the extract of *in vitro* grown plants in comparison with the *in vivo* grown plants that is in agreement with the current study.

A possible justification would be due to the formation of complexes by a part of phenolic compounds with carbohydrates and proteins, which are more extractable in methanol than in other solvents that have been emphasized in this study. The results obtained do not deviate from those of Parsaeimehr et al. (2010) who also found that wild plant, compared to the callus tissue, showed higher extracting phenolic components with regard to some medicinal plants.

The rich-flavonoid plants could manifest themselves as good sources of antioxidants that would assist in the enhancement of the overall antioxidant capacity of an organism and protection against lipid peroxidation (Sharififar et al., 2009). The total flavonoid content results were entirely synchronous with those of the total phenolic. It was

successfully shown that samples with high level of phenolic content also contain flavonoids in great amount. The rich-flavonoid plants could be a good antioxidant source that would help increase the overall antioxidant capacity of an organism and guard it against lipid peroxidation (Sharififar et al., 2009).

Phenolics as valuable natural plant compounds have health promoting bioactive properties for human nutrition because their hydroxyl group has a significant radical scavenging ability. Many studies showed that the antioxidant activities of different plant species are closely related to the total phenolic content of the plants (Kroyer & Winkler 2001; Kroyer & Hegedus 2001; Rice-Evans & Miller 1996).

2,2-diphenyl-1-picrylhydrazyl radical is a stable organic free radical with an absorption band at 517 nm. It loses this absorption when it accepts an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow. It can incorporate many samples in a short time span and is vulnerable enough to distinguish active ingredients at low concentrations (Hseu et al., 2008). The differential scavenging activities of the extract against the DPPH system that has been observed could be explained by the presence of different compounds in the fractions.

Although the DPPH radical scavenging activities of the fractions were less ($P < 0.05$) than those of the ascorbic acid, the study had made a disclosure that *in vivo* grown plants and callus tissue of red clover have free radical scavengers or inhibitors, possibly acting as primary antioxidants more than *in vitro* grown *T. pratense* (Table 4.8). There was an observation on a similar trend in a study of the antioxidant activity of the *Artemisia judaica* L. extract (Liu et al., 2009).

As it is a reactive oxygen species, superoxide has some damaging properties that can be imposed to the cells and DNA and subsequently invites various diseases. Thus, a

proposal has been established to gauge the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. In a dose dependent manner, all of the fractions had scavenging activities on the superoxide radicals. Nonetheless, when compared to the ascorbic acid, the superoxide scavenging activities of the extracts were found to be significantly lower ($P < 0.05$) (Table 4.9). This could be due to the presence of flavonoids and other antioxidants in the extract.

The ABTS radical cation decolourisation test is another widely established approach adopted to evaluate antioxidant activity. Colour reduction shows the decrease of ABTS radical. In the ABTS radical scavenging assay, significant differences among the EC_{50} values of all the examined extracts were found ($P < 0.05$). Some of the extracts did not show any ABTS radical activity in the examined concentrations (Table 4.10). The different EC_{50} levels of samples obtained from ABTS assay may reflect a relative difference in the ability of antioxidant compounds in the extracts to reduce ABTS free radicals. ABTS radical scavenging is less susceptible to steric hindrance (Maisuthisakul et al., 2007; Delgado et al., 2005)

While hydrogen peroxide itself is not very reactive, it can sometimes be poisonous to cells, since it may trigger the rise of hydroxyl radicals inside the cell (Halliwell, 1991). Extracts from *in vivo* and *in vitro* grown plants as well as callus tissue of red clover had the capability to scavenge hydrogen peroxide in a concentration dependent manner (50–300 µg/ml). The hydrogen peroxide scavenging activities of *in vivo* grown samples was proven to be more effective ($P < 0.05$) than the *in vitro* grown and callus samples, as revealed by the comparison with the EC_{50} values (Table 4.11). The chloroform fraction and methanol extract were found to be more efficient ($P < 0.05$) than that of the n-hexane and ethyl acetate fractions. EC_{50} values of all the extracts, in scavenging abilities on

hydrogen peroxide were remarkably different ($P < 0.05$) from the EC_{50} value that had been obtained for ascorbic acid.

Ability to chelate or deactivate transition metals, which in turn, has the ability to catalyze hydroperoxide decomposition and Fenton-type reactions, is a vital mechanism of antioxidant activity. It was thus, considered of importance to screen the iron (II) chelating ability of extracts (Manian et al., 2008). All the fractions had ferrous ion chelating activity but they were remarkably low ($P < 0.05$) in comparison to catechin. The sequence for the chelating power was *in vivo* grown samples > callus tissue of samples > *in vitro* grown samples (Table 4.12). The ion chelating data with different measurements of concentrations (50–300 µg/ml) suggested that ferrous ion chelating effects of all the fractions of *in vivo* and *in vitro* grown as well as callus tissue of red clover would be rather advantageous to offer protection against oxidative damage.

The reducing power of the extract, which potentially serves as a significant reflection of the antioxidant activity, was ascertained using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the colour of the test solution, which was yellow, transforms to various hues of green and blue, based on the extent of the reducing power of the samples. The presence of the antioxidants in the samples leads to Fe^{3+}/ferricyanide complex reduced to the Fe^{2+} form and Fe^{2+} can be monitored through the measurement of the formation of Perl's Prussian blue at 700 nm (Qingming et al., 2010).

In the reducing power assay, the presence of reductants (antioxidants) in the fractions would bring about the reduction of Fe^{+3}/ferriccyanide complex to the ferrous form by giving away an electron. Increasing the absorbance at 700 nm implies an increase in its ability to reduce. The sequence for this reducing power was as follows: *in vivo* samples > callus tissue > *in vitro* samples of red clover (Table 4.13). Some degrees of electron-donating capacity were found in all examined extracts.

In the earlier researches on plant extracts phenolics were found as the main antioxidant components and antioxidant activity of the extract was directly proportional to the total phenolic content of the extract (Liu et al., 2009; Chew et al., 2009).

Erkan et al. (2008) reported a close correlation between radical scavenging activity and total phenolic content of extract from various natural sources. Moreover, for the *in vivo* samples in this study, EC_{50} of hydrogen peroxide presented an important correlation with phenolics while non-significant with flavonoids, whereas for the *in vitro* samples hydrogen peroxide demonstrated a remarkable correlation with both phenolics and flavonoids. Hydrogen peroxide of callus samples did not shed light on any correlation with phenolics and flavonoids. Chelating power of *in vivo* samples pointed to a significant correlation with flavonoids but non-significant correlation with phenolics while for the callus samples it was found with both phenolics and flavonoids and for the *in vitro* samples chelating power did not point to any correlation with phenolics and flavonoids (Tables 4.14; 4.15; 4.16). The present results are consistent with those found by Sahreen et al. (2010) who reported that there is the existence of a strong relationship between phytochemical contents and DPPH, superoxide and ABTS radical scavenging.

Some of the extracts examined in this study showed a complex relationship with the total phenolic and flavonoid contents. This observation could be due to the several reasons: (1) the different extraction solvent resulted in the differences of the extract compositions and accordingly their antioxidant activities (Pinelo et al., 2004); (2) different assays for testing antioxidant activity are based on different conditions and mechanisms, thus they may show different results, each only partially reflecting the antioxidant activity (Hseu et al., 2008; Arabshahi and Urooj 2007; Delgado et al., 2005); (3) the method used to measure the total phenolic content of the extracts could be disturbed by other components (Prior et al., 2005). Also other soluble compounds such

as peptides, pigments, proteins and polysaccharides that are present in the plant extracts could be responsible for the antioxidant activity partially (Prior et al., 2005).

Cancer is one of the most threatening health problems around the world that is growing very fast. There is a long history for using of natural product to prevent and treat many diseases like cancer, so plant products are important candidates for the development and producing of anticancer drugs (Smith et al., 2000).

Bioassays offer special advantages for identification of medicinal botanical extracts. Most often, a desired biological response is not due to one component but rather to a mixture of bioactive plant components. Therefore, crude extracts must be screened for biological activity and then any active extract should be fractionated directed with bioassays to exploit the bioactive compounds (Jerry & Lingling 1998).

Based on traditional uses of *T. pratense* for cancer, some researches have been done to study the role of isoflavones from *T. pratense* in preventing and treatment of cancer. Liu et al. (2011) have reported the anticancer properties of red clover against the prostate cancer. They reported that under *in vitro* condition, isoflavones from red clover may partly prevent the prionflammatory effects of transforming growth factor β1 in human primary prostate cancer-derived stromal cells. In another study by Jarred et al. (2002), a non-randomised and non-blinded trail on 38 patients, indicated that the progression of prostate cancer may stop by dietary red clover isoflavons.

Matsingou et al. (2003) have reported that the differences in biological activities of plants in varying biological systems may be explained by the presence of different substrates and also by the variable nature of product produced by the reaction system.

Genistein isolated from *Trifolium* species is a molecule of great interest as a potentially chemotherapeutic agent or as a lead compound in anticancer drug design (Polkowski et

al., 2004). A moderate tumor growth inhibiting activities by *Trifolium fragiferum* and *Trifolium repens* extracts have been reported by Lellau and Liebezeit (2003). Khalighi-Sigaroodi et al. (2012) reported a moderate cytotoxic activity (LC_{50} between 30 and 50 µg/ml) by the extracts of *Trifolium campestre* and *Trifolium repens* against the brine shrimp. Our results also showed a limit anticancer activity of *Trifolium pratense* extract that is in agreement with previous finding of moderate anticancer activity of other *Trifolium* species.

It was found that the different four extracts of red clover (methanol extract, hexane fraction, ethyl acetate fraction and chloroform fraction) assayed present different cytotoxic activity against human cancer cell lines (MCF-7 and HCT-116), in the *in vitro* screening. The differential inhibition activities of the extracts that have been observed against the examined cancer cell lines could be explained by the presence of different compounds in the different fractions. The chloroform fraction and methanol extract were found to be more efficient ($p < 0.05$) than that of the n-hexane and ethyl acetate fractions (Tables 4.17 and 4.18).

The results of the present study is in agreement with the previous reports on higher antimicrobial activities of methanolic plant extracts than ethanolic (Eloff, 1998; Durmaz et al., 2006; Soniya et al., 2013). From the results, it is clear that the effectiveness of the extracts largely depends on the type of solvent used.

The results of this study also confirmed the study of Gami and Kothari (2011), Dhabhai et al. (1999) and Shafiqua et al. (2011) whereby, they showed that antimicrobial activities of callus and *in vitro* grown plants were higher than *in vivo* grown plants (Tables 4.19 and 4.20). The higher antimicrobial activity of the callus extract might be either related to the production of a compound produced in only undifferentiated callus cells or may be produced in higher amounts in these cells when compared to differentiated cells.

Several quantitative estimations and studies showed that the production of biocompounds can vary between differentiated and undifferentiated plant cells. For example, Tanwer et al. (2010) reported that calli of *S. acemella* produced a higher amount of sugars when compared with stem, leaves and roots of the intact plant. Jana and Shekhawat, (2010) also showed that the callus cells of *A. graveolens* produced saponins, while the *in vitro* leaf cells were not able to produce the same compound.

Some previous studies also demonstrated the antimicrobial activities of different *Trifolium* species. The antibacterial activity of leaf extract of *Trifolium alexandrinum* against seven Gram-positive and eleven Gram-negative hospital isolated human pathogenic bacteria strains has been demonstrated by khan et al. (2012). They also found that the methanol extract displayed considerably stronger antibacterial activity. Flythe and Kagan, (2010) reported that the phenolic compounds from *Trifolium pratense* possessed antimicrobial activity against hyper ammonia-producing bacteria (*Clostridium sticklandii*). Turker et al. (2009) reported that the alcoholic extracts of *Trifolium pannonicum* showed a broad antibacterial spectrum against four different fish pathogens: *Aeromonas hydrophila, Yersinia ruckeri, Streptococcus agalactia* and *Lactococcus garvieae*.

In photosynthesis process, plant leaves produced food from water and carbon dioxide in the presence of light, so they are the most important "food manufacturing" parts in all plants. Carbon dioxide is disseminated into the leaf when stomata opens in the presence of light, and converted into sugars through photosynthesis. Meantime, water vapour exits the leaf, along a diffusive gradient via the stomata, to the surrounding atmosphere via transpiration. The Optimization Theory, which was first suggested by Cowan and Farquhar (1977), proposed that when the photosynthesis of plant is maximizing at a certain average rate of transpiration, gas exchange in the plant is optimal. Meanwhile,

stomatal apertures naturally vary in reaction to the variation in light intensity, saturation deficit of ambient water vapor and soil moisture accessibility. The sizes of stomatal aperture provide a corresponding resistance to the diffusion of CO_2 and H_2O, in and out of the leaf. So the rate of photosynthesis and transpiration will be different when the size of the stomatal aperture changes. Linear relationship between E and g_s was also demonstrated in previous studies by Lakso (1984) and Düring (1987).

According to the present results the photosynthetic specifications were found significantly different in *in vivo* grown plants in contrast to the *in vitro* cultured plants. The P_{Nmax} of *in vivo* cultured plants was approximately two-folds of that found in plants of the similar age cultured *in vitro*. This finding is in agreement with the previous researches by Seelye et al. (2003), Huylenbroeck et al. (1998) and Carvalho et al. (2001), that reported although *in vitro* grown leaves are photosynthetically competent, but after acclimatization the leaves with higher photosynthetic activity would be formed to replace the *in vitro* leaves.

The lower P_N observed in *in vitro* grown plants could be resulted from the restricted air exchange between the culture vessels and outer environment and also the lower concentration of CO_2 inside the culture vessels (Hazarika, 2006; Zobayed et al., 2001). In contrast to the photosynthetic rate, lower values of transpiration rate were found in *in vivo* grown plants compared with the *in vitro* plants.

An increase in the photosynthesis rate was observed with increases in photosynthetic photon flux (area) density (PPFD) in both *in vivo* and *in vitro* grown plants. These results are in agreement with the recorded light saturation point for other species like *Alocasia macrorrhiza* and *Colocasia esculenta*. Previous studies recorded that the values of photosynthetic rate saturating PPFD can change, however, depending on the culture

condition. It is in agreement with present study that shows different values of PPFD for *in vivo* and *in vitro* grown plants.

