ESTABLISHMENT OF *IN VITRO* HAPLOIDS OF TEA (*CAMELLIA* SPP) AND THEIR POTENTIAL FOR THE PRODUCTION OF SECONDARY METABOLITES

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I have great pleasure to dedicate this thesis to my parents, brother, sister and my revered teachers.

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STATEMENT

I do, hereby, declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the supervision of Dr Rakhi Chaturvedi.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: June 24, 2013 **Vijay Kumar Mishra**

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CERTIFICATE

It is certified that the work described in this thesis entitled "***Establishment of in vitro haploids of Tea (Camellia*** **spp*****) and their potential for the production of secondary metabolites***" by Vijay Kumar Mishra for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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ABSTRACT

The tea plant is a tropical, evergreen plant of the family Theaceae and genus *Camellia*. It is one of the most economically important beverage crops in the world. Chinese were the first to use tea as medicinal drink, later as beverage and have been doing so far for more than 3000 years. The original home or 'the primary centre of origin' of tea was South-East Asia. The cultivated taxa of tea comprise three main types, which are mainly differentiated on the basis of their leaf size. Assam type has the biggest leaf size while China type has the smallest. The Cambod type has leaf in between Assam and China types. Tea plant is a highly cross pollinated and genetically complex species. The genus Camellia includes more than 325 species which indicates genetic instability and high out-breeding nature of the genus. There are 600 cultivated varieties worldwide with unique traits, such as high caffeine content, drought tolerance, blister blight disease tolerant etc. In spite of having valued properties, improvement of tea by conventional methods is very laborious and time consuming and owing to its highly heterozygous nature and long reproductive cycle. In this context, it is noteworthy that, studies utilizing gametophytic cells are in infancy, in this tree species. *In vitro* haploid production from gametophytic cells enables the establishment of completely homozygous lines in a shortened time frame compared to conventional methods and has many potential applications in plant improvement to establish inbred lines rapidly and to observe recessive traits.

Therefore, the aim of the present study was to establish in vitro androgenic lines of tea from anthers. Androgenic haploids were produced by anthers, cultured at early-to-late uninucleate stage of pollen. Haploid development occurred via callusing of microspores. TV21 and TV19 cultivars were regenerated and developed to haploid plants. Further, the androgenic lines were checked for the production of medicinally important compounds, such as (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophylline. These compounds are observed to be present in maximum amount in young leaves of parent plants, followed by in vitro embryos and the least in calli. The presence of compounds has been confirmed by chromatographic and spectroscopic techniques. Antioxidant activity assays of in vitro androgenic cultures were also investigated and found that hot water extract (80ºC for 20 min.) shows maximum activity. Batch kinetics

study in cell suspension cultures of TV21 cultivar was also performed and found that the production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophylline was observed to be growth associated and increased with an increase in fresh weight of the cells.

The thesis is divided into five chapters. Chapter 1, introduces and reviews all the major contributions and studies perform on tea until now, with regard to tissue culture, secondary metabolite production and antioxidant activity in tea. Chapter 2, contains all the protocols and methodologies used for the present work. Chapter 3, presents the results obtained in the current investigation. The tables and graphs are included within the text while all the figures have been compiled at the end of the thesis, in the form of plates. Inferences drawn from the results are discussed in chapter 4, in the light of other reports available on related aspects. Chapter 5, throws light on the major highlights of the present work and its future scope. This is followed by the appendix that mentions the taxonomic classification of the plants studied. The thesis concludes with the list of bibliography and visible research output in terms of peer-reviewed journal publications, book chapters and conference proceedings.

Indian Institute of Technology Guwahati

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LIST OF ABBREVIATIONS

ABA	➢	Abscisic acid
BAP	➢	N^6-benzylaminopurine
C	➢	(+)-Catechin ((2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol)
CAF	➢	Caffeine (1,3,7-Trimethyl-3,7-dihydro-1H-purine-2,6-dione)
CM	➢	Coconut milk
CV	➢	Coeficient of variation
2,4-D	➢	2,4-dichlorophenoxyacetic acid
DNA	➢	Deoxyribonucleic acid
DPPH	➢	2,2-diphenyl-1-picrylhydrazyl
DW	➢	Dry weight
EC	➢	(−)-Epicatechin ((2*R*,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-trio)
EGCG	➢	(−)−Epigallocatechin gallate ((2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate)
ESI	➢	Electrospray ionization
FDA	➢	Fluorescein diacetate
FL	➢	Fluorescence
FW	➢	Fresh weight
GA_3	➢	Gibberellic acid
HCl	➢	Hydrochloric acid
$HgCl_2$	➢	Mercuric chloride
HQ	➢	8-Hydroxyquinoline
HPLC	➢	High performance liquid chromatography
2-iP	➢	2-isopentenyl adenine
IAA	➢	Indole-3-acetic acid
IBA	➢	Indole-3-butyric acid
Kinetin (Kn)	➢	6-furfurylaminopurine
MS	➢	Murashige and Skoog's medium

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N_6	➢	Chu medium
NAA	➢	α-naphthaleneacetic acid
NaClO	➢	Sodium hypochlorite
NOA	➢	α-naphthoxyacetic acid
PI	➢	Propidium iodide
PVP	➢	Polyvinylpyrrolidone
RAPD	➢	Random amplification of polymorphic DNA
RNA	➢	Ribonucleic acid
Rt	➢	Retention time
SD	➢	Standard deviation
SDW	➢	Sterile distilled water
SEM	➢	Scanning electron microscopy
T	➢	Theophylline (1,3-dimethyl-7H-purine-2,6-dione)
TBA	➢	Tertiary butyl alcohol
t-CA	➢	Trans-cinamic acid
TDZ	➢	Thidiazuron
TPB	➢	Tetraphenylboron
TV	➢	Tocklai vegetative
UV	➢	Ultraviolet
WPM	➢	Woody plant medium

LIST OF UNITS

%	➢	percentage
μg/g	➢	microgram per gram
μl	➢	Microlitre
μm	➢	Micrometer
μM	➢	Micromolar
°C	➢	degree Celsius
cm	➢	centimeter
g	➢	gram
h	➢	hour
mg/g	➢	milligram per gram
mg/L	➢	milligram per liter
mg/ml	➢	milligram per milliliter
min	➢	minute
mm	➢	millimeter
mM	➢	millimolar
nm	➢	nanometer
rpm	➢	revolution per minute
SD	➢	standard deviation
μ	➢	specific growth rate
%RSD	➢	percent relative standard deviation

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Chapter 1

Introduction and Literature Review

INTRODUCTION

The majority of the people in the world consume non-alcoholic beverages from natural resources, namely, tea, coffee and cocoa. Among all, tea is the most important drink for two-thirds of the world population due to its pleasant aroma, taste and health benefits (Mondal et al., 2004; Chaturvedula and Prakash, 2011). It is a safe and affordable drink for all sections of the society. Tea is made from the leaves and buds of *Camellia sinensis* (L.) and has been safely consumed for thousands of years. It has attracted attention of people in medical community in the last few decades. This is because of the scientific evidence, indicating preventive action in a number of human ailments (Chopade et al., 2008). The tea plant, *Camellia sinensis* (L.), is a member of the Theaceae family. It is a woody, perennial plantation crop (**Figure 1 A and B**), with an economic life span of more than 60 years (Barua, 2008; Mondal, 1990).

Tea plant requires a warm, humid summer and cold winter. Climate of the tropics with a considerable amount of rainfall (above 2000 mm per year) is favorable for its plantation. The highest ambient temperature rarely rises beyond 32ºC even in the hottest time of summer and winter, minimum up to an altitude of above 2000 m, remains above freezing (Barua, 2008). Tea has become one of the leading agro-based, eco-friendly, labor-intensive, employment generating, export-oriented industries in all the tea-growing nations. It is an evergreen shrub that includes around 82 species (Banerjee, 1992).

1.1. HISTORICAL IMPORTANCE, ORIGIN AND DISTRIBUTION

The original home or “the main centre of origin” of tea is South-East Asia which is approximately the point of intersection of latitude 29°N and longitude 98°E near the source of the river of Irrawaddy, the point where currently the lands of Assam, North Myanmar, southwest China and Tibet meet (Wight, 1959). Tea is closely linked with

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Asia, herbal medicine and Buddhism. Tea was first used as a medicinal drink by Chinese, later as a beverage, and they have been doing so for the past 5000 years (Eden, 1958). It is believed that almost 4000 years ago around 2737 BC, tea plants have been discovered accidentally by the great herbalist, 'Divine Healer' and Chinese legendary emperor Shen nung (Ukers 1935). Gradually due to medicinal values, the demand for tea in China enhanced, which resulted in cultivation of tea plant in Sichuan province about 3000 years ago. Later, the knowledge of tea cultivation was popularized everywhere with fine arts of Buddhism. Although, in India, C. A. Bruce was the discoverer of wild tea plants in Assam during 1823, G. J. Gordon in 1836 brought seeds from China for establishing a commercial garden. Later, C.A. Bruce was appointed for supervising the government tea plantations. He took active interest in the indigenous tea plants. Shortly after that, commercial interests turned on in tea plantations. Then the world's first privately owned tea company (Assam Tea Company) was established in February 12, 1839, with the directives from the British Parliament. This was the foundation of present day tea industry in India.

Another centre of origin is considered to be located in southeast China, Mizoram and Meghalaya (Kingdon-Ward, 1950). These areas are, therefore, considered to be the zone of origin and dispersion of the genus *Camellia* as a whole (Sealy 1958). However, presently tea cultivation is spread within the latitudinal range of 45°N–34°S. In the early part of the eighth century, tea was introduced to Japan from China. In seventeenth century tea cultivation was extended to Indonesia from Japan. In 1839, tea was first planted in Sri Lanka by sowing seeds which were brought from Kolkata, India. Tea cultivation started in USSR (Union of Soviet Socialist Republics) when seeds were imported from China towards the end of last century. During 1939-1940, seeds were exported to Turkey from USSR. Captain Goff of East India Company, in 1740, introduced tea for the first time in Europe. In 1768, the first successful introduction of tea was achieved by a British merchant and naturalist John Ellis (Aiton 1789; Booth 1830). From Europe cultivation of tea spread to the African countries at the end of the 19th century. The tea crop has been introduced to other parts of the world with diverse climatic conditions from its native habitat ranging from a Mediterranean-type climate to hot humid tropical and subtropical regions from as far north as Georgia (42° N), and as

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far south as Argentina (27° S) and between sea level and 2,460 m altitude. Presently, more than 52 countries produce tea (Mondal, 2009) and the main producers are China, India, Sri Lanka, Kenya, Indonesia, Turkey, Iran, Georgia, Japan, Vietnam, Bangladesh, Argentina, Malawi, Uganda and Tanzania.