Several environmental factors such as light, concentration of carbon dioxide, temperature and humidity affect the changes in the guard cell turgor as well as stomatal opening and closure (Schulze & Hall 1982). Meanwhile, Collatz *et al.* (1991) suggested that according to response to the environmental factors the stomatal could be divided into two groups: first group those which are dependent on photosynthesis and second group those which are independent on photosynthesis, however, there are significant interactions between these two groups. Farquhar and Sharkey (1982), investigated the role of stomatal conductance in relation to photosynthesis. They reported that although transpiration is limited by stomatal conductance substantially but photosynthesis rarely seriously is limited by that and it is because of the other factors that they effect on photosynthesis. As indicated earlier, there is a correlation between changes in stomatal conductance and changes in the rate or capacity of photosynthesis. It can be concluded that the rates of photosynthesis, transpiration, and stomatal conductance are significantly influenced by the closing or opening of stomata.

A substantial variation seems to exist among the plants in their responses of stomata to light and consequently, photosynthesis rate, transpiration, and stomatal conductance are significantly influenced by stomatal closing or opening (Miyashita *et al*., 2005). It should be noted that an increased initial slope and maximal rate of photosynthesis was observed when leaves were exposed at a high photon flux density.

The production of many crops is inhibited by salinity stress, because of its direct and indirect harmful effects and also inducing intense physiological dysfunctions (Ashraf *et al*., 2008). The lower concentration of NaCl may have nutrient effect rather than deleterious effect on some plant growth (Kaya, 2009)

Under salt stress, some of the biochemical, morphological and physiological characteristics of plant are affected. The germination, growth and antioxidant activity of red clover germinated in MS media under different concentrations of NaCl were investigated in the presence study. The present results showed that the seed germination of red clover was inhibited under 100, 150 and 200 mM of NaCl treatments while it was higher in the presence of 50 mM of NaCl, in comparison with the control plants (Figure 4.16). The deterrence in seed germination under salt stress may be because of the efficacy of salinity on some metabolic aspects and induction of anatomical and morphological changes. The most important traits for selection under salt stress condition are root and shoot length, because of direct contact of roots with soil thus they have the main roll in absorption of water and nutrient, and shoot only grows until the root can support it. Results obtained showed that growth significantly decreased in the seedlings treated with different NaCl concentration (Figures 4.17 and 4.18). The more effective inhibitory effect on seedlings growth investigated when treated with higher concentration of NaCl (200 mM). The toxic effects of NaCl and also unbalanced nutrient uptake by seedlings may cause these growth inhibitions in roots and shoots. These results are in agreement with those obtained by Asci, (2011) and also Winter and Lauchli (1982), that previously reported a lower survival potential at higher concentration of NaCl treatment (100, 150 and 200 mM or higher) for *T. pratense*. Similar findings have been reported in several clover species (Ates & Tekeli, 2007) and also in wheat (Moud & Maghsoudi, 2008). Similarly, the growth inhibition has been reported in many plants exposed to different concentrations of NaCl (Zapata *et al.,* 2004; Tang & Newton, 2005; Carpici *et al*., 2009). Interestingly, results from the present study showed that salt stress inhibited root development more than shoot development (Figures 4.17 and 4.18).

The antioxidant activity of plants can be changed by salt stress, but this is critically dependent on the plant's capacity to tolerate and adapt to salinity conditions. Salt

tolerance seems to be favored by an increased antioxidative capacity to detoxify reactive oxygen species (Dalmia & Sawhney, 2004).

Scavenging of DPPH free radical and superoxide dismutase (SOD) activity are the basis of common antioxidant assays (Sharma & Bhat, 2009; Dibyendu, 2011) and are used to measured total antioxidant activity of aromatic and medicinal plants (Otles & Yalcin, 2012).

In the present investigation, increase in the antioxidant activity of the extract of *in vitro* grown red clover under salt stress in comparison to the control treatment was noticed at 50 mM salt stress. However, the antioxidant activity showed significant decrease under higher examined concentration of NaCl (100,150 and 200 mM). Therefore, value of antioxidant inhibitory differ among the samples treated with different concentration of NaCl. The increase in antioxidant inhibitory in the presence of 50 mM NaCl could be due to the increase in phenolic compounds because of the salt stress. It is known from the literature that decrease in antioxidant defense inhibitory at higher salt concentration is due to decline of phosphor and potassium uptake, which are principal substances of secondary metabolism such as polyphenols (Waring & Pitman, 1985). This can explain the observed reduction of antioxidant inhibitory at higher levels of salt stress in this study.

CHAPTER 6: CONCLUSION

Trifolium pratense L. as an important forage crop and also traditional medicinal herb is a temperate crop that can not grow in Malaysia. One of the tools to produce this forage crop is by using tissue culture or micropropagation. Maybe in future by using biotechnological methods such as gene transfer it may be possible to adapt this important crop to the Malaysian climate.

Trifolium pratense L. was proven to be a highly regenerative when cultured *in vitro*, where it was found that the nodal explant was the most responsive for efficient direct regeneration of this plant. Direct regeneration of *T. pratense* was best achieved when the nodal explants were cultured on MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA, with mean number of 6.05 ± 0.28 shoots per explant, where by 100% of the samples had produced shoots. Production of roots also was best achieved through the culture of nodal explants on MS media supplemented with 1.5 mg/l BAP and 0.75 mg/l IBA, with the mean number of 3.3 ± 0.21 roots per explant.

Induction and production of callus was successfully achieved when the nodal explants were cultured on MS medium containing different types of plant hormones. MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l 2,4-D was the most responsive, when 100% of the nodal explants managed to induce callus production.

All the *in vitro* grown plants were acclimatized to ensure further growth of the plantlets. Adaptation process for the transfer of *in vitro* grown plants to the ex *vitro* was succesful, with very high survival rates of plantlets (93.71 ± 4.64 %) when the plant were transferred to the combination of red soil and black soil with the ratio of 1:1.

The *in vivo* and *in vitro* grown plants were subjected to the cytological studies to detect early somaclonal variation which may occur among the regenerated plants. The

chromosome number of *in vivo* and *in vitro* grown *T. pratense* was observed to be constant (14) in all samples. However, the size of cell and nuclear areas were found to be lower in *in vitro* grown plants compared with the *in vivo* grown plants. The observation of mitotic index showed that it is higher for the *in vitro* grown plants although it is not significantly different with the mitotic index (MI) of *in vivo* grown plants.

The antioxidant, anticancer and antimicrobial potentials of *in vivo* and *in vitro* grown as well as callus tissue of *T. pratense* were investigated using different extraction solvents and different antioxidant assays. The result obtained also suggested a considerable value in terms of the antioxidant activities of methanol extract and chloroform fraction of aerial parts of the *in vivo* grown red clover. In addition, the extracts of callus tissue had demonstrated a strong antioxidant activity but the extract of aerial parts of *in vitro* cultured red clover had demonstrated a lower antioxidant activity. This primary finding of this work was that extract of *in vitro* culture of red clover, especially the callus tissue possesses an antioxidant activity comparable to the *in vivo* cultured plants' extract. It can be concluded that *in vitro* cultured plants are able to produce and accumulate many medicinally valuable secondary metabolites.

The results also suggested a considerable value in terms of inhibition activities of methanol extract and chloroform fraction of aerial parts of *in vivo* grown plants and callus tissue of *T. pratense* against MCF-7 and HCT-116 cell lines. Although these inhibition activities were lower than that of doxorubicin as a positive control.

Investigation on antimicrobial activities also revealed that the callus extract of *T. pratense* contains higher antimicrobial activities than *in vivo* and *in vitro* grown plants. Although this antimicrobial potential is significantly smaller than positive control, these findings indicate that *in vitro* culture or even undifferentiated cells of callus can improve the quality of plant materials for medicinal uses.

In leaf gas exchange and light response curve studies it was observed that high levels of differentiation in terms of photosynthesis parameters exist among the *in vivo* and *in vitro* samples, whereby *in vivo* plants were shown to have the highest net photosynthesis. According to our finding the production of phenolic compounds by plants is in a direct relation with the photosynthetic rate. As phenolic compounds are reactive toward oxidation, so they are important factors in medicine. Maybe in future work it could be possible to develop methods to produce *in vitro* plants with same photosynthetic condition in contrast to nursery plants, to improve the medicinal properties of micropropagated plants.

The effects of different concentrations of NaCl treatments on *in vitro* seed germination and also antioxidant response of *in vitro* grown *T. pratense* were examined to determine if we can get better growth or higher antioxidant activity of *in vitro* grown plants. The *in vitro* culture was successfully applied in the assessment of salinity tolerance of this plant. The results revealed that a moderate concentration of NaCl (50 mM) was favorable for higher percentage of seed germination of *T. pratense* and also higher antioxidant activity of *in vitro* grown plants compared to the control. However, all these differences are not significant. On the other hand all four tested concentration of NaCl inhibited root and shoot growth of *T. pratense* under *in vitro* conditions.

Forage crops are the most widely grown plants in the world, but there are not many researches and development on this agricultural crops. To overcome the difficulties associated with traditional breeding methods of forage crops, the potential of plant biotechnology techniques should be recognized and adopt this technology for the development and improvement of forage cultivation. Some strategies are needed to improve the forage crops productivity due to the limited cultivated lands for them, as they contribute a lot to our food supply through animals. Besides, some of these crops can be

used for medicinal purposes. The findings of this thesis can provide fundamental information to be applied in future research on micropropagation of plants for medicinal purpose.

Although micropropagation and cellular behavior studies were carried out in this project and have achieved some encouraging results, however, some investigations concerning molecular aspects are very much needed for future exploitation of this important forage and medicinal crop. Since red clover is not being grown in Malaysia yet, but due to many advantages of this forage crop, developing a new variety of this plant which can adapt to Malaysian climate is very beneficial. The development of new variety is possible through genetic engineering and biotechnological methods. The cultivation of this plant in Malaysia can save a lot of budget on livestock industry. Since Malaysia imports a lot of hay from other countries.

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Khorasani Esmaeili, A., Mat Taha, R., Mohajer, S., & Banisalam, B. (2015). Antioxidant Activity and Total Phenolic and Flavonoid Content of Various Solvent Extracts from In Vivo and In Vitro Grown Trifolium pratense L. (Red Clover). *BioMed Research International, 2015*.

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A. Khorasani Esmaeili, R. M. Taha, S. Mohajer and B. Banisalam (2015). In Vitro regeneration and comparison of phenolic content, antioxidant and antityrosinase activity of in vivo and in vitro grown Asparagus officinalis L. Sains Malaysiana. (Accepted).

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Khorasani Esmaeili, A., Mat Taha, R., Mohajer, S. & Banisalam, B. (2015). Effects of Optimal and Supra-optimal Salinity Stress on Antioxidant, Antityrosinase and In Vitro Growth Responses of Trifolium pratense L. *Chiang Mai Journal of Science*, (Submitted).

Khorasani Esmaeili, A., Mat Taha, R., Mohajer, S. & Banisalam, B. (2015). Photosynthetic and Relationship between Leaf Gas exchange and Secondary Metabolites of Red Clover (Trifolium pratense L.): a comparison between in vivo and in vitro plants. Acta Physiologiae Plantarum, (Submitted).

Paper presented/Conferences/Expo:

Effect Of Salinity Stress on Growth and Some Biological Activities of Trifolium Pratense under In Vitro Culture, International Conference on Innovative Trends in Multidisciplinary Academic Research, 20 Oct 2014 to 21 Oct 2014, ITMAR, Istanbul, Turkey.

Effects of Salinity Stress on Antioxidant Activity and Seed Germination in In Vitro Culture of Trifolium Pratense, International Conference on Biological, Chemical and Environmental Sciences (BCES), 14 Jun 2014 to 16 Jun 2014, Penang Island, Malaysia.

Antimicrobial Activities of Extracts Derived from In vivo and In vitro Grown Trifolium pratense, 3rd International Conference on Environmental and Agriculture Engineering (ICEAE), 06 Jul 2013 to 08 Jul 2013, Hong Kong.

Mass Propagation of Forage Crops (Sainfoin and Red Clover) through Synthetic Seeds Technology on MS Double Layered Medium, iCompEx'15 National Innovation and Invention Competition through Exhibition, 24 Mar 2015 to 26 Mar 2015, Jitra, Kedah, Malaysia

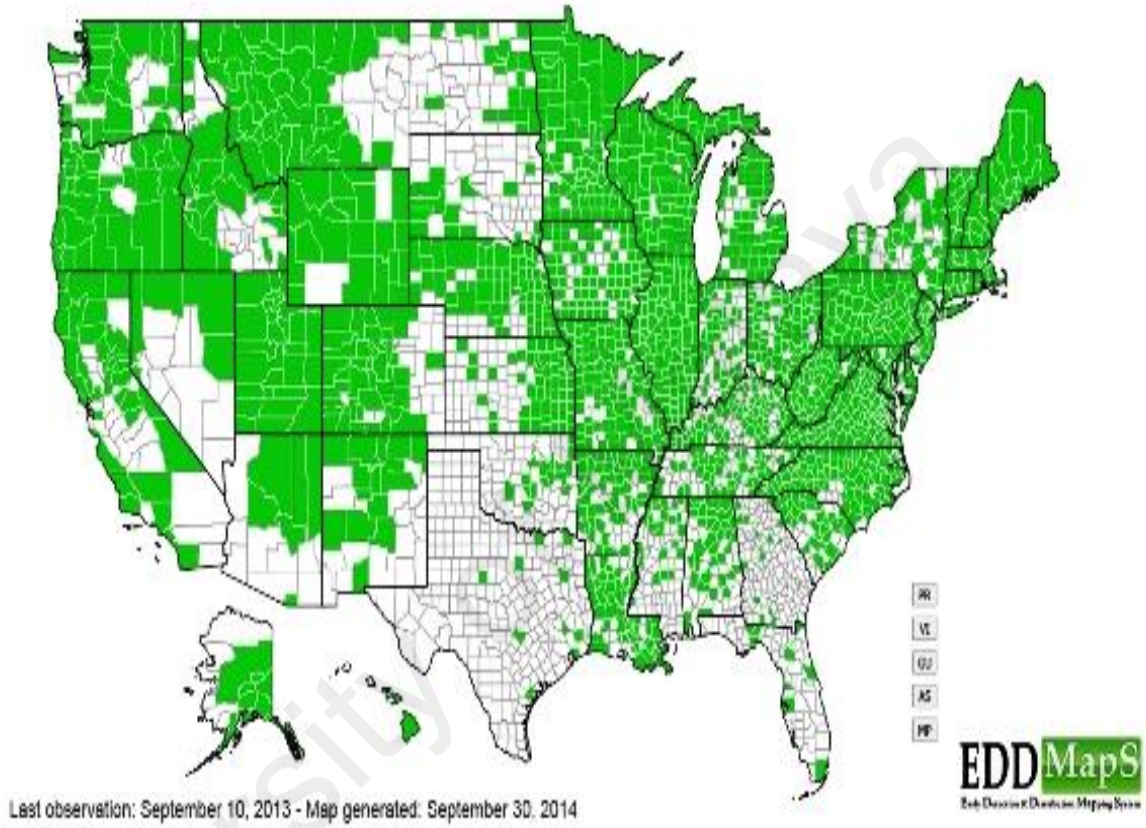
APPENDIX A: MINERAL COMPOSITION OF RED CLOVER

(From Spedding and Diekmahns, 1972)

Constituent	Content range
	(g/kg DM)
N	23.4-47.0
P	1.4-4.5
K	8.8-41.0
Ca	11.9-24.2
Mg	1.7-3.8
S	1.5-2.4
Na	0.2-2.0
Cl	3.2-6.2
	(mg/kg DM)
Fe	74-362
Mn	36-75
Zn	21-35
Cu	5.8-11.6
Co	0.10-0.36
Mo	0.44

APPENDIX B: DISTRIBUTION MAP OF RED CLOVER

Trifolium pratense



APPENDIX C: FORMULATIONS OF MS MEDIA (MURASHIGE AND SKOOG)

MS Medium Components	Content ($mg.l^{-1}$)
Macronutrients	
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
$MgSO_4.7H_2O$	370.0
KH_2PO_4	170.0
Micronutrients	
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Vitamins and other supplements	
Inositol	100.0
Glycine	2.0
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Nicotinic acid	0.5

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Research Article

Antioxidant Activity and Total Phenolic and Flavonoid Content of Various Solvent Extracts from *In Vivo* and *In Vitro* Grown *Trifolium pratense* L. (Red Clover)

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In the present study the extracts of *in vivo* and *in vitro* grown plants as well as callus tissue of red clover were tested for their antioxidant activities, using different extraction solvent and different antioxidant assays. The total flavonoid and phenolic contents as well as extraction yield of the extracts were also investigated to determine their correlation with the antioxidant activity of the extracts. Among all the tested extracts the highest amounts of total phenolic and total flavonoids content were found in methanol extract of *in vivo* grown plants. The antioxidant activity of tested samples followed the order *in vivo* plant extract > callus extract > *in vitro* extract. The highest reducing power, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging, and chelating power were found in methanol extracts of *in vivo* grown red clover, while the chloroform fraction of *in vivo* grown plants showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, superoxide anion radical scavenging and hydrogen peroxide scavenging compared to the other tested extracts. A significant correlation was found between the antioxidant activity of extracts and their total phenolic and total flavonoid content. According to the findings, the extract of *in vitro* culture of red clover especially the callus tissue possesses a comparable antioxidant activity to the *in vivo* cultured plants' extract.

1. Introduction

As a member of the family Leguminosae or Fabaceae, *Trifolium pratense* L. (red clover) is a short-lived biennial plant which serves as food for livestock, but also as a health food for humans [1].

Red clover (*Trifolium pratense* L.) has high concentrations of isoflavonoids, compounds largely distributed in the Leguminosae family [2]. Many isoflavone preparations obtained from red clover are currently available as nutritional supplements [3]. Isoflavonoids are secondary metabolites divisible into isoflavones and pterocarpans. The main isoflavones in red clover are biochanin A and formononetin, both of which are found in abundance in leaves [4]. It also has antioxidant activity that may result from the presence of different flavonoids and other phenolic compounds such as phenolic acids, clovamides, and saponins. Apart from that, it has also been adopted in traditional medicine to treat whooping cough, asthma, eczema, and eye diseases [5].

As shown in recent years, natural antioxidants discovered in plants have attracted some interest due to their widely acclaimed nutritional and therapeutic values. Antioxidant properties stand to be an essential mechanism of beneficial activity of plant-derived compounds and extracts.

Ethnopharmacological surveys have shed light on the fact that the therapeutic use of even 80% of 122 plant-derived drugs may have a link with their recommendations in traditional medicine [6]. Natural antioxidants have a diversity of biochemical activities, some of which include the inhibition of reactive oxygen species (ROS) generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential [7]. Antioxidants have functioned to inhibit apoptosis because apoptosis was at first thought to be mediated by oxidative stress [8]. It is known that many

antioxidant substances have anticancer or anticarcinogenic properties [9, 10]. Epigallocatechin-3- gallate (EGCG) in green tea, for instance, has been reported to scavenge free radicals [11] and to hinder carcinogen-induced tumors in the skin, lung, forestomach, and colon of rodents [12].

Therefore, there has been undeniable evidence of interest when it comes to finding natural antioxidants from plant materials.

Studies regarding the bioactivities of various plants have assumed an important position because of the variations in the effectiveness of the plant extract with the solvent for extraction used, plant part used, the plants' age, and geographic origin. The excessive use of medicinal plants for drug formulation also puts pressure on the need for more biomass of plants which can be met with biotechnological tools like micropropagation.

Our current work aims to evaluate the antioxidant properties of the plant extracts, prepared from the aerial parts of *in vitro* and *in vivo* grown red clover. We also seek to examine the effects of extraction solvent on total phenolics, total flavonoids, and antioxidant activities of extracts from both the *in vivo* and *in vitro* grown red clover.

2. Materials and Methods

2.1. In Vivo Plant Samples. Seeds of *Trifolium pratense* L. were purchased from Stock Seed Farms (Murdock, US) and planted in polybags filled with coco peat, sand, and compost in the ratio of 1 : 1 : 1. The organic fertilizer was then added to each polybag and kept in the growth room (at temperature of 25 ± 1°C, 16 hours photoperiod, 40 μmol/m^2/s of photon flux density, and 60% relative humidity).

2.2. In Vitro Plant Samples. Some of the purchased seeds were surface sterilized by first placing them under the running tap water for 30 minutes and were rinsed once using sterile distilled water and soaked in 50% Clorox for 2 minutes. The seeds were then washed 5 times using sterile distilled water and soaked in 10% Clorox for one minute. Again, the seeds were rinsed 5 times using sterile distilled water and dipped in 70% ethanol for 1 minute (inside laminar flow chamber). As the final step, the seeds were rinsed 5 times using sterile distilled water and blotted on sterilized filter paper. The MS (Murashige and Skoog) medium, without the addition of any hormones, served to germinate the sterilized seeds. Cultures were stored in growth room (at 25 ± 1°C, 16 hours photoperiod, 40 μmol/m^2/s of photon flux density, and 60% relative humidity) until the grown plants are deemed suitable to be used for further investigation.

The *in vitro* regenerated red clover after six weeks of culture was used as one of the plant materials in our current study.

The callus tissue of red clover was also produced using MS medium containing 1.5 mg/L BAP (6-benzylaminopurine) and 0.5 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid). The node explants of 6 weeks old *in vitro* grown plants were cultured in the media. The six-week-old callus was collected as one of the *in vitro* plant materials/samples.

2.3. Extract Preparation. The aerial parts of *in vivo* and *in vitro* grown plants and also the callus obtained from *in vitro* culture were collected and dried in the dark. The dried samples were powdered in an electronic blender before being used for solvent extraction. For the preparation of extract, 25 g of fine powder was extracted with 100 mL of 95% methanol at room temperature for 48 hours. The extracts were filtrated through Whatman number 1 and combined and this was followed by a concentration using rotary evaporator under pressure that was reduced at 45°C. The filtrate obtained was suspended in distilled water (25 mL) and n-hexane (50 mL) was added to it, where the mixture was shaken well and the layers were enabled to be separated for 6 h. After separation of n-hexane layer again 50 mL of n-hexane was added to get the n-hexane fraction. In a similar manner, the protocol was repeated with the rest of the solvents (ethyl acetate and chloroform). Each fraction obtained was dried using a rotary evaporator [13]. The dry extract obtained with each solvent was weighed and the percentage yield was expressed in terms of the air-dried weight of plant materials. Samples were stored in an airtight container at −20°C until it was time to conduct further analysis.

2.4. Determination of Antioxidant Activities

2.4.1. Total Phenolic Content. The total phenolic content of the obtained extracts was spectrometrically analyzed in adherence to the Folin-Ciocalteu method [14]. In short, 100 μL of each extract (dissolved in methanol) or gallic acid standard solution was mixed with 2 mL of 2% (w/v) sodium carbonate solution. The mixture was then incubated for 5 minutes, and afterwards 100 μL of Folin-Ciocalteu reagent was added. After being left for 30 min at room temperature for color development, absorbance was measured at 750 nm using a spectrophotometer. Results are expressed as mg gallic acid equivalents (GAE) per gram dry matter of sample.

2.4.2. Total Flavonoid Content. Total flavonoid content was ascertained based on the method by Zhishen et al. [15]. In brief, 50 mg of each fraction was dissolved in 10 mL of 80% aqueous methanol and filtered through Whatman filter paper number 42 (125 mm). In a 10 mL test tube, 300 μL of each extract, 3.4 mL of 30% methanol, 150 μL of 0.5 M $NaNO_2$, and 150 μL of 0.3 M $AlCl_3{\cdot}6H_2O$ were added and mixed, followed by 5 min incubation and the addition of 1 mL of NaOH (1 M). Absorbance was measured at 510 nm with a spectrophotometer. The standard curve for total flavonoids was made using rutin standard solution (0–100 mg/L) using the same aforementioned procedure. The total flavonoid content was shown as milligram of rutin equivalents (CTE) per gram dry matter of extract.

2.4.3. Antioxidant Assays. Each sample was dissolved in 95% methanol at a concentration of 1 mg/mL and then diluted in order to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparative purposes in all assays.

2.4.4. DPPH Radical Scavenging Activity Assay. The DPPH radical scavenging assay was conducted following the method of Zhu et al. [16]. In brief, 2 mL of DPPH solution (0.1 mM, in methanol) was blended with 2 mL of the samples at varying concentrations (50, 100, 150, 200, 250, and 300 μg/mL). As the next stage, the reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank. Ascorbic acid and rutin standard as positive controls were prepared in a similar manner, as for the test group except for the antioxidant solution's replacement. The inhibition of the DPPH radical by the sample was calculated based on the formula below:

$$\text{DPPH scavenging activity (\%)} = \left[\frac{(\text{absorbance of control} - \text{absorbance of sample})}{(\text{absorbance of control})}\right] \times 100. \quad (1)$$

2.4.5. Superoxide Anion Radical Scavenging Assay. The assay for superoxide anion radical scavenging activity leaned on a riboflavin-light-NBT system [17]. The reaction mixture had 0.5 mL of phosphate buffer (50 mM, pH 7.6), 0.3 mL riboflavin (50 mM), 0.25 mL phenazine methosulphate (PMS) (20 mM), and 0.1 mL nitro blue tetrazolium (NBT) (0.5 mM), before 1 mL sample solution was added at varying concentrations (50, 100, 150, 200, 250, and 300 μg/mL). Reaction began as the reaction mixture was illuminated with different concentrations of the extracts using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The percentage of inhibition of superoxide anion generation was calculated based on the following formula:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100. \quad (2)$$

2.4.6. ABTS Radical Scavenging Assay. The ABTS radical scavenging assay was performed adhering to the method of Re et al. [18] with slight modification. The ABTS radical was generated through the oxidation of ABTS with potassium persulfate. In brief, the ABTS solution (7 mM) had reacted with potassium persulfate (2.45 mM) solution and was stored in the dark for 12–16 h to produce a dark coloured solution containing ABTS radical cation. Before being used in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.70 (±0.02) at 745 nm, with the temperature control fixed as 30°C. Free radical scavenging activity was evaluated by mixing 3 mL of ABTS working standard with 300 μL of test sample (50, 100, 150, 200, 300, 400, and 500 μg/mL) in a microcuvette. The decrease in the absorbance was measured at the exact time of 1 min after mixing the solution until it reached 6 min. The final absorbance was noted then. The inhibition percentage was calculated based on the following formula:

$$\text{Scavenging effect (\%)} = \left[\frac{(\text{absorbance of control} - \text{absorbance of sample})}{(\text{absorbance of control})}\right] \times 100. \quad (3)$$

2.4.7. Hydrogen Peroxide Scavenging Activity. The capability of scavenging hydrogen peroxide by the extract was determined based on the method of Ruch et al. [19]. A hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 mL) of the extracted sample (different concentration of 50, 100, 150, 200, 250, and 300 μg/mL) were transferred into the test tubes and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After adding 0.6 mL hydrogen peroxide solution, tubes were vortexed and the absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. The abilities to scavenge the hydrogen peroxide were calculated based on the following equation:

$$\text{Hydrogen peroxide scavenging activity} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100. \quad (4)$$

2.4.8. Reducing Power Assay. The reducing powers of the samples were determined following the method of Atmani et al. [20]. Two millilitres of each extract solution (300 μg/mL) was mixed with 2 mL of phosphate buffered saline (0.2 M, pH 6.6) and 2 mL of potassium ferrocyanate (10 mg/mL). The incubation for this mixture was set at 50°C for 20 min. In the next stage, 2 mL of trichloroacetic acid (100 mg/L) was added to the mixture. In a test tube, a volume of 2 mL from each of the above mixtures was mixed with 2 mL of distilled water and 0.4 mL of 0.1% (w/v) ferric chloride. The absorbance was measured at 700 nm after reaction was started for 10 minutes. The increased absorbance of the reaction mixture suggested that the reducing power was high.