1.2. BOTANICAL ASPECTS

Total 82 species of the genus *Camellia* have been described (Sealy, 1958), but other system of taxonomic classification have also been proposed. Chang (1981, 1998) described 284 species, and Ming (2000) documented 119 species. Out of all these *Camellia* species, only three have contributed particularly to the entire genetic pool of the cultivated tea worldwide (Sharma et al., 2010):

- Assam type, *C. assamica* ssp. *assamica* (Masters);
- China type, *Camellia sinensis* (L.) O. Kuntze; and
- Cambod type, *C. assamica* ssp. *lasiocalyx* (Planch MS).

Barua (1963) described morphological and anatomical description of three races of tea; which were later elaborated by Bezbaruah (1971). A summary of morphological features of these three types of tea, using White's nomenclature is mentioned below:

i. Assam type (*C. assamica* spp. *assamica* (Masters): By habit, Assam type tea plants are 10 -15 m in height. It has a very robust branching system. Length of the trunk is about one third of its height. Leaves are large, thin, glossy with more or less acuminate apex having distinct marginal veins and broadly elliptic leaf blades that are generally 8-20 cm long and 3.5-7.5 cm wide. Flower is single or paired pedicellate with smooth and green scars of three bracteoles on cataphyllary axils, numerous stamens, 5-6 persistent unequal sepals with 7-8 white petals. Occasionally, pale yellow pigmentation may be seen at the base of petals.

ii. China type (*C. sinensis* (L) O. Kuntze): China type tea is a big shrub (1-3 m tall) with many virgate stems arising from the base of plant. Leaf is hard, thick with a matt leathery surface and sunken stomata. Marginal veins are indistinct and appear sunken in lamina. Leaf blade is elliptic in shape with obtuse or broadly obtuse apex. Petioles are short and stout which gives the leaf an erect pose. Pedicellate flowers are borne singly or

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in pairs in the cataphyllary axils. Pedicel is 6-10 mm long, clavate, glabrous with 2-3 subopposite scars. Petals are 7-8 in numbers, showing cup shaped 1.5-2.0 cm long and broad-oval to sub-orbicular shapes and generally white in color. Style are 3-5 in number and generally free for greater part of their length but, occasionally free, up to the base of the ovary. Capsules have 1, 2 or 3 locules. This locule is nearly spherical about 10-15 mm in diameter and contains 1-3 nearly spherical seeds.

iii. Cambod type (*C. assamica* spp. *lasiocalyx* (Planch MS)): Cambod type tea is also known as *Cambodiensis* or Southern form of tea. Cambod type is a small fastigiated and upright tree (6-10 m tall), with several, almost equally developed branches and more or less erect, glossy, light green to coppery-yellow or pinkish red leaves, the size of which is intermediate between *C. sinensis* and *C. assamica*. Although the flowers are more or less similar to the Assam type, they have 4 or more bracteoles found on the pedicels, 3–4 ovaries, sometimes with 5 locules and 3–5 styles that are free up to half the length.

A summary of special anatomical and chemical characteristics of all the three types of tea races are described in **Table 1.1**.

Table 1.1: Distinguished anatomical features and chemical composition of the three type of tea (Barua, 2008).

Anatomical/chemical characters	Assam type	China type	Cambod type
Sclereids	Numerous	Absent or rare	Numerous
Sclereids shape	Stout	Slender	Stout
Spicules in sclereids	Few spicules	Almost without spicul	Numerous spicules
Lumen of sclereids	Irregular width, close in place	Almost completely closed	Irregular width but not closed
Triglycosides	Absent	Present in fairy large amount	Absent
Unknown phenolic substance (designated as IC)	IC absent	IC absent	IC present in fairly large amount

The chromosome number in tea was determined by many investigators (Morinaga et al., 1929; Janaki Amal, 1952; Ackerman, 1971; Bezbaruah, 1971). Except for a few natural

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triploids and polyploids reported by Simura (1935), Janaki Amal (1952) and Bezbaruah (1971),, the cultivated tea plant is diploid (2n=30; basic chromosome number, X=15) (Barua, 2008). The previous authors used root-tip methods for mitotic studies which required digging up the roots. In order to avoid this, Bezbarua (1968) used shoot-tip method for examination of mitotic chromosomes in tea and other related species. In 1971, Bezbarua investigated detailed karyotype of 30 tea clones belonging to three races of tea. The somatic chromosome number of 30 was common to all 30 clones. The chromosomes of tea are short but there is gradation in size from longest homologous to shortest pair. Karyotype of tea chromosomes ranges from 1.28 μ to 3.44 μ (Bezbarua, 1971). Naturally evolved polyploidy is very rare in tea. Only a few natural triploids (2n=45) (e.g. TV 29, UPASI-3), tetraploids (2n=60), pentaploids (2n=75) and deviation from natural normal chromosome number (2n±1) have been identified among the natural tea populations (Singh, 1980; Zhan et. al., 1987).

1.3. ECONOMIC IMPORTANCE, HEALTH AND OTHER BENEFITS

Tea is the second most widely consumed beverage in the world after water (Chaturvedula and Prakash, 2011). Earlier, tea was used as a medicine and subsequently as a beverage (Kuroda and Hara, 1999; Suzuki et al., 2012). The phase of research of the present hour shows a future potential of the plant as an important raw material for the pharmaceutical industry (Mondal et al., 2004). From various manufacturing processes more than 300 different kinds of tea can be produced using the tea leaves. Based on different mode of processing, mainly the extent of fermentation, tea is usually divided into three basic types: green tea (non-fermented), oolong tea (semi fermented) and black tea (fully fermented) (Mondal et al., 2004; Sang et al., 2011). Worldwide production of black tea is around 78 %, whereas green tea, mainly consumed in China and Japan, constitutes around 20%. Partially fermented oolong tea is produced around 2 %. The fermentation of tea results in oxidation and polymerization of polyphenols, changing the nature of the chemical constituents of tea leaves and forming theaflavin and thearubigin. These polyphenols are responsible for the briskness, strength, color, taste, aroma, and pungency associated with black tea. The infusion of black tea is bright red or copper color and has astringent taste and characteristic aroma (Chaturvedula and Prakash, 2011). Tea leaves

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are also used as vegetables like the "leppet tea" in Burma and "meing tea" in Thailand. *C. sinensis* seeds yield about 17.3 % of oil as compared to that of *C. sasanqua* and *C. japonica* having 58 % and 66 % oil, respectively (Mondal, 2009). Extraction of oil from tea seed is not economically viable, while the oil is used as a lubricant (Wealth of India, 1950). Tea seed cake also contains saponins but has poor value to be used as fertilizer as well as unfit for animal feed due to the low traces of nitrogen, phosphorus, and potassium. It is successfully utilized in the manufacture of nematocide.

Archeologists have proved that people consumed tea leaves steeped in boiling water as many as 500,000 years ago. Botanical evidence indicates that India and China were among the earliest countries to cultivate tea (Chopade et al., 2008). Tea is currently a hot topic of discussion in both nutritional and therapeutic research worldwide. This is not because tea is the most preferred drink after water, but because of the presence of crucial therapeutic compounds in tea which are more biostable and act directly as compared to those found in other plants (Chaturvedula and Prakash, 2011). First epidemiological reports on tea and cancer were published by Higginson (1966). From ancient times, Chinese value tea for its pleasant flavor and medicinal benefits, some of which have scientific value even today (**Table 1.2**). Many widespread extensive studies on the chemopreventive effect of tea have been carried out. Several reports are available for action of tea against various types of cancer including oesophagus, breast, pancreas, prostate and colon cancer (Crespy & Williamson, 2004; Gupta et al., 2004; Baliga, Meleth, & Katiyar, 2005; Nihal et al., 2005; Chopade et al., 2008; Wei et al., 2011). The majority of the beneficial effects of tea is attributed to its polyphenolic contents and purine alkaloids (Peng et al., 2008). Polyphenols, particularly flavonoids and flavonols, which represent 30% of fresh, leaf dry weight. The higher content of tea phenolic compounds are gallic acid and eight naturally occurring tea catechins, including (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-catechin gallate (CG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (Peng et al., 2008; Wei et al., 2011). EGCG is the most studied and most active polyphenol component in green tea. (Nakachi et al., 1998; Chopade et al., 2008). The major tea purine alkaloids present in tea are caffeine,

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Table 1.2: Traditional beneficial effects of tea

Traditional health claims	Possible scientific description
Enhanced blood flow	Vasodilation and decrease platelet activity
Removal of alcohol and toxins	Increased activity of phase I and phase II enzymes
Clear urine and improve flow	Diuretic effects
Relieves joint pain	Anti inflammatory activity
Improved resistance to diseases	Prevention of cancer and coronary heart disease

(This table has been adapted from Balentine et al., 1997)

theobromine, theophylline and theacrine (Axel et al., 1996; Ye et al., 1997; Ashihara et al.,1998; Ye et al., 1999; Zheng et al., 2002; Peng et al., 2008). Theanine is the main amino acid found in tea and it has been found to downregulate cerebral function, reduce blood pressure and inhibit the excitatory effects of caffeine (Yokogoshi et al., 1995; Terashima et al., 1999; Kakuda et al., 2000; Peng et al., 2008). Tea has also been known as an elixir of life and is commonly used as an antidote to mental fatigue. This effect may be due to the presence of caffeine, which is less in tea than in coffee, but the amount of caffeine present in a cup of tea is enough to dilate the brain's blood vessels. The two main active ingredients are the tea polyphenols and theanine (an unusual amino acid found in green but not black tea beverage). Like vitamins C and E, the tea polyphenols are antioxidants that may slow the onset of atherosclerosis, some forms of cancer, and the onset and severity of arthritis. Tea catechins have been found to be superior antioxidants than vitamin C, E, and carotene. The polyphenols block free radicals damage to lipids (found in cell membranes and serum lipids), nucleic acids, and proteins (like those found as cellular enzymes and structural proteins). Tea polyphenols and flavonoids have been reported to inhibit either enzymatic or non enzymatic lipid peroxidation, an oxidative process implicated in several pathological conditions including atherosclerosis (Chen et al., 2000). Non-antioxidant properties of tea polyphenols also may contribute to their overall effectiveness in disease prevention. Evidence suggest that theanine aids in triggering anti-cancer chemicals (such as doxorubicin) that kill tumor cells more specifically, but the mechanism is still unknown. In addition to this, polyphenols have antimicrobial (Hamilton-Miller, 1995), antiplaque (Yu et al., 1995), antidiabetic (Gomes

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et al., 1995), anti-AIDS (Hashimoto et al., 1996), antiviral (Okubo and Juneja, 1997), anorectic (Kwanashie et al., 2001), antiarthritic (Tapiero et al., 2002), anticarcinogenic (Vasisht et al., 2003) and several other properties, such as neuromuscular, antiangiogenic, antihepatotoxic, antiproliferative/ apoptotic, and immunomodulatory effects (Sueoka et al., 2001).

1.4. CONVENTIONAL BREEDING APPROACHES IN TEA

Conventionally, *C. sinensis* is propagated either through seeds or by vegetative means through cuttings. Tea seeds have low viability period, therefore, they are collected either directly from the plants while attached or immediately after shedding from the plants (Mondal, 2009). For the selection of good seeds, collected seeds are passed through rotary type sifter to eliminate very small size seeds. Big size seeds are subjected to the sinker floater test for which they are transferred to a tank or trough filled with water and soaked for 2-3 hours. Thereafter, they are planted in sand bed with scar-mark down for stratification. After 1½ month, the germinated seeds are transferred to shade for 12-18 months and finally, to the field (Mondal et al., 2004; Barua, 2008; Mondal, 2009).

In recent times, grafting has emerged as an alternative approach for propagation of tea. In this technology fresh, healthy single leaf internode cutting of both, shoot (scion) and root-stock, are selected. Shoots used for grafting are either drought tolerant or high yielding varieties. Recently, a modified and improved 'second generation' grafting technology has been developed, in which tender shoots are grafted on the young seedlings of tea which have an additional advantage over conventional grafting, due to presence of taproot system (Prakash et al., 1999; Mondal et al., 2004). Vegetative propagation by single node cutting is considered as the best choice in tea industry worldwide (Mondal, 2009).

Tea breeding mainly consists of two stages, hybridization and selection (Mondal et al., 2004). Hybridization is one of the major methods of obtaining genetic variation, and it is an important method of breeding new varieties. Hybridization in tea can be either natural or by hand pollination method. Natural hybridization method is based on identification of best character, such as best performance of yield, quality of tea or efficiency for disease resistance. Two parent plants are planted, adjacent to each other, in an isolated place and allowed to bear fruits. Subsequently, F1 seeds are harvested from these parents

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and grown to plants. The quality of these plants is monitored and if found superior to parent pants, it is termed as hybrid plant. The F_1 seeds are then released as hybrid seeds or biclonal seeds. However, some of the excellent performances among these progenies are marked and confirmed for multi-location trials. If found suitable after the trial, they are released as clone. These released clones are specific for particular location and most of the tea research institutes of the world have generated the clones for their own regions. In the above mentioned process, sometimes more than two parents are used and the seeds are known as polyclonal seeds. The motive behind this is to bring in more variability among the F1 seeds. While it is complicated to know about the pedigree of the cultivar (as pollen may come from any male), the possibility of reproducibility is low. Due to this reason, presently, this process is less preferred (Mondal, 2004).

Artificial or controlled crossing or hand pollination are an important approach but it has limited success in tea breeding. The reasons identified could be the following:

- Short flowering season in tea (2-3 months)
- Long time required for seed maturation (12-18 months)
- Difference in flowering time of various clones

In tea breeding, selection is a well known and popular technique. Commercial gardens were developed from seeds. The seed-grown plants are not uniform as they are the product of cross pollination and do not show characters of their parents. In some cases quality and yield are also unpredictable. As a result, commercial gardens, established from seeds, have great variability existing among them. Many elite plants have been recognized among the existing brushes, multiplied by vegetative means and released as clones. The majority of the tea clones are developed by selection methods but pedigree of the clones remains unknown.

Although, conventional tea breeding has contributed a lot for tea improvement, it is time consuming and labour intensive. In addition to this there are some additional bottlenecks that make the improvement process slow (Mondal et al., 2004). These are (1) tea is perennial in nature, (2) has long growth period, (3) high inbreeding depression, (4) self-incompatibility, (5) inaccessibility of distinct mutant of different biotic and abiotic stresses, (6) lack of distinct selection criteria, (7) low success rate of hand pollination, (8) short (2–3 months) flowering period (9) long duration of seed maturation ca. 12–18

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months, (10) clonal difference of flowering time and fruit bearing potential of some clones.

Likewise, propagation of tea can be effectively achieved by vegetative propagation but this process also, has its own limitations, like low rate of propagation, the rooting of cuttings is seasonal, lack of suitable planting materials in some tea growing areas due to winter dormancy, drought etc. and poor root development in some clones.

Thus, micropropagation is considered to be a better choice, besides transgenic method can also be used as means of crop improvement.

1.5. TISSUE CULTURE STUDIES

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotent nature of plant cells. This concept was first developed by Gottlieb Haberlandt (1902). In 1935, for the first time true plant tissue culture on agar was established and afterwards research in this field was hastened. But research progress on tissue culture aspects of tea is relatively slow for the last 20 years because tea is less amenable to these techniques, due to high level of polyphenols, the presence of systemic bacterial contamination and its recalcitrant nature in tissue culture (Dodd, 1994). Tissue culture has been employed for large-scale propagation of disease-free clones and gene pool conservation. A few reviews have been published on micropropagation of tea and related species (Kato, 1989; Vietez et al., 1992; Dood, 1994; Das, 2001; Mondal, 2003; Mondal et al., 2004;). On the basis of literature, pioneer work on tea tissue culture was performed by Forrest (1969). Later, Kato (1985) did systematic studies on micropropagation of tea. The biotechnological investigation on tea through tissue culture is depicted in **Scheme 1.1**.

1.5.1. Micropropagation

Initial establishment of culture and the rate of multiplication usually depend upon the type of explant used. Nodal segments, shoot-tip from early to late stages are commonly used explants for micropropagation of tea (Vietez et al., 1992). However, Kato (1985) reported regeneration of shoots using epidermal layer of stems or intact segment of stems

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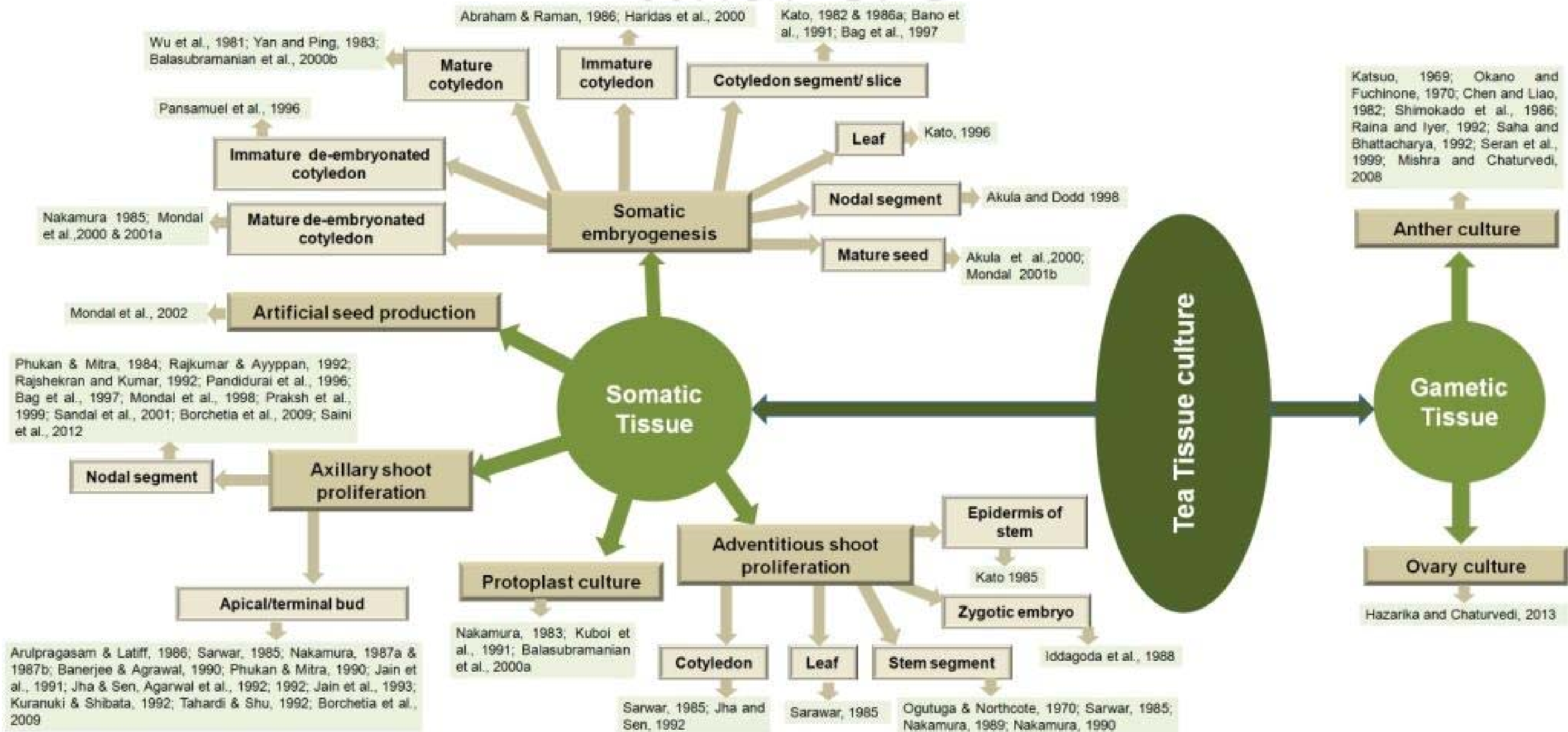

Scheme 1.1: Biotechnological investigation in tea through tissue culture

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while Jha and Sen (1992) reported the use of immature and matured cotyledons from zygotic embryos for adventitious shoot-bud development. Among all explants, callus from epidermal layer is more rapid compared to others. Micropropagation studies have been summarized in **Table 1.3**.

Various media combinations have been tried to increase the multiplication rate in tea. Commonly used medium is MS (Murashige and Skoog, 1962), besides this other media, such as WPM (Lioyd and McCown, 1980) and Heller's (1969) medium were also used. Nakamura (1987a) confirmed MS media to be the best for tea micropropagation. Several research have concluded that MS medium is highly suitable for initiation of multiple shoots. Moreover ½MS (major salts reduced to half strength) has also been found suitable for multiplication and proliferation of shoots of tea (Phukan and Mitra, 1984; Banerjee and Agarwal, 1990; Agarwal et al., 1992). Modification in composition of vitamins along with inorganic and organic salts of MS medium also favored induction and multiplication of axillary shoots (Arulpragasam and Latiff, 1986).