2.4.9. Chelating Power. The ability of the extract to chelate iron (II) was estimated based on the method of Dinis et al. [21] with minimal modification. Various sample solutions (50–300 μg/mL) were prepared with dissolving the extracts in the methanol. An aliquot of each sample (200 μL) was mixed with 100 μL of $FeCl_2{\cdot}2H_2O$ (2 mM) and 900 μL of methanol. After 5 min incubation, an initial reaction was fuelled by the addition of 400 μL of ferrozine (5 mM). After 10 min incubation, the absorbance at 562 nm was recorded. The percentage of the chelating activity was calculated based on the following equation:

$$\text{Chelating activity (\%)} = \left[\frac{(\text{absorbance of control} - \text{absorbance of sample})}{(\text{absorbance of control})}\right] \times 100. \quad (5)$$

Table 1: Total phenolic, total flavonoid, and extraction yield of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants (aerial parts) and also callus tissue of *Trifolium pretense*.

Plant extract	Total phenolic (mg gallic acid/g dry sample)	Total flavonoid (mg rutin equivalent/g dry sample)	Extraction yield (%)
In vivo			
Methanol extract	46.88 ± 1.07^{a}	26.61 ± 0.92^{a}	13.89 ± 0.54^{a}
n-Hexane fraction	30.52 ± 0.72^{cd}	16.06 ± 1.58^{d}	10.79 ± 0.59^{c}
Ethyl acetate fraction	27.57 ± 0.49^{e}	11.71 ± 1.43^{fg}	6.39 ± 1.12^{g}
Chloroform fraction	40.17 ± 0.88^{b}	19.56 ± 1.11^{b}	12.23 ± 0.54^{b}
In vitro			
Methanol extract	31.94 ± 1.68^{c}	13.03 ± 0.79^{ef}	10.84 ± 0.72^{c}
n-Hexane fraction	20.06 ± 0.41^{g}	8.23 ± 0.96^{h}	5.20 ± 0.54^{i}
Ethyl acetate fraction	16.90 ± 1.19^{h}	6.11 ± 0.79^{i}	2.35 ± 0.5^{g}
Chloroform fraction	25.43 ± 0.87^{f}	10.39 ± 0.79^{g}	7.18 ± 0.82^{g}
Callus			
Methanol extract	40.82 ± 1.5^{b}	17.84 ± 0.53^{c}	11.36 ± 0.55^{bc}
n-Hexane fraction	31.77 ± 1.14^{c}	12.70 ± 0.94^{ef}	6.13 ± 0.34^{gf}
Ethyl acetate fraction	19.23 ± 0.66^{g}	7.21 ± 0.74^{hi}	2.98 ± 0.66^{g}
Chloroform fraction	29.25 ± 1.62^{de}	14.02 ± 1.08^{e}	8.70 ± 0.52^{d}

Each value is represented as mean ± SD ($n = 3$).
Means in the same column not sharing the same letters are significantly different at (Duncan) $P < 0.05$.

2.5. Statistical Analysis. All assays were carried out in triplicate and their results were expressed as mean ± standard deviation. The EC_{50} (half-maximal effective concentration) of various fractions for different antioxidant assays were analysed using ANOVA test with least significant difference (LSD) $P < 0.05$ as a level of significance. Experimental results were examined further for Pearson correlation coefficient of phenolic and flavonoids with different antioxidants assays and its significance was tested using Student's t-test ($P < 0.05$).

3. Results and Discussion

3.1. Total Phenolic, Total Flavonoid, and Extraction Yield. Good extraction methods prove to be a crucial step for getting extracts with acceptable yields and strong antioxidant activity [22]. The percentage yields of the methanol extract and different fractions of *in vivo* and *in vitro* grown plants as well as the callus tissue of red clover are shown in Table 1. The extraction yield of these samples came in a range from 2.35 ± 0.5% to 13.89 ± 0.54%. The results showed that the methanol extraction of *in vivo* grown plants had illustrated the highest amount of extraction yield, and conversely the extraction yield of *in vitro* grown plants with ethyl acetate was prominently lower ($P < 0.05$) when compared to that of the other samples. Being plant secondary metabolites, the phenolics or polyphenols are very important judging from the virtue of their antioxidant activities by chelating redox-active metal ions, inactivating lipid free radical chains, and avoiding the hydroperoxide conversions into reactive oxyradicals. The total phenolic contents of the extracts, expressed as gallic acid equivalents, varied from 46.88 ± 1.07 mg GAE/g for the methanol extract of *in vivo* grown plants to 16.9 ± 1.19 mg GAE/g for the ethyl acetate extract of *in vitro* grown plants (Table 1). In all *in vivo* and *in vitro* plants and also callus tissue, the methanolic extract had illustrated the highest total content of phenolic, whereas the content obtained with ethyl acetate was much lower ($P < 0.05$), which is similar to the reports compiled by Sahreen et al. [23] and Ao et al. [24]. A possible justification would be due to the formation of complexes by a part of phenolic compounds with carbohydrates and proteins, which are more extractable in methanol than in other solvents that have been emphasized in this study. Our results do not deviate from those of Parsaeimehr et al. [25] who also found that wild plant, compared to the callus tissue, showed higher extracting phenolic components with regard to some medicinal plants. The rich-flavonoid plants could manifest themselves as good sources of antioxidants that would assist in the enhancement of the overall antioxidant capacity of an organism and protection against lipid peroxidation [26]. The content of total flavonoids is expressed as mg of rutin equivalents per g of dry sample that ranged from 6.11 ± 0.79 to 26.61 ± 0.92, the amounts of which were comparable with results verified in the literatures for other extracts produced [24, 27]. The methanol extract of *in vivo* grown red clover significantly contained ($P < 0.05$) higher flavonoids concentration as compared to other samples involved. The lowest flavonoid value in this study was recorded in ethyl acetate fraction of *in vitro* grown plants (6.11 ± 0.79 mg CTE/g dry sample). The total flavonoid content results were entirely synchronous with those of the total phenolic. It was successfully shown that samples with high level of phenolic content also contain flavonoids in great amount. The rich-flavonoid plants could be a good antioxidant source that would help increase the overall antioxidant capacity of an organism and guard it against lipid peroxidation [26].

3.2. DPPH Radical Scavenging Activity. 2,2-Diphenyl-1-picrylhydrazyl radical is a stable organic free radical with an

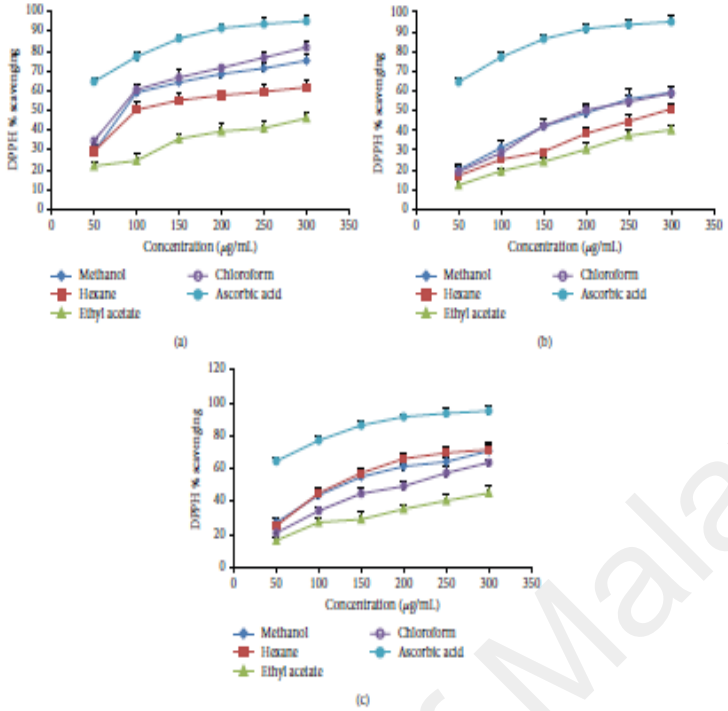

FIGURE 1: DPPH radical scavenging activity of different extracts from the methanol extract of *Trifolium pratense* by different solvent at different concentration. (a) *In vivo* grown plants, (b) *in vitro* grown plants, and (c) callus tissue. Each value is represented as mean ± SD ($n = 3$).

absorption band at 517 nm. It loses this absorption when it accepts an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow. It can incorporate many samples in a short time span and is vulnerable enough to distinguish active ingredients at low concentrations [28]. Figure 1 highlights the DPPH radical scavenging ability of *in vivo*, *in vitro*, and callus samples with different extraction solvents. The differential scavenging activities of the extract against the DPPH system that has been observed could be explained by the presence of different compounds in the fractions. The chloroform fraction of *in vivo* grown plants showed a reading of the lowest EC_{50} of DPPH radical scavenging (81.04 ± 2.33 μg/mL), while the highest EC_{50} belonged to the ethyl acetate fraction of *in vitro* grown plants (»300 μg/mL) (Table 2). Although the DPPH radical scavenging activities of the fractions were less ($P < 0.05$) than those of the ascorbic acid, the study had made a disclosure that *in vivo* grown plants and callus tissue of red clover have free radical scavengers or inhibitors, possibly acting as primary antioxidants more than *in vitro* grown red clover. There was an observation on a similar trend in a study of the antioxidant activity of the *Artemisia judaica* L. extract [29].

3.3. Superoxide Radical Scavenging Activity. As it is a reactive oxygen species, superoxide has some damaging properties that can be imposed to the cells and DNA and subsequently invites various diseases. Thus, a proposal has been established to gauge the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical [30]. The superoxide radical scavenging effect of these varying fractions of methanol extract of *in vivo* and *in vitro* grown plants as well as the callus tissue of red clover was drawn in comparison with the same doses of ascorbic acid in a range from 50 to 300 μg/mL as shown in Figure 2. The results had suggested that the lowest EC_{50} value (70.69 ± 1.76 μg/mL) belongs to the chloroform fraction of *in vivo* samples, whereas the highest value (260.01 ± 1.46 μg/mL) belongs to the ethyl acetate fraction of *in vitro* samples (Table 3). In a dose dependent manner, all of the fractions had scavenging activities

Table 2: DPPH radical scavenging activity of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants and callus tissue of red clover (*Trifolium pratense*).

Plant extract	EC_{50} of DPPH radical scavenging activity (μg/mL)		
	In vivo	*In vitro*	Callus
Methanol extract	94.25 ± 1.15^{cA}	$205.47 \pm 2.90^{bB**}$	$128.42 \pm 2.40^{cA*}$
n-Hexane fraction	$131.42 \pm 3.20^{dA*}$	$291.95 \pm 2.98^{cB**}$	120.74 ± 3.45^{bA}
Ethyl acetate fraction	$>300^{eA}$	$>300^{dB**}$	$>300^{dA*}$
Chloroform fraction	81.04 ± 2.33^{bA}	$208.86 \pm 2.20^{bB**}$	$155.17 \pm 2.80^{dA*}$
Ascorbic acid	21.17 ± 0.76^{aA}	21.17 ± 0.76^{aA}	21.17 ± 0.76^{aA}

Each value in the table is represented as mean ± SD ($n = 3$).
Means not sharing the same symbols (*, **, and * * *) are significantly different (Duncan) at $P < 0.05$ in the same row.
Means not sharing the same letters are significantly different (Duncan) at $P < 0.05$ in the same column.

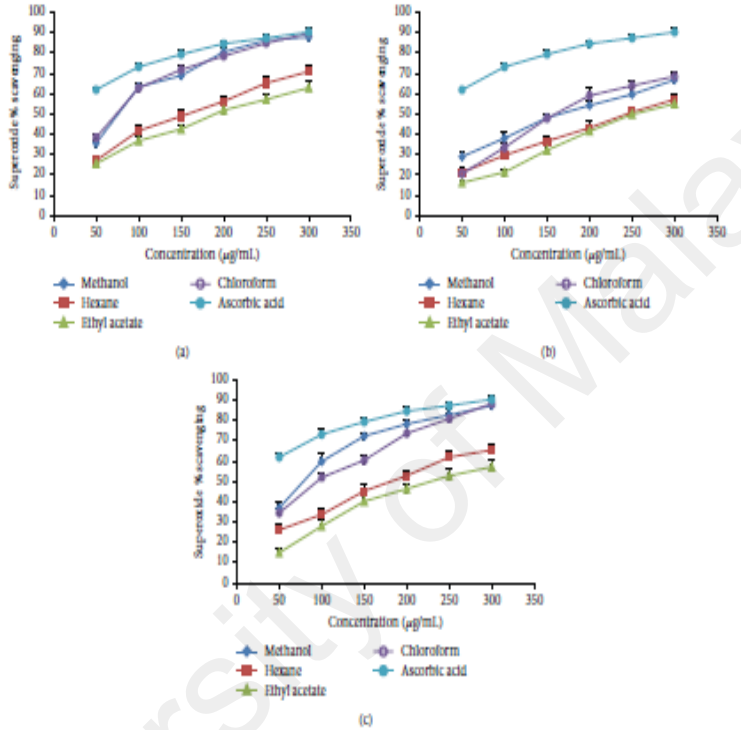

Figure 2: Superoxide radical scavenging activity of different extracts from the methanol extract of *Trifolium pratense* by different solvent at different concentration. (a) *In vivo* grown plants, (b) *in vitro* grown plants, and (c) callus tissue. Each value is represented as mean ± SD ($n = 3$).

on the superoxide radicals. Nonetheless, when compared to the ascorbic acid, the superoxide scavenging activities of the extracts were found to be significantly lower ($P < 0.05$). This could be due to the presence of flavonoids and other antioxidants in the extract.

3.4. ABTS Radical Scavenging Activity. The ABTS radical cation decolourisation test is another widely established approach adopted to evaluate antioxidant activity. Colour reduction shows the decrease of ABTS radical [31]. The samples' ABTS radical scavenging capability can be ranked

Table 3: Superoxide anion activity of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants and callus tissue of red clover (*Trifolium pratense*).

Plant extract	EC_{50} of superoxide anion activity (μg/mL)		
	In vivo	*In vitro*	Callus
Methanol extract	$74.68 \pm 1.59^{c*}$	$179.77 \pm 1.60^{c**}$	$74.36 \pm 1.42^{b*}$
n-Hexane fraction	$139.83 \pm 2.40^{d*}$	$246.59 \pm 2.09^{b**}$	$190.06 \pm 2.56^{d**}$
Ethyl acetate fraction	$201.55 \pm 1.94^{e*}$	$260.01 \pm 1.46^{a**}$	$228.41 \pm 1.52^{e**}$
Chloroform fraction	$70.69 \pm 1.76^{b*}$	$155.91 \pm 1.73^{b**}$	$104.32 \pm 1.45^{c**}$
Ascorbic acid	23.44 ± 0.84^{a}	23.44 ± 0.84^{a}	23.44 ± 0.84^{a}

Each value in the table is represented as mean ± SD ($n = 3$).
Means not sharing the same symbols (*, **, and * * *) are significantly different (Duncan) at $P < 0.05$ in the same row.
Means not sharing the same letters are significantly different (Duncan) at $P < 0.05$ in the same column.

Table 4: ABTS radical scavenging of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants and callus tissue of red clover (*Trifolium pratense*).

Plant extract	EC_{50} of ABTS (μg/mL)		
	In vivo	*In vitro*	Callus
Methanol extract	$111.84 \pm 1.46^{b*}$	$351.46 \pm 2.02^{c**}$	$164.32 \pm 1.61^{b**}$
n-Hexane fraction	$210.76 \pm 1.71^{d*}$	$>500^{d**}$	$225.63 \pm 2.05^{d**}$
Ethyl acetate fraction	$>500^{f*}$	$>500^{f**}$	$>500^{f**}$
Chloroform fraction	$122.34 \pm 2.29^{c*}$	$297.68 \pm 1.68^{b**}$	$172.18 \pm 1.55^{c**}$
Ascorbic acid	34.67 ± 0.53^{a}	34.67 ± 0.53^{a}	34.67 ± 0.53^{a}

Each value in the table is represented as mean ± SD ($n = 3$).
Means not sharing the same symbols (*, **, and * * *) are significantly different (Duncan) at $P < 0.05$ in the same row.
Means not sharing the same letters are significantly different (Duncan) at $P < 0.05$ in the same column.

as follows: *in vivo* > callus > *in vitro* samples. The methanol extract of *in vivo* grown samples had demonstrated the highest radical scavenging activity when it reacted with the ABTS radicals. By contrast, the ethyl acetate fraction of *in vivo*, *in vitro*, and callus samples and also the n-hexane fraction of *in vitro* samples did not illustrate any leveling effect at the highest concentration, but it was a fact that their radical scavenging effects were much less ($P < 0.05$) than that of the other extracts tested (Table 4). Significant differences among the EC_{50} values of all the fractions and ascorbic acid had also been noted ($P < 0.05$).

3.5. Hydrogen Peroxide. While hydrogen peroxide itself is not very reactive, it can sometimes be poisonous to cells, since it may trigger the rise of hydroxyl radicals inside the cell [32]. Extracts from *in vivo* and *in vitro* grown plants as well as callus tissue of red clover had the capability to scavenge hydrogen peroxide in a concentration dependent manner (50–300 μg/mL). The hydrogen peroxide scavenging activities of *in vivo* grown samples were proven to be more effective ($P < 0.05$) than the *in vitro* grown and callus samples, as revealed by the comparison with the EC_{50} values. The chloroform fraction and methanol extract were found to be more efficient ($P < 0.05$) than those of the n-hexane and ethyl acetate fractions. EC_{50} values of all the extracts, in scavenging abilities on hydrogen peroxide, were remarkably different ($P < 0.05$) from the EC_{50} value that had been obtained for ascorbic acid (Table 5).

3.6. Chelating Activity. Ability to chelate or deactivate transition metals, which in turn has the ability to catalyze hydroperoxide decomposition and Fenton-type reactions, is a vital mechanism of antioxidant activity. It was thus considered of importance to screen the iron (II) chelating ability of extracts [27]. All the fractions had ferrous ion chelating activity but they were remarkably low ($P < 0.05$) in comparison to catechin. The sequence for the chelating power was *in vivo* grown samples > callus tissue of samples > *in vitro* grown samples. The highest chelating power was shown by the methanol extract of *in vivo* grown plants with the EC_{50} value of 49.11 ± 0.97 μg/mL, whilst the lowest was found in ethyl acetate fraction of *in vitro* grown samples with the EC_{50} value of 183.44 ± 2.48 μg/mL (Table 6). The ion chelating data with different measurements of concentrations (50–300 μg/mL) suggested that ferrous ion chelating effects of all the fractions of *in vivo* and *in vitro* grown plants as well as callus tissue of red clover would be rather advantageous to offer protection against oxidative damage.

3.7. Reducing Power Activity. The reducing power of the extract, which potentially serves as a significant reflection of the antioxidant activity, was ascertained using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the colour of the test solution, which was yellow, transforms to various hues of green and blue, based on the extent of the reducing power of the samples. The presence of the antioxidants in the samples leads to Fe^{3+}/ferricyanide complex reduced to the Fe^{2+} form

Table 5: Hydrogen peroxide scavenging of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants and callus tissue of red clover (*Trifolium pratense*).

Plant extract	EC_{50} of hydrogen peroxide (μg/mL)		
	In vivo	*In vitro*	Callus
Methanol extract	$103.44 \pm 1.47^{Cc*}$	$219.86 \pm 1.57^{Bb***}$	$141.74 \pm 1.93^{Cc**}$
n-Hexane fraction	$270.56 \pm 1.72^{Ba*}$	$>300^{Ba***}$	$287.63 \pm 1.59^{Ba**}$
Ethyl acetate fraction	$138.16 \pm 1.93^{Dd**}$	$>300^{Dd***}$	$132.26 \pm 2.03^{Db*}$
Chloroform fraction	$88.35 \pm 1.27^{Eb*}$	$231.49 \pm 2.02^{Cc***}$	$146.31 \pm 1.67^{Dd**}$
Ascorbic acid	26.84 ± 0.83^{Fa}	26.84 ± 0.83^{Fa}	26.84 ± 0.83^{Fa}

Each value in the table is represented as mean ± SD ($n = 3$).
Means not sharing the same symbols (*, **, and * * *) are significantly different (Duncan) at $P < 0.05$ in the same row.
Means not sharing the same letters are significantly different (Duncan) at $P < 0.05$ in the same column.

Table 6: Chelating activity of methanol extract and soluble fractions of *in vivo* and *in vitro* grown plants and callus tissue of red clover (*Trifolium pratense*).

Plant extract	EC_{50} of chelating power (μg/mL)		
	In vivo	*In vitro*	Callus
Methanol extract	$49.11 \pm 0.97^{Db*}$	$142.87 \pm 1.71^{Cc***}$	$72.56 \pm 1.16^{Db**}$
n-Hexane fraction	$52.33 \pm 1.97^{Cc*}$	$131.84 \pm 2.05^{Bb***}$	$67.52 \pm 2.15^{Cc**}$
Ethyl acetate fraction	$105.67 \pm 1.56^{Ba*}$	$183.44 \pm 2.48^{Ba***}$	$118.23 \pm 2.02^{Ba**}$
Chloroform fraction	$86.32 \pm 1.89^{Dd**}$	$161.75 \pm 1.36^{Dd***}$	$60.17 \pm 1.42^{Eb*}$
Catechin	22.76 ± 0.37^{Ea}	22.76 ± 0.37^{Ea}	22.76 ± 0.37^{Ea}

Each value in the table is represented as mean ± SD ($n = 3$).
Means not sharing the same symbols (*, **, and * * *) are significantly different (Duncan) at $P < 0.05$ in the same row.
Means not sharing the same letters are significantly different (Duncan) at $P < 0.05$ in the same column.

and Fe^{2+} can be monitored through the measurement of the formation of Perl's Prussian blue at 700 nm [33].

In the reducing power assay, the presence of reductants (antioxidants) in the fractions would bring about the reduction of Fe^{+3}/ferricyanide complex to the ferrous form by giving away an electron. Increasing the absorbance at 700 nm implies an increase in its ability to reduce. The sequence for this reducing power was as follows: *in vivo* samples > callus tissue > *in vitro* samples of red clover. Some degrees of electron-donating capacity were found in all examined extracts. Among the examined extracts the methanol extract of *in vivo* samples showed the highest reducing power with the absorbance of 1.05 ± 0.07 at 700 nm, so this extract could act as electron donors and also could convert free radicals to more stable products [34], although, in comparison with the positive control (ascorbic acid), it is significantly lower (Table 7).

3.8. Correlation with Antioxidant Activities and Phytochemical Contents. Through the correlation analysis for phytochemical contents with EC_{50} values of radical scavenging activity and antioxidant ability of the extract of red clover and its various soluble fractions, the phenolic and flavonoid contents had exhibited excellent association with DPPH, superoxide, ABTS radical scavenging activities, and reducing power of *in vivo*, *in vitro*, and callus samples (Tables 8, 9, and 10). Erkan et al. [35] reported a close correlation between radical scavenging activity and total phenolic content of extract from various natural sources. Moreover, for the *in vivo* samples, EC_{50} of hydrogen peroxide presented an important correlation with phenolics while nonsignificant with flavonoids, whereas for the *in vitro* samples hydrogen peroxide demonstrated a remarkable correlation with both phenolics and flavonoids. Hydrogen peroxide of callus samples did not shed light on any correlation with phenolics and flavonoids. Chelating power of *in vivo* samples pointed to a significant correlation with flavonoids but nonsignificant correlation with phenolics while for the callus samples it was found with both phenolics and flavonoids and for the *in vitro* samples chelating power did not point to any correlation with phenolics and flavonoids. Our results are consistent with those found by Sahreen et al. [23] who reported that there is existence of a strong relationship between phytochemical contents and DPPH and superoxide and ABTS radical scavenging.

4. Conclusion

The present work shows the antioxidant effects of *in vivo* and *in vitro* grown plants and callus tissues of red clover. The results also suggested a considerable value in terms of the antioxidant activities of methanol extract and chloroform fraction of aerial parts of the *in vivo* grown red clover. The fraction's activity is due to the phenolic and flavonoid contents. It is further suggested by our results that the extract of red clover can be adopted as an effective and safe antioxidant source, despite the fact that the antioxidant activities of methanol extract and chloroform fraction of red clover were lower than that ascorbic acid and rutin as positive controls. It is safe to sum up that red clover is consumed

Table 7: Reducing power of methanol extract and soluble fraction (300 μg/mL) of *in vivo* and *in vitro* grown plants and callus tissue of red clover (*Trifolium pratense*).

Plant extract	Reducing power absorbance at 700 nm		
	In vivo	*In vitro*	Callus
Methanol extract	$1.05 \pm 0.07^{Ba*}$	$0.72 \pm 0.03^{Bb**}$	$0.82 \pm 0.03^{Cc**}$
n-Hexane fraction	$0.88 \pm 0.04^{Ca*}$	$0.52 \pm 0.04^{Cc**}$	$0.65 \pm 0.02^{Db**}$
Ethyl acetate fraction	$0.59 \pm 0.03^{Da*}$	$0.38 \pm 0.01^{Db**}$	$0.52 \pm 0.03^{Ec**}$
Chloroform fraction	$0.95 \pm 0.03^{Ca*}$	$0.71 \pm 0.04^{Bb**}$	$0.91 \pm 0.03^{Bc*}$
Ascorbic acid	2.45 ± 0.03^{A}	2.45 ± 0.03^{A}	2.45 ± 0.03^{A}

Each value in the table is represented as mean ± SD ($n = 3$).
Means not sharing the same symbols (*, **, and * * *) are significantly different (Duncan) at $P < 0.05$ in the same row.
Means not sharing the same letters are significantly different (Duncan) at $P < 0.05$ in the same column.

Table 8: Correlation between the antioxidant activity and total phenolic and flavonoid of the extract of *in vivo* grown *Trifolium pretense*.

Assays	Correlation R^2	
	Total flavonoid	Total phenolic
DPPH radical scavenging activity	0.759**	0.745**
Superoxide radical scavenging activity	0.856**	0.903**
ABTS radical scavenging ability	0.814**	0.801**
Hydrogen peroxide radical scavenging activity	0.414	0.598*
Chelating power	0.670*	0.500
Reducing power	0.884**	0.861**

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

Table 9: Correlation between the antioxidant activity and total phenolic and flavonoid of the extract of *in vitro* grown *Trifolium pretense*.

Assays	Correlation R^2	
	Total flavonoid	Total phenolic
DPPH radical scavenging activity	0.893**	0.876**
Superoxide radical scavenging activity	0.793**	0.806**
ABTS radical scavenging ability	0.825**	0.827**
Hydrogen peroxide radical scavenging activity	0.784**	0.833**
Chelating power	0.502	0.441
Reducing power	0.862**	0.862**

**Correlation is significant at the 0.01 level (2-tailed).

as a traditional medicine and food stuff in various parts of the world and that it can be used as an achievable source of natural antioxidants with consequent beneficial properties for health. There is indeed a pressing need to make available new plant-derived bioactive molecules; thus, red clover may be a great natural source for the establishment of new drugs.

In earlier reports, such properties have been named as essential for the extracts of red clover [36]. Based on the results obtained, the extracts of callus tissue had demonstrated a strong antioxidant activity but the extract of aerial parts of *in vitro* cultured red clover had demonstrated a lower antioxidant activity. Matsingou et al. [37] have reported that the differences in antioxidant properties of plants in varying biological systems may be explained by the presence of different substrates and also by the variable nature of product produced by the reaction system. It was observed that, due to a variety of antioxidant compounds present in red clover, the activity of the extract was attributed to the samples extraction method and the assay method.

Table 10: Correlation between the antioxidant activity and total phenolic and flavonoid of the extract of callus tissue of *Trifolium pretense*.