Among various plant growth regulators used, addition of 6-Benzylaminopurine (BAP) 4.44 µM - 26.67 µM and Indole-3-butyric acid (IBA) (4.44 µM - 17.76 µM) has been found suitable for shoot induction and further multiplication of tea (**Table 1.3**). Kato (1985) achieved indirect organogenesis using epidermal layer from stem as explant and cultured on MS medium supplemented with BAP (44.4 µM). Auxin, such as NAA, in combination with BAP either produced callus or shoot-buds with 4-5 shoots/explant within 8-12 weeks of culture (Phukan and Mitra, 1984; Bag et al., 1997). Sandal et al. (2005) reports rhizogenic calli with 2,4-D. When the concentration of 2,4-D was decreased from 53.6 µM to 8.04 µM, shoot-buds were induced. For elongation of shoots, Picloram and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) had also been used (Arulpragasam and Latiff, 1986; Jain et al., 1991; Iddagoda et al., 1988; Nakamura, 1987a,b ; Nakamura 1989a). After performing a test experiment on 30 clones it was found that high auxin concentration (53.6 µM) is critical for shoot elongation (Kuranuki and shibata, 1993). On the other hand, IAA (5.71– 11.42 µM) and Kinetin (0.93– 37.2 µM) proved better for multiplication of axillary shoots (Phukan and Mitra, 1984; Sarwar, 1985; Das and berman, 1988). A report on comparison between Thidiazuron (TDZ) and BAP for their effect on micropropagation has also been available (Mondal et al. in 1998).

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They found that low concentration of TDZ (1 pM-100 pM), when used alone, was effective for shoot multiplication. Besides this, higher concentration of BAP (1-10μM) was continuously required for shoot proliferation. Calli were produced from different explants when MS medium was supplemented with higher concentrations of TDZ (5, 10 and 15 μM) in combination with 2,4-D or IBA at 5, 10, and 15 μM. More number of shoots were obtained on the medium containing TDZ compared to that on medium supplemented with BAP, but multiplication rate was similar in both the media. This study lead to a conclusion that TDZ results in higher rate of shoot proliferation and is, therefore, a more effective cytokinin for tea micropropagation. Sandal et al. (2001) standardized an efficient liquid culture system for shoot proliferation of tea. They found that MS medium along with 2.5 to 5.0 μM TDZ was best for shoot proliferation at static condition. They also observed that 20 ml medium in 250 ml Erlenmeyer flask was best for culture, more than 20 ml induced hyperhydricity in cultures. Borchetia et al. (2009) reported role of GA_3 on shoot multiplication from different explants. They obtained a maximum of 32-33 shoots from cotyledonary node explants cultured on ½MS medium supplemented with BAP (26.64 μM), GA_3 (2.89 μM) and IBA (2.45 μM). For shoot multiplication, ½MS + Kn (4.65 μM) were found best. Role of Betaine was also studied on micropropagation of tea by Sainie et al. (2012). They reported that Betaine enhanced the water/ nutrient uptake and assimilation of carbon/ nitrogen. The vital role of growth adjuvants for tea micropropagation is established. These adjuvants includes, Coconut milk (Phukan and Mitra, 1984; Sarwar, 1985; Sarathchandra et al., 1988; Banerjee and Agarwal, 1990; Phukan and Mitra, 1990), Casein acid hydrolysate (Chan and Liao, 1983; Jha and Sen, 1992), Glutamine and Serine as nitrogen source (Chan and Liao, 1983). Sucrose (3-6%) is the best carbon source for adventitious-bud formation in comparison to other sugars, such as Lactose, Glucose, Galactose and Maltose (Nakamura, 1990). Based on these studies, it can be summarized that MS medium with low concentrations of cytokinin, mainly BAP, seems to be the best for micropropagation of tea.

Acclimatization of tea plants depends upon the rooting of micropropagated shoots. Both in vitro and in vivo, root proliferation media are reported in tea. In vitro rooting depends upon the concentration of auxin treatments and concentration of major inorganic salts of basal medium, and also physiological conditions, such as light and pH. Hardening is

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determined by humidity in hardening chamber and pH of the hardening media (Mondal et al., 2004). The concentration of major MS salts when reduced to half strength favored not only root formation but also the elongation. (Kato, 1985; Banerjee and Agarwal, 1990). IBA (2.45 µM to 39.2 µM) was preferred over NAA in tea (**Table 1.3**). Roots induced by NAA were shorter and thicker producing calli simultaneously, causing difficulty in subculture. Rooting appeared much later when treated with IBA but were long and fibrous (Nakamura, 1987a; Banerjee and Agarwal, 1990). Liquid medium with filter paper was used for rooting of tea (Tiang and Ling, 1982; Kato, 1985; Nakamura, 1987a). Jain et al. (1993) reported that in vivo rooting was better than all types of in vitro rooting techniques, like liquid shake culture, agar solidified medium or filter paper bridge. They received 97% rooting from microshoots in which cut end was dipped in IBA (245 µM) for 2 h before transplanting in to potting mixture. Banerjee and Agarwal (1990) achieved induction of rooting in tea in low light and low pH (4.5 – 4.6). Same was reported by Nakamura (1987a). He observed that dark treatment after dipping the shoots in auxin promoted rooting in tea. Murali et al. (1996) concluded that in vitro rooting also depends upon the genotype of tea.

Most critical phase of micropropagation is the transfer of in vitro plants to greenhouse. Green house conditions as well as internal microclimate (local environmental) conditions influence the uniform growth and survival rate of plant. Hardening can be done in tea by conventional, biological and micrografting means. Conventionally, microshoots of tea were hardened for six months on soil mixture containing several supplements, such as cow dung, soilrite etc. After this they were kept inside indigenously developed polytunnel of various shapes and sizes. Rooted plants (5-6 cm long) were transferred to plastic pots containing fumigated soil and kept in humidity chamber for 7 to 10 days (Arulpragasam et al., 1988). Das et al. (1990) reported hardening after pre-conditioning the plant at low temperature (22ºC) and low light intensity (250 $\mu mol.m^{-2}.s^{-1}$) after transferring to soil. Before transferring to field, the plants were covered with polybags and kept in thatch-house for a few weeks and subsequently exposed to sun light. Usually, the well optimized method for hardening is to transfer the rooted plantlets of tea to potting mixtures having equal ratio of peat and soil under high humidity (Banerjee and Agarwal, 1990; Agarwal et al., 1992; Jha and Sen, 1992; Jain et al., 1993). Combination of Vermiculite and soil

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(1:1) also gave satisfactory results (Tian-Ling, 1982; Kato, 1985). Sharma et al. (1999) concluded that pH of the soil coupled with CO_2 enrichment gave a higher percentage of survival. Mondal et al. (2004) mentioned that treatment of micropropagated shoots with IBA (2450 µM for 30 min) before transferring to Hikkotrays with pre-sterilized cow dung and soil (1:1) gave 90% survival of plants when these Hikkotrays were kept in poly-tunnel with intermediate watering for 90 days inside an indigenously developed polyhouse. They then transferred plants into polythene sleeves for a year containing black virgin soil in same house (Mondal et al, 1998; Rajshekaran and Mohankumar 1992). A new concept of biological hardening was reported in tea by Pandey et al. (2000). They isolated four antagonistic bacterial isolates, *Bacillus subtilis*, *Bacillus* sp., *Pseudomonas corrugata* 1 and *P. corrugata* 2 from the rhizosphere of tea plants growing in different geographical locations in India and tested them for hardening of tissue culture raised tea plants, prior to transfer in open land. These bacterial isolates enhanced survival up to 100 %, 96 %, and 88 % as against 50 %, 52 %, and 36 % survival observed in the corresponding control plants, in rainy, winter and summer seasons, respectively. Micrografting technique for hardening of in vitro raised tea plants is also published (Prakash et al., 1999; Mondal et a al., 2003; Borchetia et al., 2009). Micrografted plant growth rate was higher than micropropagation due to higher root volume. The higher volume of root helps to absorb more nutrients and water from soil due to presence of tap root in seedlings (Mondal et al., 2004). Prakash et al. (1999) suggested that plant growth regulators, assessment of computability and age of root-stock as well as season on the graft union are three most important factors for micropropagation. Mondal et al. (2003) used same technique for hardening and received higher survival percentage (i.e. 98%) without using any plant growth regulators. Growth of the grafted plants was much higher than the ungrafted in vitro plants or seedlings. Borchetia et al. (2009) micrografted in vitro grown shoots having 4-5 leaves on root stock of 12 month old juvenile seedling of TS506 and TS463 tea plants. The cut surfaces of root stocks and the microshoots were treated with 22.2 µM BAP for 30 min, and this was grafted on the stock so that the cambium tissues of both the stock and microshoots come in contact. The grafted area was covered with moist cotton and sealed with parafilm. The whole grafted plants were covered with polythene bags. The entire setup was kept under diffused light at room

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temperature for 3 weeks. Misting was done when required. Polythene bags were removed when grafting union was done and kept in net house for hardening. After 3-4 weeks, microshoot grafted sleeves were transferred to the hardening chambers, followed by transfer to fields. Major bottlenecks in tea micropropagation are phenolic exudation from the explants, that leads to browning and death (Sarwar, 1985; Agarwal et al., 1992; Iddagoda et al., 1998) and microbial contamination (Debergh and Vanderschaeghe, 1998). Phenolics exudation occurs due to high amount of phenols which when exuded in medium undergoes enzymatic oxidation to form some toxic compounds. These products decrease the pH of the medium. To avoid this problem, Sarwar (1985) gave various chemical treatments, such as ascorbic acid, catechol, l-cysteine, phloroglucinol, phenyl-thiourea, polyvinylpyrolidone-10, sodium diethyl dithio-carbamate, sodium fluoride and thiourea along with different strength of MS medium. He observed that 1/20 MS strength medium was the best for preventing explant browning. Ascorbic acid had been also reported to avoid this problem (Iddagoda et al., 1988; Agrawal et al., 1992). Creze and Beauchesne (1980) recommended use of polyvinyl-pyrolidone to prevent explant browning of in vitro cultures. Explants procured from field grown tea plants cause severe losses at every stage of micropropagation due to the presence of various epiphytic and endophytic organisms (Debergh and Vanderschaeghe, 1988; Mondal et al., 2004). This problem can be avoided by surface sterilization as reported by several workers. Ogutuga and Northcote (1970a) used 70 % alcohol followed by 7% sodium hypochlorite but Ghanati and Ishka (2009) used 20 % sodium hypochlorite for 20 min. Kato (1989a) used 7% calcium hypochlorite for 20 min. Arulpragasam and Latiff (1986) used 10-15% Clorox solution for 15 min and 0.04 to 1% mercuric chloride has been used by several scientists (Agrawal et al., 1992; Rajkumar and Ayyappan , 1992; Jhan and Sen, 1992; Borchetia et al., 2009; Saini et al 2012).

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Table 1.3: Reports on in vitro micropropagation of tea (*Camellia* spp).

Species/ cultivar	Explant source	Medium	Growth response	Remark	Reference
C. sinensis, TV1	Terminal buds and nodal segments (0.7-1.0 cm) from field grown plants	MS + IAA (1.4 µM) + BAP (17.8 µM) + CW (10%) + YE (200 mg/l)	Little elongation of the explants and proliferation of leaves and very slight multiplication.	• 490 µM IBA pre-treatment increased rooting response • Shoot proliferation and rooting in 60% cultures • Hardening and rooting of in vitro developed shoots.	Agarwal et al., 1992
		½ MS + IAA (2.99 µM) + BAP (17.8 µM) + CW (10%) + YE (200 mg/l)	Shoot multiplication (proliferation time was 4-5 weeks).		
		½MS (4% Sucrose) + IBA (34.5 µM) + Ascorbic acid (11.4 µM)	Rooting of shoots.		
C. sinensis (L.) O. Kuntze	Nodal segments	MS + IBA (1.07 µM) + BAP (9.99 µM)	Multiplication of axillary shoot.	• Response time was 10 weeks. • Rooted plants were transferred to pot with plastic cover	Bag et al., 1997
		½ MS + NAA (19.33 µM)	Rooting of shoots.		
C. sinensis, TV1	Shoot-tips and nodal segments (0.7-1cm) from mature plant	MS + YE (200 mg/l) + CM (10%) + IAA (2.85 µM) + BAP (17.76 µM)	Elongation of shoots from shoot-tip explants.	• No shoot multiplication from nodal segments. • 30 min pretreatment of aqueous 490 µM IBA to shoots favored root induction. • Acidic soil (pH is 4.5 - 4.6) favored rooting • Plant transferred to pots containing peat & soil and irrigated with ½ MS. After 30 days plants were transfer to greenhouse.	Banerjee and Agarwal, 1990
		½ MS + CM (10%) + IAA (1.43 µM) + BAP (17.76 µM)	Shoot multiplication .		
		½ MS + modified vit + IBA (34.3 µM)	Rooting of in vitro grown shoots (60 %).		

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Species/ cultivar	Explant source	Medium	Growth response	Remark	Reference
C. sinensis TV25, TV21 and T78.	Shoot-tips and nodal explants from field grown plants and in vitro plants, cotyledonary nodes from in vitro germinated seedlings	MS + GA_3 (2.89 µM)	Seed germination	• Rate of shoot multiplication was better from nodal explants than the shoot-tips. • 90% in vitro derived micropropagated shoots were micrografted into rootstocks.	Borchetia et al., 2009
		½MS + BAP (26.64 µM) + GA_3 (2.89 µM) + IBA (2.45 µM)	Multiple shoots (32-33 number) from cotyledonary node		
C. sinensis L.	In vitro shoot	MS + BAP (26.64 µM) + NAA (2.68 µM) + GA_3 (5.78 µM)	Shoot multiplication	• Response time was 2-3 weeks • Soil mixture was soil:peat (1:1) with light humidity	Jain et al., 1991 ; 1993
		Shoot were treated with IBA (245 µM) then transferred to pots containing soil mixture.	Rooting from shoots (97 %)		
		½ MS + IBA (24.5 µM) without agar in dark incubation	Rooting (in 50% of the cultures) from shoots		
		Filter paper bridge on ½ MS + IBA (2.45 µM)	Rooting (in 44% of cultures) from shoots		
C. sinensis (L.) O. Kuntze, T-78	Shoot-tips and cotyledonary nodes from germinated seedlings. Nodal segments from field grown plants	½ MS + CW (10 %) + CH (1 g/l) + BAP (4.44 µM) + IBA (0.49 µM)	8 shoots from shoot-tips and 35 axillary buds were proliferated from cotyledonary nodes.	• Treatment with IBA (2450 µM) for 30-40 min induced rooting from shoots. • Cytological analysis was performed to know genetic uniformity.	Jha and Sen, 1992
		MS + IBA (490 µM) for 10 days then transferred to MS Basal medium	Rooting (80 % to 90 %)		

Species/ cultivar	Explant source	Medium	Growth response	Remark	Reference
C. sinensis cv. Yabakita	Epidermal layer from stem, intact stem segments, stem segment without epidermal layer	MS + BAP (8.88 µM) + IBA (19.6 µM)	Callusing (duration 4 weeks)	• 20% organogenesis appeared from epidermal layer explants. • Time duration for callusing was 4 weeks and for organogenesis, it was 8 weeks. • After 6 weeks rooted plants transferred to vermiculite and soil in pot.	Kato, 1985
		MS + BAP (44.4 µM) + IBA (2.45 µM)	Shoot regeneration (duration 8 weeks)		
		Filter paper bridge of liquid ½ MS + IBA (2.45 µM) medium	Rooting of shoots		
C. sinensis cv. China hybrid	Nodal culture from in vitro grown seedlings	MS Basal Medium	Multiplication from axillary buds	Axillary shoot proliferation occurred and shoot multiplication was achieved in 24 weeks.	Mondal et al., 1998
		Shoots treated with 2450 µM IBA for 30 min	Rooting of in vitro developed shoots		
C. sinensis (Tuckdah-78, UPASI-9 & Kangra Jat)	Nodal segments (0.5 – 1 cm)	MS + TDZ (5µM) without agar (static and agitated condition)	Shoot induction and multiplication	As compared to BAP which induced hyperhydricity in the proliferated shoots in liquid medium, a progressive increase in the multiplication rate together with healthy shoot growth was achieved when thidiazuron (2.5 to 5.0 µM) was used instead of BAP.	Sandal et al, 2001
		4.92µM IBA treatment of 3.0 cm long shoots for 30 minutes prior to their transfer to uncovered jars containing potting mix (sand:soil:2:1)	Rooting observed		

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Species/ cultivar	**Explant source**	**Medium**	**Growth response**	**Remark**	**Reference**
C. sinensis UPASI-9 and UPASI-10	1.5–2.0 cm size Nodal segments	MS Basal Medium for 15 days and then transferred to MS + Betaine (8.5 mM)	Maximum growth response observed in 60 days	• In the presence of Betaine, water/nutrient uptake was faster and carbon & nitrogen assimilation was higher. • Nutrient uptake, assimilation and growth were significantly higher in UPASI-10 as compared to UPASI-9 shoots.	Saini et al., 2012
C. sinensis, Kangra jat	In vitro leaf explants	MS + BAP (8.88 µM) + IBA (0.98 µM)	Culture establishment from nodal segments	After 3 month of culture establishment, three types of leaves were used as explants (completely folded, half opened and fully expanded)	Sandal et al., 2005
		MS + 2,4-D (53.6 µM)	Rhizogenic calli obtained after prolonged culture, after 6 weeks from fully expended leaf		
		MS + 2,4-D (8.04 µM)	From rhozogenic calli shoot bud initiate after prolong culture		

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1.5.2. Somatic embryogenesis

Somatic embryogenesis is a process in which plant somatic cells are differentiated into somatic embryos. Somatic embryos are morphologically similar to zygotic embryos. They are bipolar and bear typical embryonic organs. Wu et al. (1981) were pioneer for somatic embryogenesis in tea. It has been reported as the most efficient system for the regeneration of tea (Jain and Newton, 1990; Vieitez, 1994). Somatic embryogenesis offers many advantages over organogenesis, such as single cell origin, possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds (Mondal et al., 2000b). Furthermore, the bipolar nature of embryos allows their direct development into complete plantlets without the need of a rooting stage, as required for plant regeneration via organogenesis (Bhojwani and Razdan 1996). Somatic embryos can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds. Furthermore, epidermal single cell origin of embryos favors the use of this process for plant transformation and to maintain the genetic fidelity (Bano et al., 1991; Mondal et al., 2001a; 2001c). Few reviews are available on somatic embryogenesis of tea (Vieitez, 1994; Akula and Akula, 1999; Mondal et al., 2004).

Induction of somatic embryos

Somatic embryo induction depends on several factors, such as type of explant, physiological status of parent plant, genotype, type of medium, concentration and time of application of different plant growth regulators, and some other factors, such as nitrate salts of potassium and ammonium, together with sulphate salts of aluminum, potassium, magnesium and ammonium, sucrose, maltose as well as concentration of trans-cinamic acid (t-CA) (Akula and Akula, 1999; Mondal et al., 2004; Vieitez, 1994).

In tea, mature cotyledons and zygotic embryos are the most popular explants to obtain embryogenesis (**Table 1.4**). Other selected explants have also been used, like immature cotyledons (Abraham and Raman, 1986; Nakamura, 1988a; Bano et al., 1991), de-cotylenated embryos (Nakamura, 1985; Paratasilpin, 1990; Mondal et al., 2000b), de-embryonated cotyledons (Ponsamuel et al., 1996; Rajkumar and Ayappan, 1992a), nodal cuttings (Akula and Akula, 1999), juvenile leaves (Sarathchandra et al., 1988) and leaf

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stalks (Hua et al., 1999) (**Scheme 1.1**). Genotype and physiological status of the parent plants have direct influence on somatic embryogenesis (Vieitez, 1994; Mondal et al., 2000b). Nakamura (1988a) reported that late September to mid October is the best time for induction of embryogenesis in cotyledon cultures, because by this time seeds were physiologically mature in Japan.

Type and strength of media also influences induction of somatic embryogenesis in tea. MS Basal medium (Murashige and Skooge, 1962) is the most commonly accepted medium for somatic embryogenesis in tea (**Table 1.4**). Besides, Woody plant medium (Lloyd and McCown, 1980) and Nitsch and Nitsch (1969) media have also been used. Type of plant growth regulators and their concentrations also play a significant role in induction of somatic embryogenesis in tea. Normally, high cytokinin-to-low auxin ratio or low cytokinin alone was found necessary for induction of somatic embryogenesis in tea. In few cases reduction of cytokinin in subsequent subcultures has also been reported (**Table 1.4**). Cytokinin BAP (up to 44.4 μM) has been extensively used (Kato, 1982; Nakamura, 1988a; Jha et al., 1992; Rajkumar and Ayyappan , 1992; Bag et al., 1997; Akula and Dood, 1998; Balasubramanian et al., 2000; Haridas et al., 2000; Mondal et al., 2001a; Aoshima, 2005; Seran et al., 2006). In few other cases, Kinetin (0.23- 46.5 μM) has also been used for the induction of embryos (Wu et al., 1981; Bano et al., 1991; Wachira and Ogado, 1995). Among various auxins, IBA (upto 9.8 μM) is commonly used for somatic embryogenesis (Kato, 1982 and 1986a; Jha et al., 1992; Mondel et al., 2001a). Use of other auxins, such as NAA (Bag et al., 1997; Seran et al., 2006), 2,4-D (Kato , 1996; Balasubramanian et al., 2000) and IAA (Wu, 1986; Sood et al., 1993) have also been reported. Ponsamuel et al. (1996) used a novel auxin α-napthoxyacetic acid (NOA), tetraphenylboron (TPB), phenylboronic acid, etc., for induction of somatic embryogenesis from cotyledon explants of *C. sinensis* cultivar UPASI-10. Akula et al. (2000) reported that presence of ABA (28.35 μM) in induction medium enhanced the rapid development of direct somatic embryogenesis from seeds of tea cultivar TRI-2025. Suganthi et al. (2012) evaluated the impact of abscisic acid (ABA) alone or in combination with osmotica on induction of somatic embryogenesis. They tested various concentrations of osmoticum viz., Polyethylene glycol (PEG) or Mannitol or Glycine Betaine. ABA 18.5 μM and PEG 3 %, increased the number of somatic embryo

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formation compared to the other osmoticum coupled with ABA. Results suggested that the use of osmoticum along with ABA was quite effective to induce somatic embryogenesis in tea. It has been found that growth adjuvants also influence somatic embryogenesis but in case of tea, its requirement is very less (Vieitez, 1994). Use of some growth adjuvants, such as Adenine sulfate (Akula and Dood, 1998; Akula et al., 2000), Activated charcoal (Bano et al., 1991), Coconut water (CW) (Sarathchandra et al., 1988; Rajkumar and Ayyappan, 1992), Yeast extract (YE) (Arulpragasam et al., 1988), Betaine (Akula et al., 2000) etc. have also been reported.