Assays	Correlation R^2	
	Total flavonoid	Total phenolic
DPPH radical scavenging activity	0.849**	0.822**
Superoxide radical scavenging activity	0.912**	0.917**
ABTS radical scavenging ability	0.903**	0.876**
Hydrogen peroxide radical scavenging activity	0.023	0.020
Chelating power	0.775**	0.734**
Reducing power	0.777**	0.753**

**Correlation is significant at the 0.01 level (2-tailed).

Our primary finding of this work was that extract of *in vitro* culture of red clover, especially the callus tissue, possesses an antioxidant activity comparable to the *in vivo* cultured plants' extract. It can be concluded that *in vitro* cultured plants are able to produce and accumulate many medicinally valuable secondary metabolites.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Micropropagation of Bioencapsulation and Ultrastructural Features of Sainfoin (*Onobrychis viciifolia*) Grown *In Vivo* and *In Vitro*

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To explore the potential of *in vitro* rapid regeneration, three varieties (Golpaygan-181, Orumieh-1763, and Gorgan-1601) of sainfoin (*Onobrychis vicifolia* Scop. syn. *Onobrychis sativa* L.) were evaluated. For the first time, an encapsulation protocol was established from somatic embryogenic callus in torpedo and cotyledonary stages to create artificial seeds. Callus derived from different concentrations of Kinetin (0–2.0 mg L^{-1}) and Indole-3-acetic acid (0–2.0 mg L^{-1}) was coated with sodium alginate and subsequently cultured either in Murashige and Skoog (MS) medium or in soil substrate. Adventitious shoots from synthetic beads developed into rooting in full and half strength MS medium supplemented with various concentrations of auxin and cytokinin. Prolonged water conservation of black and red soils (1 : 1) had the highest rate of survival plantlets in the acclimatization process. Diverse resistance techniques in *Onobrychis vicifolia* were evaluated when the plants were subjected to water deficiency. Higher frequency of epicuticular waxes was observed in *in vivo* leaves compared to *in vitro* leaves. Jagged trichomes nonsecreting glands covered by spines were only observed in the lower leaf side. Ultimately, stomata indices were 0.127 (abaxial), 0.188 (adaxial) in *in vivo* and 0.121 (abaxial), 0.201 (adaxial) in *in vitro* leaves.

1. Introduction

Despite the fact that sainfoin (*Onobrychis vicifolia*) is an important forage species, it has received little attention and assessment for *in vitro* studies. Among the attributes of sainfoin, it improves soil fertility, where the environmental conditions limit the cultivation of alfalfa, and produces safe bloat forage. Therefore, the progress of this species by genetic engineering techniques will contribute significant advantages for plant breeding objectives.

A basic prerequisite of genetic engineering is advance of an efficient adventitious shoot regeneration system for the desired species. Rapid multiplication of shoot tips is notable to reduce the cost and genetic purity of micropropagated plants. Indeed, different auxins and cytokinins concentrations in MS medium play an important role in achieving a desired rate of multiple shoot formation. Ratio of regeneration depends on culture type, composition of the medium, and the variety used [1].

Plant regeneration via somatic embryogenesis is usually investigated for important objectives of somaclonal propagation and multiplication in particular genetic transformation. In reality, the critical conversion of somatic embryos into plants is attained through maturation and germination stages [2, 3]. Nowadays, production of artificial seeds or synthetic seeds, consisting of enclosed somatic embryos or shoot buds, is a highly common propagation technique. This system is an outstanding proficiency used to propagate and preserve plants and assess many species for microshoots production from somatic embryogenesis [4]. This facile and unique propagation system deliberated by Bapat et al. [5] can be utilized on both difficult to root species and worthwhile varieties. In order to promote root induction of sainfoin and overcoming the effect of cytokinin hormones during rooting,

two auxin solutions, 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), with different concentrations have been suggested [6, 7].

In vivo cultivation is totally different compared to *in vitro* growth culture during the acclimatization process. Relative humidity (RH) is an important criterion that promotes the morphological, physiological, and biochemical features of plantlet when plantlets are transferred to *in vivo* condition for acclimatization [8, 9]. Moreover, nutrient retained in *in vitro* leaves is another important factor in the process of acclimatization [10]. The exclusive novelty of the current study is successful *in vitro* regeneration from synthetic seeds coated consisting of embryogenic callus, while previous studies evaluated the adventitious shoot regeneration from a range of explants, including mature [11] and immature embryos, root, leaf, and stems [12].

Wide varieties of plant microstructures, including light reflection and water absorption structures, have been already defined by the scanning electron microscope (SEM). The most significant threat in plant life can be referred to high temperature, because of intense radiation and decrement of water loss as a growth limiting factor. Physiological activity of land plants mainly depends on conservation of water which is carried out via plant roots. In order to hold the water and avoid the filtration of ions from interior structure in plants, a protective waxy layer called cuticle is developed that covers the epidermis cells from inside the plant [13, 14].

Whilst the intracuticular waxes are the main transport barrier to prevent the water loss and leach the molecules from inside of the living cells [15, 16], the epicuticular waxes have also an outstanding role in different plants as an interface layer. Epicuticular waxes are the cause of irritability control, self-cleaning [17], sliding of insects [18], reflection of visible light, absorption of UV radiation [19, 20], and adhesion reduction of particles [21]. Water loss might be influenced by trichomes function and affected on surface wettability [22].

In the present study, short-term stability and regeneration capacity of synthetic seeds containing embryogenic callus of *Onobrychis viciifolia* were investigated. Comparison of intact (*in vivo*) and *in vitro* leaf morphological structures based on epicuticular waxes, convex cells, and trichomes was also carried out using scanning electron microscope (SEM). Ultimately, this study suggests that evaluation of different resistance strategies of intact plant can be analyzed against water loss.

2. Materials and Methods

2.1. Explant Source. Seeds of three superior varieties (Golpaygan-181, Orumieh-1763, Gorgan-1601) of *Onobrychis viciifolia* were selected from the natural resources existing of gene bank in Iran. The best sterilization procedure of sainfoin's seeds was achieved when 50% Chlorox (outside the laminar chamber-1 min) and 70% of alcohol (inside the laminar chamber-1 min) were treated, respectively. After a couple of weeks, all seeds were almost germinated in the Murashige and Skoog medium (MS) supplemented with 3% (w/v) sucrose and 0.75% (w/v) agar. Explant sources were derived from aseptic seedlings to leaf, stem, and root segments.

2.2. Embryogenic Callus Induction. After inoculation of seeds in MS medium, stem and leaf explants of sainfoin were cut into small pieces (2-3 mm) from aseptic seedlings using a sharp sterile blade. To induce the callus, prepared explants were inoculated in MS medium fortified with different concentrations of Kinetin (0–2.0 mg L^{-1}) and Indole-3-acetic acid (0–2.0 mg L^{-1}). All explants were placed in culture room at 25 ± 1°C, 70% humidity and 16 h light photoperiod provided by cold fluorescent lamps for 3 weeks. Double staining method was used to ensure that the callus has truly regeneration capacity and contains the embryonic cells [23]. Fresh weight and percentage of produced callus from leaf and stem explants were measured. Different callus textures (compact and friable) were also evaluated after 3 weeks. Five various stages of somatic embryogenesis were observed using Dinocapture camera. In addition, stem and leaf explants produced adventitious shoots directly in some hormone concentrations which were calculated from 30 explants.

2.3. Encapsulation of Embryogenic Callus. Fresh calluses were collected in torpedo and cotyledonary stages of somatic embryogenesis from leaf and stem explants of the three sainfoin varieties (Gorgan-181, Orumieh-1763, and Gorgan-1601). Embryogenic calluses were isolated and mixed with/without 1 mg L^{-1} 6-benzylaminopurine (BAP) of autoclaved sodium alginate (5%) prepared from MS solution after adjusting the pH to 5.8. Then, the samples (3–5 mm) were dropped into solution of $CaCl_2 \cdot 2H_2O$ (1% w/v). Subsequently, the beads were retained in $CaCl_2 \cdot 2H_2O$ solution for 30 min and transferred to distilled water after the incubation period.

2.4. Germination Medium/Substrate. The beads (without BAP) of three varieties were germinated on various media and substrates: (1) MS basal medium + 3% sucrose + 0.8% agar (MSO) as control, (2) MS + 3% sucrose + 0.8% agar + 1 mg L^{-1} IBA, (3) MS + 3% sucrose + 0.8% agar + 1 mg L^{-1} NAA, (4) black sterilized soil (50% white peat + 50% black peat + 1.0 kg NPK fertilizer) + distilled water, (5) black nonsterilized soil + tap water. The beads with 1 mg L^{-1} BAP were cultured on MSO as well. All synthetic seeds were maintained in the culture room at 25 ± 1°C, 16 hours of light, and 8 hours of dark. The germination rate of artificial seeds was recorded after a couple of weeks. The beads were also cold-stored in the fridge at 4°C. Then, the beads were sown in MS basal medium for every 15-day interval to compare the viability of synthetic seeds.

2.5. Root Production and Acclimatization. Different auxins (NAA, IBA, and IAA) and cytokinin (BAP) concentrations were used to produce roots in regeneration process. Adventitious shoots (4-5 cm) obtained from synthetic seeds were transferred to full and half strength MS medium containing 3% sucrose and 0.75% agar. Cultures were preserved at 25 ± 1°C, 16 hours of light, and 8 hours of dark for one month. Then, the number of roots per shoot, callus percentage,

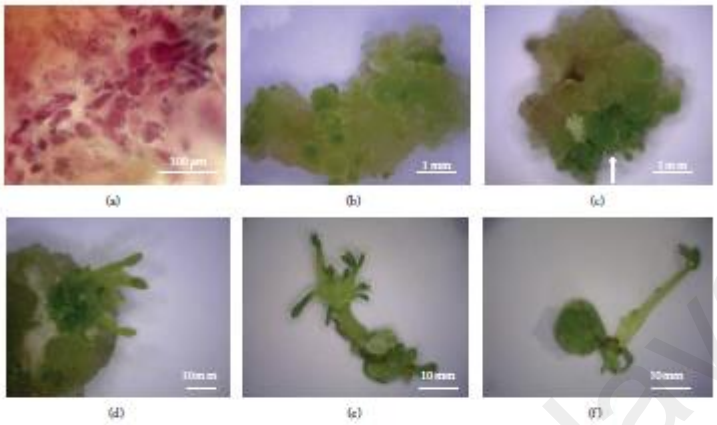

FIGURE 1: Stages of somatic embryogenesis in *Onobrychis victifolia* (a–f). (a) Embryogenic callus in double staining method with camera lens, magnification of 40x; (b) globular stage and friable callus in root explants; (c) heart-shaped stage; (d) torpedo stage; (e) cotyledonary embryo stage; (f) shoot formation.

microshoots, and dry weight of plantlets were recorded. The 4-week-old plantlets with well-developed roots were transferred to plastic pots containing different black and red (clay) soil combinations. Plantlets were maintained inside a growth chamber at 25 ± 1°C, 16-hour light for 2 weeks before being transferred to the greenhouse.

2.6. Scanning Electron Microscopy (SEM). Leaf specimens of both *in vitro* and *in vivo* grown cultures were treated with the following solutions: 1 : 1 (v/v) glutaraldehyde (4%) and phosphate buffer solution at room temperature for 1 h, phosphate buffer solution and distilled water in 1 : 1 mixture for 30 min, and osmium tetroxide (4%) at 48°C for 14 h. After rinsing the samples with distilled water, the tissues were immersed in an ethyl alcohol series (10–100%) at 15 min intervals, followed by (1) 3 : 1 ethyl alcohol and acetone for 20 min, (2) 1 : 1 ethyl alcohol and acetone for 20 min, (3) 1 : 3 ethyl alcohol and acetone for 20 min, and (4) 100% acetone for 20 min. The final step was repeated four times. The replacement of acetone with carbon dioxide was carried out several times using a critical point dryer. Eventually, the samples were coated with gold for 1 min, before the observation by SEM (JEOL 6400).

Epidermal peel was evaluated to assess trichomes on the anticlinal walls, types of stomata, epicuticular waxes, convex cells, trichomes, and stomata index of the both abaxial and adaxial leaf surfaces.

Stomata index: (total numbers of stomata/(total numbers of epidermal cells + number of stomata)).

3. Results

Leaf and stem explants of *O. victifolia* were cultured in MS media supplemented with different concentrations of Kinetin and IAA. Double staining method was used to detect and differentiate the embryogenic from nonembryogenic callus. Embryonic cells had large nuclei with dense cytoplasms which were stained bright red with acetocarmine (Figure 1(a)). Generally, callus was formed in stem and leaf explants after 2-3 weeks, respectively. Two types of compact and friable callus were observed with cream, green, and light green colors. Best Kinetin and IAA concentrations were chosen based on the highest percentage and fresh weight of callus. Callus induction of three sainfoin varieties from stem and leaf explants is shown in Tables 1 and 2, respectively. Somatic embryos were enlarged into distinct bipolar structures and passed through typical developmental stages, including globular, heart, torpedo, and cotyledonary stages (Figures 1(b), 1(c), 1(d), and 1(e)). Although callus percentage was low in the control culture, MS medium supplemented with 1.5 mg L^{-1} Kinetin and 2 mg L^{-1} IAA had the highest percentage in both stem and leaf explants.

Pregerminated torpedo and cotyledonary shaped somatic embryos were used for encapsulation (Figure 1(d)). Encapsulated somatic embryos derived from stem explants induced the highest percentage of microshoots from the Golpaygan-181 variety. Conversion into adventitious shoots increased from the beads cultured in MSO (control culture) to MS medium supplemented with 1 mg L^{-1} NAA. However, MS medium supplemented with 1 mg L^{-1} IBA had an optimum

Table 1: Effect of Kinetin and IAA on mean weight and callus percentage of three O. vicifolia varieties: Golpayegan-181, Orumieh-1763, and Gorgan-1601 (stem explants).

Kinetin (mg L^{-1})	IAA (mg L^{-1})	Weight (g)	Callus (%)	Colour	Texture	Embryo stage	Shoot/plant
0	0	$0.064^{e} \pm 0.001$	$12.00^{e} \pm 0.82$	Green	CO	PE	—
0.5	0	$0.337^{b} \pm 0.019$	$11.25^{e} \pm 0.68$	Green	CO	GL	—
1	0	$0.133^{de} \pm 0.014$	$35.00^{de} \pm 1.24$	Light G.	FR	GL	—
1.5	0	$0.312^{b} \pm 0.021$	$73.75^{ab} \pm 2.54$	Cream	FR	PE	—
2	0	$0.192^{de} \pm 0.018$	$23.75^{de} \pm 1.06$	Light G.	CO	PE	—
0	0.5	$0.088^{e} \pm 0.002$	$41.25^{de} \pm 1.32$	Light G.	FR	CT	10
0.5	0.5	$0.156^{de} \pm 0.012$	$68.33^{b} \pm 1.95$	Green	CO	GL	—
1	0.5	$1.083^{a} \pm 0.082$	$51.67^{b} \pm 1.75$	Cream	FR	GL	—
1.5	0.5	$0.155^{de} \pm 0.011$	$61.67^{b} \pm 1.84$	Light G.	FR	GL	—
2	0.5	$0.450^{b} \pm 0.028$	$68.33^{b} \pm 1.94$	Light G.	FR	CT	—
0	1	$0.032^{e} \pm 0.001$	$6.25^{e} \pm 0.14$	Green	CO	PE	—
0.5	1	$0.842^{ab} \pm 0.057$	$63.33^{b} \pm 1.65$	Light G.	FR	GL	—
1	1	$0.502^{b} \pm 0.023$	$76.25^{ab} \pm 2.21$	Light G.	CO	GL	—
1.5	1	$0.089^{e} \pm 0.002$	$71.25^{ab} \pm 2.15$	Green	CO	CT	3
2	1	$0.122^{de} \pm 0.009$	$40.00^{de} \pm 1.41$	Light G.	FR	PE	—
0	1.5	$0.203^{de} \pm 0.014$	$77.50^{ab} \pm 2.98$	Light G.	FR	GL	—
0.5	1.5	$1.003^{a} \pm 0.086$	$100.0^{a} \pm 3.21$	Light G.	FR	CT	7
1	1.5	$0.187^{de} \pm 0.019$	$57.50^{b} \pm 1.65$	Green	CO	CT	2
1.5	1.5	$0.430^{b} \pm 0.028$	$92.50^{a} \pm 3.02$	Green	CO	CT	8
2	1.5	$0.819^{a} \pm 0.068$	$78.33^{ab} \pm 2.68$	Green	CO	GL	—
0	2	$0.077^{e} \pm 0.005$	$30.22^{de} \pm 1.54$	Green	CO	PE	—
0.5	2	$0.771^{ab} \pm 0.024$	$76.25^{ab} \pm 2.45$	Light G.	FR	GL	—
1	2	$0.432^{b} \pm 0.035$	$73.75^{ab} \pm 2.42$	Green	CO	CT	—
1.5	2	$1.076^{a} \pm 0.098$	$87.50^{a} \pm 2.68$	Light G.	FR	CT	—
2	2	$0.176^{de} \pm 0.006$	$95.00^{a} \pm 3.47$	Green	CO	CT	—

The means of the populations with the same small letters were not significantly different as per Duncan's multirange test at $P < 0.05$.
CO: compact, FR: friable, PE: preembryo, GL: globular, CT: cotyledon, and G: green.

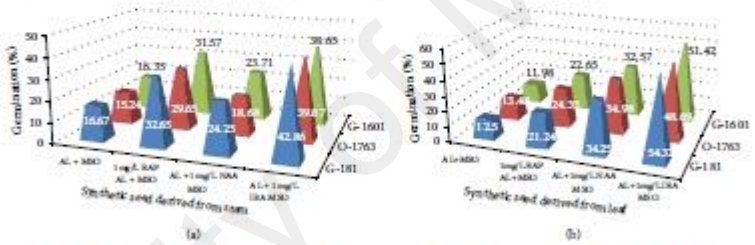

Figure 2: Percentage of germination from encapsulated somatic embryos of Onobrychis vicifolia on matrice of medium and variety: (a) stem and (b) leaf.

effect on germination rate of synthetic seeds (Figure 2(a)). The survival rate of plantlets increased significantly when the beads derived from leaf explants were cultured in MS medium supplemented with 1 mg L^{-1} IBA. In line with this, 1 mg L^{-1} BAP and 1 mg L^{-1} NAA had also the positive influence in germination rates of the synthetic seeds, respectively. The survival rates of plantlets varied from 11.98% to 54.32% in the three sainfoin varieties, with maximum survival rate of beads obtained from leaf explants, which was observed in MS medium supplemented with 1 mg L^{-1} IBA in Golpayegan-181 variety (Figure 2(b)).

Sterilized soil showed the least preferred germination substrate in both stem and leaf synthetic seeds. In this regard, Golpayegan-181 had the maximum germination with 8.97% (Figures 3(b) and 3(d)). Although the survival rate was increased from control MS medium to nonsterilized soil in stem beads, soil substrates had a lower survival percentage in the leaf synthetic seeds (Table 3). Temperature

Table 2: Effect of Kinetin and IAA on mean weight and callus percentage of three *O. viciifolia* varieties: Golpaygan-181, Orumieh-1763, and Gorgan-1601 (leaf explants).

Kinetin (mg L^{-1})	IAA (mg L^{-1})	Weight (g)	Callus (%)	Colour	Texture	Embryo stage	Shoot/plant
0	0	$0.046^{c} \pm 0.002$	$26.67^{bc} \pm 0.94$	Green	CO	PE	—
0.5	0	$0.339^{b} \pm 0.028$	$55.00^{b} \pm 1.45$	Green	CO	GL	—
1	0	$0.198^{bc} \pm 0.019$	$86.60^{a} \pm 2.35$	Light Gl.	FR	GL	—
1.5	0	$0.731^{ab} \pm 0.034$	$97.50^{a} \pm 3.58$	Cream	FR	GL	—
2	0	$0.292^{b} \pm 0.016$	$52.50^{b} \pm 1.98$	Light Gl.	FR	PE	—
0	0.5	$0.219^{b} \pm 0.024$	$70.02^{ab} \pm 2.14$	Light Gl.	CO	CT	15
0.5	0.5	$0.142^{bc} \pm 0.09$	$68.33^{ab} \pm 2.35$	Light Gl.	FR	GL	—
1	0.5	$0.639^{ab} \pm 0.034$	$85.06^{a} \pm 3.24$	Cream	FR	GL	—
1.5	0.5	$0.484^{b} \pm 0.042$	$85.20^{a} \pm 3.05$	Light Gl.	CO	GL	—
2	0.5	$0.121^{bc} \pm 0.008$	$70.47^{ab} \pm 2.45$	Cream	FR	CT	—
0	1	$0.179^{bc} \pm 0.006$	$26.25^{bc} \pm 0.98$	Green	CO	PE	—
0.5	1	$0.489^{b} \pm 0.025$	$73.33^{ab} \pm 2.45$	Light Gl.	FR	GL	—
1	1	$0.291^{b} \pm 0.018$	$85.51^{a} \pm 2.87$	Light Gl.	CO	CT	—
1.5	1	$0.163^{bc} \pm 0.012$	$78.75^{ab} \pm 2.34$	Green	CO	CT	6
2	1	$0.217^{bc} \pm 0.015$	$68.33^{ab} \pm 2.17$	Light Gl.	FR	GL	—
0	1.5	$0.174^{bc} \pm 0.016$	$82.50^{ab} \pm 3.78$	Light Gl.	FR	CT	14
0.5	1.5	$0.826^{ab} \pm 0.057$	$100.0^{a} \pm 3.45$	Light Gl.	FR	CT	7
1	1.5	$0.311^{b} \pm 0.027$	$73.75^{ab} \pm 3.15$	Green	CO	CT	2
1.5	1.5	$0.724^{ab} \pm 0.065$	$96.25^{a} \pm 3.54$	Green	CO	GL	—
2	1.5	$1.182^{a} \pm 0.102$	$76.67^{ab} \pm 2.97$	Green	CO	GL	—
0	2	$0.051^{c} \pm 0.002$	$11.25^{c} \pm 0.54$	Green	CO	PE	18
0.5	2	$1.015^{a} \pm 0.098$	100.0^{a}	Light Gl.	FR	CT	—
1	2	$0.288^{b} \pm 0.017$	$92.50^{a} \pm 2.54$	Green	CO	GL	—
1.5	2	$1.351^{a} \pm 0.114$	$95.00^{a} \pm 2.58$	Light Gl.	FR	CT	—
2	2	$0.266^{b} \pm 0.016$	$88.33^{a} \pm 2.41$	Green	CO	GL	—

The means of the populations with the same small letters were not significantly different as per Duncan's multirange test at $P < 0.05$.
CO: compact, FR: friable, PE: proembryo, GL: globular, CT: cotyledon, Gr: green.

Table 3: Effect of storage durations and soil substrates on mean synthetic seed germination of control condition[*].

Varieties	Nonsterilized soil (%)	Sterilized soil (%)	Storage at 4°C		
			15 days	30 days	45 days
Leaf					
Golpaygan-181	10.25 ± 0.78	3.21 ± 0.12	6.24 ± 0.59	0.98 ± 0.08	—
Orumieh-1763	9.65 ± 0.67	4.87 ± 0.23	5.14 ± 0.46	0.35 ± 0.02	—
Gorgan-1601	6.23 ± 0.46	1.29 ± 0.11	3.24 ± 0.21	—	—
Stem					
Golpaygan-181	24.68 ± 1.35	8.97 ± 0.68	9.87 ± 0.12	3.54 ± 0.01	—
Orumieh-1763	23.54 ± 1.42	5.24 ± 0.58	6.74 ± 0.06	1.87 ± 0.01	—
Gorgan-1601	19.74 ± 1.28	3.66 ± 0.19	3.34 ± 0.02	—	—

[*]Control condition: sodium alginate + MS medium.

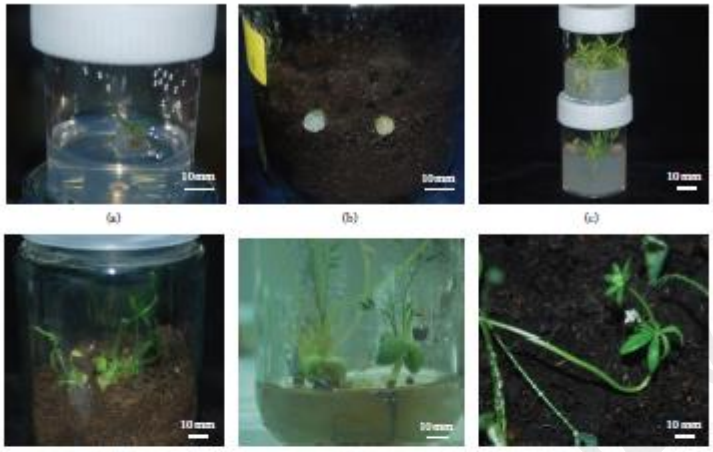

Figure 3: Micropropagation of *O. victifolia*: (a) synthetic seeds in MS medium, (b) artificial seeds in black soil, (c) microshoots induction from synthetic seed, (d) seed germination in black soil, (e) adventitious roots induction, and (f) acclimatization and complete plantlets of *O. victifolia*.

and storage period are important factors to determine the regeneration frequency of the encapsulated somatic embryos. Approximately, 50–60% viability of stem and leaf synthetic seeds fell after 15-day storage at 4°C (Table 3).

Root production was a difficult stage after adventitious shoot induction in sainfoin synthetic seeds. MS medium supplemented with IBA showed no significant root production in this recalcitrant species. Concentrations of IBA and NAA with BAP did not induce high adventitious roots as well. Micropropagated shoots induced 52.62% root formation in half strength MS medium supplemented with 1 mg L^{-1} NAA. Shoot cultured on full MS medium supplemented with 1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP had the highest percentage of rooting with 82.35% (Figure 3(e)). Among the three types of auxin, NAA was superior in comparison with IBA and IAA in terms of root number induction per shoot (Table 4).

The survival percentage of plantlets was affected by different soil substrates. It was revealed that black soil had lower efficiency as compared with red soil in acclimatization stage (Figure 3(f)). Combined substrates of red and black soils (ratio 1 : 1) had the highest survival percentage (98%) of plantlets (Table 5). Subsequently, plantlets were transplanted to the greenhouse with 100% survival rate and grown to 30 cm after 2 months.

Epidermal peels of *in vitro* and *in vivo* (intact plant) leaves were studied thoroughly. In order to achieve this, properties of both lower (abaxial) and upper leaf sides (adaxial) were assessed. Anticlinal walls and polygonal epidermal cells were exposed in the abaxial leaf sides of *in vivo* and *in vitro* (Figures 4(a) and 5(a)). Additionally, basic outline of the epidermal cells was elongated to polygonal cells with more than four edges, whereas cell boundaries were U-undulated in both *in vivo* and *in vitro* adaxial leaves (Figures 6(a) and 7(a)). Jagged trichomes nonsecreting glands covered by spines (botanically thorns) were only observed in the lower leaf side. Mean length of trichome in *in vitro* leaves was more than that in *in vivo* leaves (Figures 4(d) and 5(d)). Cuticle folding was induced by an undulated morphology of the underlying cellulose cell wall. Unlike the epidermal cells, folding or tubercular (verrucate) patterns were recognized in trichomes (Figures 4(d) and 5(d)). The cell sculptures or curvature of the outer epidermis wall (periclinal wall) has a great influence on the surface roughness in the micrometer scale.

Among the three basic forms of cell curvatures (tabular, convex, and concave), convex cells shaped cupolas were observed on the adaxial epidermal surface of both *in vivo* and *in vitro* leaves (Figures 6(b) and 7(b)). Some research has demonstrated that the impact of water loss leads to collapse cells. Along this line, sufficient humidity and water were the cause of convex cells shrinking in *in vitro* leaves (Figure 6(c)). Moreover, hierarchical surface structures including cuticle folding were not observed in the convex cells.

In the classification of wax morphologies, several three-dimensional structures such as crusts, threads, plates,

Table 4: The responses of multiple shoots derived from encapsulated seeds on MS medium supplemented with different auxins and cytokinins concentrations.