Maturation and germination of somatic embryo

The aim of embryogenesis is high rate of germination and plant regeneration. The process of embryogenesis is a sequential process which passes through various developmental phases, such as globular, heart shape, torpedo shape, complete mature embryos, hypocotyls root axis elongation and emergence of root (Bhojwani and Razdan, 1996). In comparison to other woody tree species, the somatic embryogenesis in tea has progressed well but the main bottleneck is the low frequency of germination (Vieitez, 1994). Other problems associated with tea somatic embryos are their precocious and abnormal germination because of lack of stored reserved food and improper balance of endogenous hormone levels. So several factors such as, external nutrients, PGRs supply and culture conditions will improve conversion rate of somatic embryo of tea (Mondal et al., 2004).

Type of sugar and its concentration have major role in maturation and germination of somatic embryos. Sugar has dual role which acts as carbon source and increases desiccation tolerance as well at the time of maturation and germination of somatic embryos (Lecouteux et al., 1993) by acting as osmoticum (Tremblay and Tremblay, 1995). Mondal et al. (2002b) reported that sucrose concentration (3 %) in the medium and duration of treatment of cultures (5 weeks), profusely affects the germination (35.2 %) of somatic embryo. Higher concentration of sucrose (6 or 9 %) for varying duration (3, 4 and 5 weeks) did not show any response. They suggested that reason behind this is readily available reserve food in the form of carbohydrate during maturation which is important for subsequent successful germination of embryos. Thus, the embryo cultured on 3 % sucrose for the period of 5 weeks germinates normally to the extent of 35.2 % but

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declines afterward. It is seen that desiccation treatment enhances the germination capacity of somatic embryos (Roberts et al., 1990) Tea cultures have also been shown sensitive to desiccation treatment (Mondal et al., 2004).

Additionally, the role of plant growth regulators in maturation and germination of somatic embryos has been substantiated by many workers. Abscisic acid (ABA) prevents precocious germination and promotes normal development by suppression of secondary embryogenesis and pluricotyledony (Ammiro rato, 1974, 1983; Bhojwani and Razdan, 1996). So this hormone is very commonly used in woody plant species (Roberts et al., 1990). This growth regulator is also known to promote accumulation of storage lipids (Avgioglu and Knox, 1989) and proteins (Roberts et al., 1990). In case of tea, no significant germination of somatic embryos were noted when ABA treatment was given at different concentrations (1.89, 9.41, 47.24 and 236.44 µM) for various duration (3, 4, and 5 weeks), but (Mondal et al., 2002b). Likewise GA_3 (1.45-14.45 µM), promotes germination in various woody plant species and hybrids of *Camellia*, but was not effective in case of tea. Jha et al. (1992) reported that 20% somatic embryos germinated on B_5 medium (Gamborg et al., 1968) supplemented with BAP (13.32 µM) and IAA (11.42 µM). It is found that high cytokinin, like BAP to low auxin, such as IBA and IAA, is the best for of maturation and germination of somatic embryos in tea (Jha et al., 1992; Sood et al., 1993; Kato, 1996; Mondel et al., 2001a; Thiep, 2012). Application of other growth regulators has also been reported for maturation and germination of embryos, such as Brassin (synthetic analog of Barassinoids) that was found to induce 50% embryos for maturation and germination of tea (Ponsamuel et al., 1996). This is due to an inherent sensitivity of tissues to brassinoide which is also present in tea leaves (Ikekawa, 1991). Mondal et al. (2002b) evaluated different concentrations of maltose and trans-cinamic acid (t-CA) either alone or in combination, for improvement of germination efficiency in tea somatic embryos. Very low percentage of embryo (3-6 %) germination was observed at all concentrations of maltose and t-CA individually. The investigators obtained significant percentage of (70.6 %) germination of embryos on medium supplemented with both maltose (4 %) and t-CA (3 mg/l), for 4 weeks, which were subsequently transferred to GA_3 containing medium. They also reported that maltose, as compared to

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Table 1.4: Summary of somatic embryogenesis in tea, *Camellia* spp

Species/ cultivar	Explant used	Medium	Response	Remark	Reference
C. sinensis TRI-2025	Nodal segment	MS + BAP (2.22 µM) + GA_3 (8.67 µM) + Adenine sulfate (100 mg l^{-1}) + L-Glutamic acid (100 mg l^{-1})	Induction of embryo	Platelets conversion rate 60%	Akula and Dood,1998
		½ MS basal medium	Multiplication and germination of plantlets		
		in small pot pre-sterilised potting mix (sand : peat : vermiculite, 1:2:1)	90% survival rate in greenhouse		
C. sinensis Surugawase cultivar	shoot apex-derived callus tissue	MS + BAP (8.67 µM) + Gellan (0.3%)	Callusing from shoot apex	Addition of high osmotic stress or treatment with antibiotics was effective on somatic embryogenesis	Aoshima, 2005
		MS + Hygromycin (5 mg/l)	Induction of embryos		
C. sinensis	Mature seed	½ MS macro salt + Full MS micro salt + Adenine hemisulphate (100 mg/l) + Glutamine (100 mg/l)	Induction, maturation and germination of embryos in the same medium	Germination rate was 50-20%	Akula et al.,2000
C. sinensis	Cotyledon segment	MS + BAP (5 µM) + NAA (121.9 µM)	Induction and multiplication of somatic embryos	Plantlet conversion rate 60% Rooted plantlets were subsequently transferred to community pots.	Bag et al.,1997
		MS + BAP (10 µM) + NAA (212.9 µM) + GA_3(0.35 µM)	Germination of embryo		
C. sinensis	Mature cotyledon, leaf	MS+ BAP (8.88 µM) + 2,4-D (26.8 µM)	Induction of embryo	14% from induction from cotyledon and 2% from leaf 12 % embryo germination	Balasubramanian et al., 2000
		MS+ BAP (8.88 µM) + NAA (16.11 µM)	Germination of induced embryo		

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Species/ cultivar	Explant used	Medium	Response	Remark	Reference
Thea sinensis (L.), B-61	Cotyledon slice	MS + Kn (0.23 μM) + 2,4-D (2.68 μM)	Embryo induction	40 % germination of somatic embryo. For germination high light intensity (5000 Lux) was used. Sporadic germination was the major limitation.	Bano et al., 1991
		½ MS + Kn (0.23) + Activated charcoal (0.2%) + Glucose (1.5%)	Maturation and germination of embryo		
C. sinensis	Immature cotyledon	MS (modified) + BAP (13.32 μM) + NAA(10.74 μM)	Induction of embryo		Haridas et al., 2000
		MS+ BAP (22.2 μM)	Germination of embryo		
C. sinensis T-78	Cotyledon	MS + BAP (44.4 μM) + IBA (2.45 μM) + Adenine (80 mg/l)	Induction and maturation of embryo	Secondary embryogenesis also occurred	Jha et al., 1992
		B_5 + BAP (13.32 μM) + IAA (11.42 μM)	Germination		
		In pot peat : soil (1:1)	Acclimatization (70% survival)		
C. sinensis Yabukita	Cotyledon Segments	MS + BAP (17.76 μM) + IBA (9.8 μM)	Induction of direct embryogenesis		Kato, 1982 & Kato, 1986a
		MS + BAP (44.4 μM) + IBA (2.45 μM)	Germination of embryo and plantlet development		
C. sinensis Yabukita, Sayamamidori, Benikaori, Akane	Immature leaves from in vitro grown shoots	MS (Liquid) + 2,4-D (2.68 μM)	Induction of embryos	Younger leaves were better than older leaves to product somatic embryo	Kato , 1996
		MS + BAP (44.4 μM) + IBA (2.45 μM), or MS +BAP (17.76 μM) + IBA (9.8 μM)	Maturation and germination of embryo		
C. sinensis	Mature de-emryonated cotyledon	MS + BAP (8.88 μM) + IBA (0.98 μM)	Induction	Embryo conversion rate was 70%	Mondel et al., 2001a
		MS (Modified) + BAP (8.88 μM) +	Maturation		

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Species/ cultivar	Explant used	Medium	Response	Remark	Reference
		IBA(0.98 µM) + Glutamine (1 mg/l) +K_2SO_4 (1.5 mM)			
		Modified MS +BA (8.88 µM) + IBA (0.98 µM) + Glutamine (1 mg/l)	Germination		
C. sinensis with 13 cultivars, *C. Japonica* 3cultivars, *C. brevistela* , *C. nokoensis, C. japonica* (cv kosyougatu) *x C. granthamian*	Half sliced cotyledon	MS + BAP (4.44-22.2 µM)	Induction of embryos	Best time for maximum embryo induction was from late September to mid October. 93% of adventitious embryos were formed in *C. brevistela*.	Nakamura, 1988a
C. sinensis, UPASI-10	De-embryonated immature cotyledon	MS (6 % agar) + PBOA (1 µM) + BAP (0.5 µM)	Induction of embryo	For rooting, shoots were treated with MS (liquid) + IAN (1 µM) + Brassin (1 µM) + Phloroglucinal (1 µM) for 5 days. Plantlets were transferred in vermiculite in greenhouse. After 200 days plants were transferred to red acidic soil.	Ponsamuel et al., 1996
		MS (0.8 % agar) + Brassin (1 µM)	Maturation and 50% germination of embryos occurred.		
C. sinensis, UPAKI-10	Cotyledon, cotyledon without embryogenic axis	MS (Modified) + BAP (1.11 µM)	Induction of embryos		Rajkumar and Ayyappan , 1992
		MS + BAP (3-5 µM) + CW (10 %) + GA_3 (0.72-2.89 µM)	Maturation and 80-90 % of germination of embryos.		

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Species/ cultivar	Explant used	Medium	Response	Remark	Reference
C. sinensis	In vitro leaf	MS + BAP (13.32 µM) + NAA (5.37 µM)	Callusing		Seran et al., 2006
		MS + BAP (4.44 µM) + NAA (5.37 µM)	somatic embryogenesis		
C. sinensis Kangra Jat	Cotyledon Slice	½MS + BAP (8.88 µM) + IAA (1.14 µM)	Induction of embryos	Describes hypertrophied structure under correct media combination might gave somatic embryos.	Sood et al., 1993
		½MS + BAP (8.88 µM) + IAA (1.14 µM) + GA_3 (0.57 µM)	Germination of embryos		
"China" hybrids, UPASI-9, UPASI-10 & ATK-1 and "Cambod" variety UPASI-17	Mature cotyledons without embryo	MS + L-Glutamine (200 mg/L)	Globular shape, tiny, somatic embryos were emerged from the surface of the cotyledons (After 3-4 weeks).		Suganthi et al., 2012
		MS + ABA (18.9 µM) + PEG (3 %)	Increased number (19.9%) of somatic embryo		
		MS + BAP (8.88 µM) + GA_3 (14.45 µM)	Germination of embryo		
C. sinensis Shan Chat Tien variety	Cotyledon	MS + BAP (26.64 µM)	96.36 % bud break from somatic embryos		Thiep, 2012
		MS + BAP (13.32 µM) + IBA (1.47 µM)	Shoots multiplication		
		MS + IBA (14.7 µM)	Rooting		

sucrose, is not only broken down gradually, but also can provide a steady supply of more readily metabolisable carbon source (glucose) to the embryos which probably have reserve carbohydrates. It was further reported that t-CA is an important compound involved in a wide variety of metabolic pathways, like flavonoid biosynthesis, the phenolic synthesis pathway and, most importantly, in the synthesis of Malonyl-CoA, the key pre-cursor of fatty acid synthesis pathway. Suganthi et al. (2012) obtained enhanced frequency in the germination and growth of embryos on BAP and GA_3 containing medium.

1.5.3. Haploid production

Haploid plants are sporophytes having only a single set of chromosomes (n; gametophytic number of chromosome). This is in contrast to diploid plants, which contain two sets (2n) of chromosomes (Kasha and Maluszynsky 2003; Germanà 2011a). Haploids have attracted great interest of plant physiologists, embryologists, geneticists and breeders since the first discovery of haploid plants in *Datura stramonium* as early as in 1922 (Blakeslee et al., 1922). Subsequently, haploidy was reported in many species, such as,in *Nicotiana tabacum* (Clausen and Mann, 1924), *Triticum aestivum* (Gains and Aase, 1926) and in several other plant species (Kimber and Riley, 1963) but at low and variable frequencies and was regarded as a special biological phenomenon (Mishra and Chaturvedi, 2012). The remarkable discovery that haploid embryos and plants can be produced by in vitro culture of anthers of *Datura* (Guha and Maheshwari 1964, 1966) brought renewed interest to haploidy. Various in vitro haploid production techniques are shown in **Scheme 1.2**.

In vitro androgenesis is the most efficient, fast and dependable technique to produce haploid plants. It is a common tool for producing doubled haploids, more so for tree species, because majority of the trees are outbreeding in nature, highly heterozygous and have long generation cycle (Hazarika et al., 2013). The prevalent heterozygosity and absence of pure lines in woody plants make selection and genetic studies rather difficult to conduct. Conventional breeding programmers are unpredictable, high labor cost and time consuming where several generations of selfing is required to obtain homozygous pure lines, which is difficult to raise in woody plants due to long generation cycle. The

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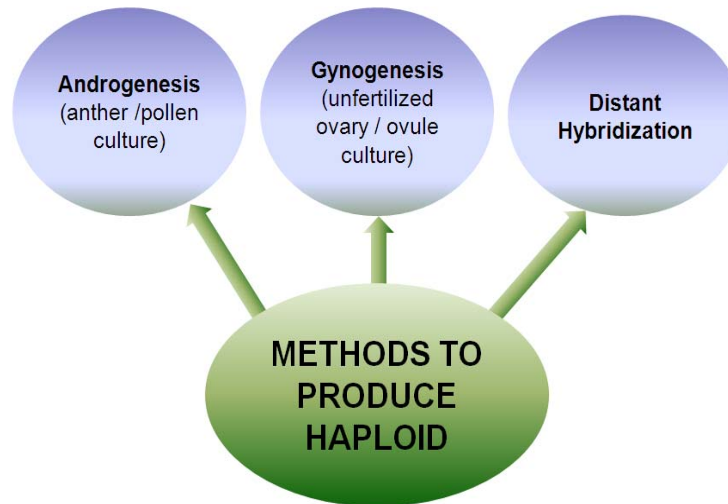

Scheme 1.2: Methods to produce haploids under in vitro conditions

perspective of raising haploid plants through in vitro androgenesis offers scores of foreseeable advantages, like shortening of breeding period, production of homozygous diploid lines in a single step through chromosome doubling and isolation of valuable recessive traits at sporophytic level which otherwise, remain accumulated and unexpressed in natural heterozygous diploid population (Srivastava and Chaturvedi 2008).

The initial attempts to produce haploids through anther culture of tea were pioneered by Katsuo (1969) and Okano and Fuchinone (1970). They obtained roots from anther derived callus. However, it was Chen and Liao in 1982, who produced complete plantlets from tea anthers of cultivar Fuyun No-7 out of nine different tea cultivars on which they worked. The plantlets were obtained when the anthers were cultured on N_6 medium supplemented with Kinetin (9.3µM), 2,4-D (2.26µM), L-Glutamine (800 mg/l) and Serine (100 mg/l), followed by sub culturing on N_6 medium supplemented with Zeatin (9.12 µM), adenine (148 µM) and Lactoalbumin hydrolysate (10 mg/l). On this medium, either the calli continued to proliferate into shiny masses or shoots. These shoots were subsequently rooted on medium containing IAA (0.57 µM). While 3 out of 4 plants were haploids, the rest were aneuploids with a chromosome number 2n=18. Later, Saha and Bhattacharya in 1992 reported formation of globular structures in tea which failed to differentiate further on MS medium (with 7% sucrose) was supplemented with NAA (0.53 µM), 2, 4-D (0.45 µM), Kinetin (0.46 µM), and Glutamine (400 mg/l). However,

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the differentiation of true pollen embryos and regeneration of haploid plants were described by Shimokado et al., 1986 and Raina and Iyer in 1992. Pedroso and Pais (1994) tested 17 different media combinations based on MS and N_6 with various concentrations of carbon source, growth regulators and amino acids, such as Serine and Glutamine for *C. japonica*. The embryogenic calli from microspores were obtained when isolated microspores were cultured on 2, 4-D (4.53 µM) and Kinetin (0.46 µM) and subsequently on MS medium supplemented with BAP (2.22 µM). However, further growth ceased at maturation stage. Seran et al. (1999) reported the highest response in terms of micro calli formation in a Sri Lankan Tea clone TRI-2043 (78-98 %) out of 5 different clones selected for study. On ½ MS + 2, 4-D (9.06 µM) + Kinetin (4.65 µM) + IAA (5.71 µM) under dark condition, 98% anther response was achieved. Determination of ploidy levels in the callus cells showed that the frequency of haploid cells was greater (68 %) in comparison to diploid cells (6 %). However, plantlets could not be regenerated. For the regeneration of haploid plants in tea several attempts have been made but the success remained only upto the development of haploid calli (Seran et al. 1999; Mondal et al. 2004; Hazarika et al., 2013).

Gynogenic development of plants from unfertilized cells of female gametophyte (embryo-sac) in ovary/ovule cultures is one of the available alternatives for haploid production. It was first reported in barley San Noeum (1976). This method of haploid production is more tedious than androgenesis. The reasons being that there is a single egg cell (female gamete) per flower for gynogenic haploid production, which too, is deep seated within the embryo-sac (female gametophyte), compared to indefinite number of microspores (male gametes) within the anther wall available for androgenesis. However, the technique is useful where anther culture has been unsuccessful, male gametes are sterile or androgenesis is confronted with the problem of albino or non-haploid formation. Hazarika and Chaturvedi (2013) has obtained for the first time, successful callus induction from unpollinated ovules of 4 different cultivars of tea, TV1, TV7, TV18 and TV19, when thin sections of ovary cultures were subjected to various regimes of temperature and light treatments. Out of the four experimental tea cultivars, TV18 gave the highest percentage of callus induction. Within a week of culture, the ovules were swollen to almost double their original size and callusing was observed. In few cultures,

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white friable callus emerged from within the cultured ovules, whereas in others profuse callusing was seen from the entire section. Flow cytometric analysis revealed that most cells were haploids. This was further supported by histological evidence that the egg cell was intact at the time of culture, and after 2 week of culture the synergid cell shrunk while the egg cell enlarged and become multicelled.

1.6. SECONDARY METABOLITES

Plants and their derivatives form important part of our everyday diet, and as such nutritional value of these have been extensively studied for decades. In addition to essential primary metabolites (e.g. carbohydrates, lipids and amino acids), higher plants also synthesize a wide variety of organic compounds collectively termed as 'secondary metabolites'. These secondary compounds do not participate in vital metabolic functions of plants (eg. photosynthesis, respiration, protein and lipid biosynthesis, growth and reproduction) but they act primarily as defense molecules that facilitate plant to adapt to new environment. Plant secondary metabolites are biosynthesized from primary metabolites by specific genetically controlled, enzymatically catalyzed reactions that lead to the formation of complex compounds. Higher plants are a major source of natural products, like pharmaceuticals, agrochemicals, flavor and fragrances, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant-derived chemicals should, thus, be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson, 1990). However, it is becoming increasingly clear that secondary metabolites may play important roles in plant signaling and defense mechanisms (which are shown in **Scheme 1.3**) (Wink, 2010). In addition, the plants constitute important UV absorbing compounds, thus, preventing serious leaf damages from the light (Li et al. 1993). These secondary metabolites are explained as being antioxidant, antibiotic, antifungal, antiviral and, therefore, are able to protect plants from pathogens (phytoalexins). They are also anti-germinative or toxic to other plants (allelopathy). The plant metabolites act on insects as anti-feeders or even cattle for which forage grasses can express estrogenic properties and interact with fertility (Bourgaud et al. 2001). Due to their diverse biological activities, plant secondary metabolites have been used for centuries in traditional medicine. Based on their biosynthetic pathways

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these compounds are usually classified into three large families: 1. Phenolics, 2. Terpenes and Steroids, and 3. Alkaloids (Harborne 1999).