MS + hormones (mg L^{-1})		Observations (%)				Number	
	BAP	Shoot	Callus	Necrosis	Root	Root/plant	Shoot/plant
Control		NR	86.47	12.24	NR	—	—
IBA							
1	0.5	72.24 ± 3.24	22.48 ± 1.12	2.34 ± 0.15	NR	—	18
2	0.5	48.76 ± 1.57	33.37 ± 1.57	16.66 ± 1.10	3.23 ± 0.24	1	12
0.5	—	NR	23.24 ± 1.68	75.94 ± 2.68	NR	—	—
1	—	NR	78.23 ± 2.87	5.35 ± 0.24	12.84 ± 1.25	2	—
0.25	0.25	24.26 ± 1.14	12.35 ± 0.27	52.95 ± 1.68	NR	—	14
2	—	NR	42.35 ± 1.22	32.95 ± 1.27	18.24 ± 1.36	2	—
NAA							
1	0.5	NR	NR	12.48 ± 0.95	82.35 ± 2.68	3	—
0.5	—	21.35 ± 1.24	72.14 ± 3.07	5.87 ± 0.14	NR	—	7
0.25	—	NR	10.24 ± 0.68	48.41 ± 1.26	42.68 ± 1.63	2	—
0.5	—	NR	28.35 ± 1.34	61.84 ± 2.65	10.24 ± 1.15	—	—
2	—	NR	9.87 ± 0.36	35.24 ± 1.14	58.95 ± 1.74	—	—
IAA							
0.25	0.25	6.24 ± 0.24	46.23 ± 1.84	2.45 ± 0.14	47.68 ± 1.36	4	4
1	0.25	NR	66.24 ± 2.16	32.54 ± 1.36	NR	—	—
2	0.5	NR	75.62 ± 3.27	12.87 ± 1.08	NR	—	—
1	0.5	NR	72.32 ± 3.16	15.84 ± 1.24	NR	—	—
1	0.25	12.35 ± 0.47	32.24 ± 1.47	10.23 ± 0.84	46.98 ± 1.42	2	6
2	—	NR	58.62 ± 1.88	42.32 ± 2.15	NR	—	—
1	—	NR	42.35 ± 1.65	48.95 ± 2.06	NR	—	—
Half strength							
1 mg/L IBA	—	NR	42.65 ± 2.14	10.25 ± 1.02	48.01 ± 1.55	3	—
1 mg/L IAA	—	78.95 ± 2.57	NR	12.52 ± 1.24	10.65 ± 0.84	1	11
1 mg/L NAA	—	NR	23.65 ± 1.06	24.51 ± 1.28	52.62 ± 2.47	2	—

NR = no response was obtained.

Table 5: Acclimatization of plantlets of *O. viciifolia* in different soil substrates.

Methods	Observations	Survival rate (%)
Autoclaved black soil + 1/2 MS	Most plantlets were weak with low vigour	45%
Non-autoclaved black soil	Most plantlets became weak after 3 weeks	65%
Non-autoclaved red soil	Normal growth but some plots contaminated due to high humidity	72%
Non-autoclaved black soil : red soil at ratio 1 : 1	The best situation of water adjustment, normal growth	98%

platelets, filaments, rods, and tubules have been distinguished. Both *in vivo* and *in vitro* leaves had 3D and platelet waxes on their epidermal cells (Figure 7(c)). Platelet waxes on adaxial side were more than the underside of leaf in both growth cultures. In reality, the epidermal surface of *in vivo* leaves was exposed to a higher amount of platelet waxes in comparison with *in vitro* leaves in a specific pattern around stomata and subsidiary cells (Figures 5(c) and 7(c)).

Some microstructures of epidermal cells arising from subcuticular inserts of mineral crystals were identified in upper leaf side which were clear in intact specimens. In this manner, stomata and their surrounding cells had a micropattern of small enhanced spots, formed by subcuticular inserts of calcium oxalate (Figure 5(b)). To regulate both the water evaporation and gas exchanges, leaves developed specialized breathing pores called stomata which were anomocytic in this research (Figure 6(d)). Stomata indices were 0.127 (abaxial), 0.188 (adaxial) in *in vivo* and 0.121 (abaxial), 0.201 (adaxial) in *in vitro* leaves, respectively. Stomata interrupted the cuticular layer but could be closed (intact plants) when the humidity and water reduced in high temperature days of *in vivo* growth culture (Figures 5(b) and 7(d)). However, this barrier limits

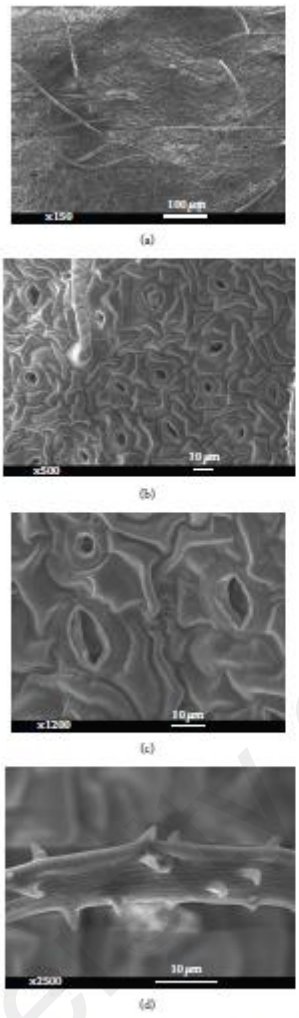

FIGURE 4: Abaxial side of *in vitro* leaf: (a) basic outlines of epidermal cells, (b) elongated polygonal cells with more than four edges, (c) open anomocytic stomata, and (d) folded jagged trichomes.

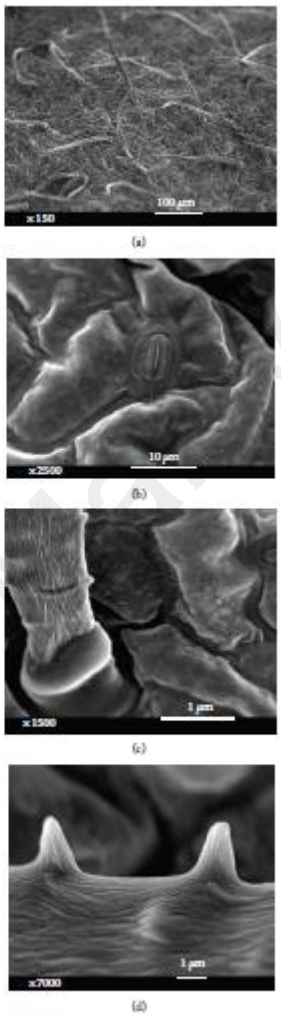

FIGURE 5: Abaxial side of *in vivo* leaf: (a) basic outlines of epidermal cells, (b) micropattern of small enhanced spots, formed by calcium oxide, (c) basal cell and stalk cell, and (d) folded jagged trichomes.

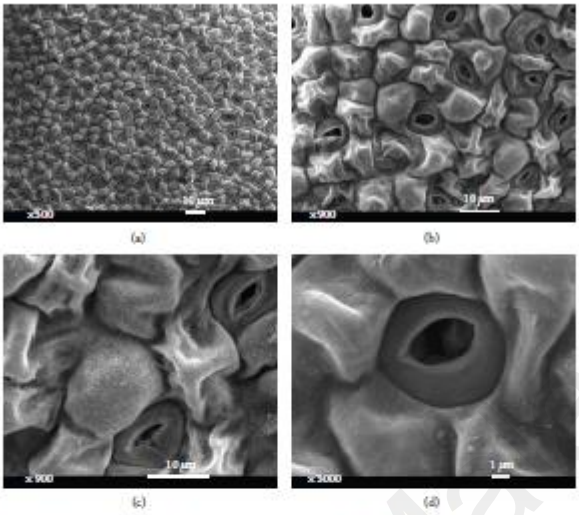

FIGURE 6: Adaxial side of *in vitro* leaf: (a) basic outlines of epidermal polygonal cells, (b) convex cell form with irregular cuticular shrinking, (c) guard cells and inner wall, and (d) open anomocytic stomata.

the uptake of carbon dioxide for photosynthesis from the atmosphere.

4. Discussion

Sainfoin seed production is not economical for farmers, since it should be harvested at flowering stage when the crop has the highest yield and best fodder quality. In order to overcome this situation, synthetic seeds technology might be the solution, due to the fact that the cost of seed production can be lowered through synthetic seed method compared to graining. Artificial seed induced through tissue culture is free from pathogens. Therefore, avoiding the bulk transportation of plants, quarantine, and spread of diseases are significant advantages of encapsulated propagules. In this study, either propagation of *Onobrychis viciifolia* was obtained in the large number or genetic uniformity of plants was preserved. Vegetative propagation method is recommended highly for preservation of uniformity and unique characteristics of sainfoin, while sexual propagation methods make heterogeneity varieties due to the outbreeding nature of this species [24].

Callus induction was achieved on MS medium supplemented with different concentrations of Kn and IAA. In this regard, the obtained embryogenic callus can be considered as the source of explants for further experiments. Encapsulation technique has sufficient potential for adventitious shoot production with high germination rate, which has been successfully applied in some species, like sandalwood, *Valeriana wallichii*, *Guazuma crinita*, and *Paulownia elongate* [25, 26].

Although a number of plants produced adventitious roots spontaneously in tissue culture, sainfoin lacks efficient root systems in *in vitro* culture. Therefore, rooting procedure from the shoot was carried out in a separate step by subculturing in full and half strength MS medium containing different concentrations of auxin and cytokinin. Root initiation process is critical mainly to provide sufficient stimulus by the concentration of required hormones. High doses of cytokinin used in the current study prevented the adventitious shoots for the normal rooting proliferation. The main restrictive parameters in vegetative propagation of sainfoin have been reported with very low frequency to be the establishment of rooted plantlets [6, 7]. Most of the previous researches used full strength MS medium supplemented with NAA to induce root [27, 28]. Besides, excised rooting from adventitious shoots was studied in both half and full strength MS medium in the present research. Subsequently, the highest rooting percentage was identified in full strength MS medium supplemented with 1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. Prolonged water conservation of red soil was significantly higher than black soil to increase the survival rates of plantlets in acclimatization. Deficit of

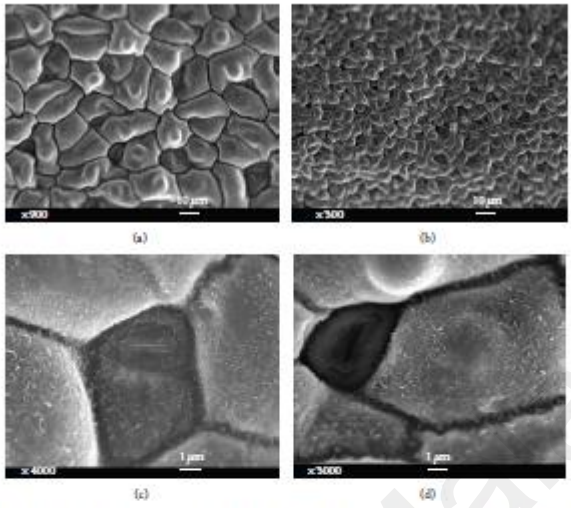

FIGURE 7: Adaxial side of *in vivo* leaf: (a) outlines of U-undulated and convex cell, (b) basic outlines of epidermal polygonal cells, (c) close anomocytic stomata, and (d) three-dimensional structures waxes.

water storage in black soil, which is required for further photosynthesis process, was inevitably compensated via the function of *in vivo* leaves. Since the black soil was lack of appropriate texture to maintain water, plantlet leaves were severely affected to wilting and necrosis of leaf blades. Despite the fact that plantlets indicated the notable survival rate in the red soil, equal mix ratio of black and red soil was recommended for acclimatization with 98% success rate.

It was observed that sainfoin beads were not cold resistant due to the low percentage of germination after storage at 4°C. In reality, other techniques should be contemplated to preserve the sainfoin artificial seeds from cold tension. Elvax 4260 (ethylene vinyl acetate acrylic acid terpolymer, Du Pont, USA) inoculation can be suggested to prevent the rapid water loss when the artificial beads are exposed to the surrounding environment [29].

Water transpiration is a natural and self-cooling mechanism for plants [30]. Subsequently, to minimize the water loss during drought season, leaves of nonsucculent plants use various mechanisms. In this respect, carbon dioxide absorption is raised and consequently photosynthesis process is enhanced due to the existence of stomata in amphistomatous (stomata on both sides) leaves [31]. To explore more clarifications, diverse resistance techniques used by *Onobrychis victifolia* were evaluated when the plants were subjected to water deficiency. Transpiration barrier feature of leaves originates from hydrophobic material made up by a polymer called "cutin" and integrated and superimposed lipids called "waxes" [32, 33]. Additionally, to lessen the water deprivation, the cuticle prevents leaching of ions from inside the cells to outside. In fact, the cuticle and waxes are the main mentioned factors in transpiration barrier. Gibson [34] stated that thicknesses of both cuticle and waxes were increased in order to reduce the water loss in the dry regions. Similar observations in our research indicated that epicuticular waxes had higher frequency in *in vivo* leaves compared to *in vitro* leaves.

Convex cells morphology of microstructured surface which is found on the leaves and stems of flowering plants is originated by expansion of the outer side (periclinal wall) of the epidermis cells [35, 36]. Based on the comparison of *in vivo* and *in vitro* leaves, cells shrinking were induced by water loss due to convex cell morphology and sufficient water of MS media in *in vitro* growth culture (Figure 6(b)). Convex cells that contained water were observed in the epidermal layer of *in vivo* leaves when the water was scarce in soil (Figure 7(a)). Water evaporation rate was controlled by opening (Figure 6(b)) and closing (Figure 7(c)) functions of stomata. Closing of stomata took place in order to reduce the water evaporation when plants could receive insufficient

amount of water through the roots (Figure 7(c)). Adversely, stomata were opened when the gas exchange process was the main objective (Figure 6(d)).

The functions of trichomes are to protect the plants from herbivores, heat, and sunlight. They also control leaf temperature as well as water loss through glandular trichomes. They produce various substances, which are stored at the plant surface. Moreover, leaf trichomes can protect plants against drought by reducing absorption of solar radiation, which in turn reduces the heat load and minimizes the need for transpirational cooling. Tolerance to drought can be also related to plant traits such as shoot and root morphology, root/shoot ratio, leaf wax production, the leaf area to volume ratio, and leaf area per stem [36, 37]. The cuticle folding of tissue was observed in the trichome (Figure 5(d)). Plant cuticle folds are used to (1) stabilize thin cell walls, (2) decrease wettability with water and contamination by both cuticle hydrophobicity and its microscopic sculpture (contact area reduction), and (3) set up surface reflection properties [38, 39]. Physiological and mechanical characteristics of species are influenced by bioactive elements such calcium [36]. Calcium oxalates are widespread in plants, including both dicotyledons and monocotyledons. They may represent storage forms of calcium and oxalic acid, and there has been some evidence of calcium oxalate resorption at times of calcium depletion. Fauteux et al. [40] reported the resistance increment of plants against pathogenic fungi by silica function. In the current study, calcium oxalates were clearly observed in intact leaves, but not in leaves of *in vitro* grown plants (Figure 5(b)).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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