Among the three classes, phenolics are the largest group of secondary metabolites present in tea. The composition of the tea leaves depends on several factors, such as climate, season, horticulture practices, type, age and physiology of tea plant (Bansal et al., 2012). The tea leaves contain more than 700 chemical constituents (Mondal et al., 2004). The plucked tea shoots consist of two to three tender leaves and an apical bud which contains 10-35% (dry weight basis) polyphenolic compounds (Balentine et al., 1997; Chaturvedula and Prakash, 2011; Sabhapondit et al., 2011; Bansal et al., 2012). Flavonoids are plant secondary metabolites widely distributed in the plant kingdom, and can be subdivided into six classes: flavones, flavanones, isoflavones, flavonols, flavanols, and anthocyanins, based on the structure and conformation of the heterocyclic oxygen ring (C ring) of the basic molecule (**Scheme 1.4**). The main classes of flavonoids found in tea are flavanols and flavonols (Balentine et al., 1997; Wang et al., 2000). Flavonoids are basically C15 units consisting of two benzene rings (A and B) connected by three carbon chain. This chain is closed to most flavonoids to form the heterocyclic ring (C). The natural flavonoids are divided into classes based generally on the oxidation state of their C-ring (Stafford, 1990). The major flavonoids present in tea are chatechins. These eight naturally occurring catechins present in tea are (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-catechin gallate (CG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (**Scheme 1.4**). Maximum percent of catechins in tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC) and (+)-catechin (C) (Balentine et al., 1997; Liang, et al., 2006; Wei et al., 2011). These are colorless, water soluble compounds that contribute the bitterness and astringency to green tea (Balentine et al., 1997). Catechins are considered to be synthesised through phenylpropanoid and flavonoid biosynthetic pathway. The formation of dihydroquercetin and dihydromyricetin, which are the precursors of dihydroxylated catechins (EC and ECG) and trihydroxylated catechins (EGC and EGCG), respectively, is genetically controlled (Gerats & Martin, 1992; Wei et al., 2011). EGCG is most abundant (50 – 80% of total catechin) of all catechins in green tea (Bansal

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et al, 2012). Almost all of the characteristics of manufactured tea, including its taste, color, and aroma, are linked directly or indirectly with modifications to the catechins (Wang et al., 2000). Moreover, it was found that the total catechin of tea leaves increased with exposure to sunlight, suggesting that catechin biosynthesis is also environmentally dependent (Mariya et al., 2003; Wei et al., 2011). It has been published that Assam type cultivars contain higher amount of polyphenols (Bhuyan et al., 2009; Sabhapondit et al., 2012). China variety cultivars generally have quercetin and kaemferol-3-glucosides but these are totally absent or are present only in negligible amount in Assam variety (Hazarika and Mahanta, 1984; Sabhapondit et al., 2012). Flavonols are mainly present as glycosides rather than as their non-glycosylated forms (aglycones). At least 14 glycosides of myricetin, quercetin and kaempferol in fresh tea shoots, green and black teas have been reported (Engelhardt et al., 1992; Wang et al., 2000). Flavonol glycosides are present in tea upto 2 to 3 % of the water soluble extracts (Balentine et al., 1997). Tea leaf contains 2.5 to 4 % of 1,3,7-trimethylxanthine (caffeine) (dry weight basis) and much less quantity of related methylxanthine theobromine (3,7-dimethylxanthine). Theophylline also has been reported as a tea constituent. Theanine is the characteristic and main amino acid in tea (Peng et al., 2008).

In search of alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue culture, is found to have a promising potential as a supplement to traditional agriculture (Rao and Ravishankar, 2002). The explorations of production of secondary metabolites in tea were started from 1821, when caffeine was first prepared in pure form from tea leaves (Spedding and Wilson, 1964). Ogutuga and Northcote (1970a) reported production of caffeine from callus tissue. As high as 30% catechins have been produced from cell culture of tea (Hao et al., 1994). First report on the formation of polyphenols in tea plant as well as in callus tissue was reported by Forrest (1969). He described that the production of simplest catechin and leuco-anthocynin was highly dependent on the original explants and inversely co-related with growth rate of cultured cells of tea. Zaprometov and Zagoskina (1979) reported that callus on medium supplemented with NAA had more soluble phenols, flavans as well as phenolic polymer lignin compared to medium with 2,4-D. They also reported in 1987, that abscisic acid inhibits the cell growth and production

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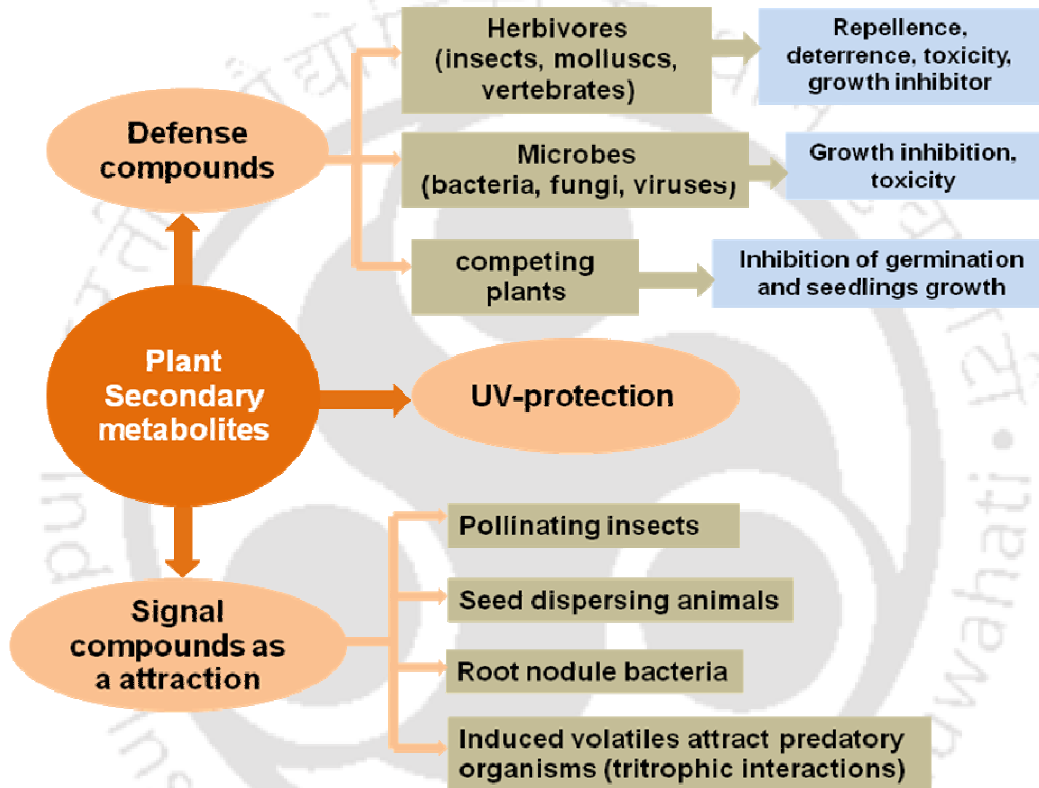
from all form of soluble phenolic compounds. Carbon source as 5% glucose is best for catechin and pro-anthocyanin (Mondal et al., 2004). NAA as a plant growth regulator stimulates cell growth as well as polyphenol production in tea (Bagratishilli et al., 1979). Tea has theanine and ethyl L-Glutamine as the major free amino acids. Orihera and Furuya (1990) published that on MS medium with 9.8 µM IBA and 0.467µM Kn, production of L-glutamine was high and when ethylamine was added to the medium, production of theanine was found increased.



Scheme 1.3: Role of plant secondary metabolites

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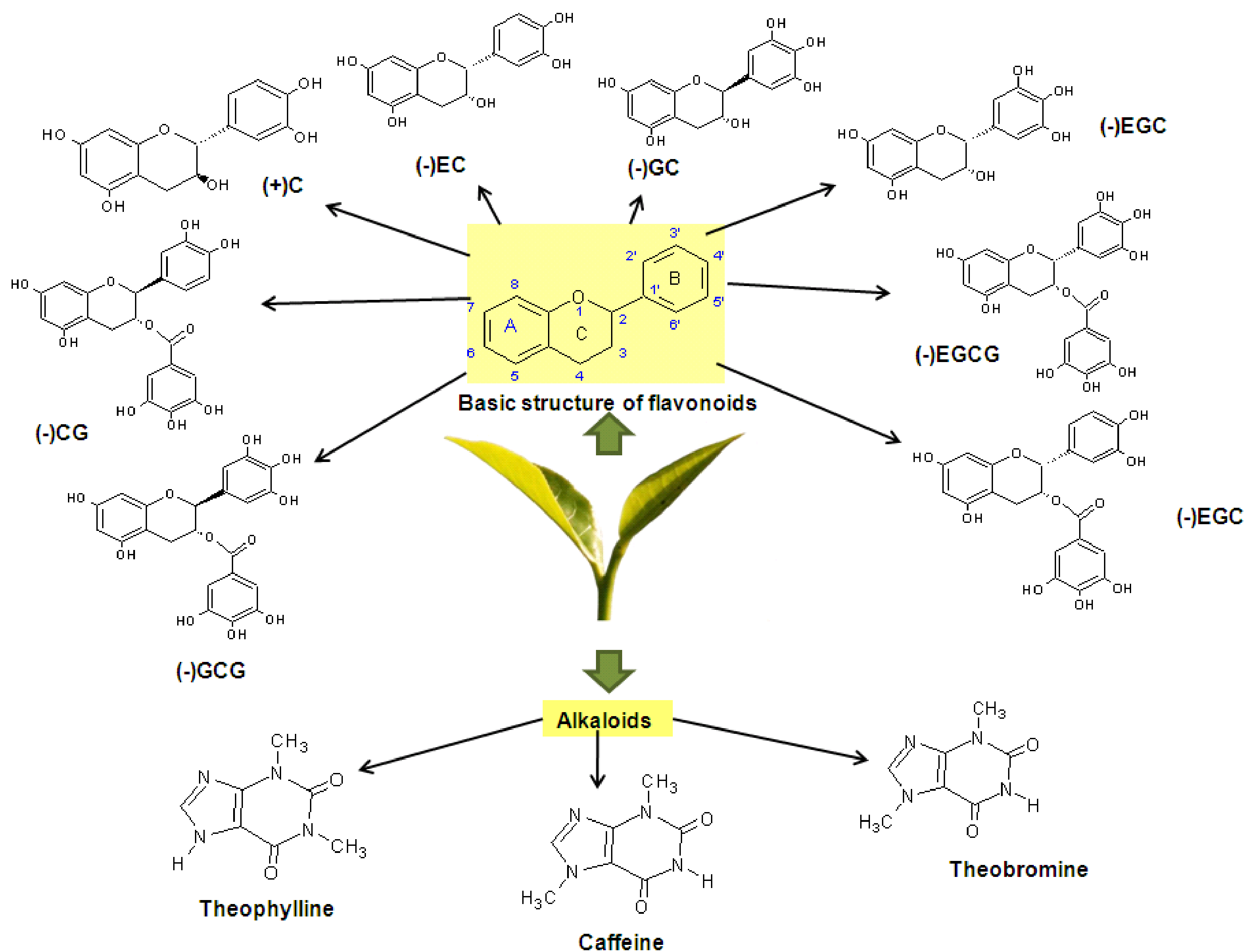

Scheme 1.4: Structure of eight catechins and three alkaloids from tea: (+) catechin (C); (-)-epicatechin (EC); (-)-gallocatechin (GC); (-)-epigallocatechin (EGC); (-)-catechin gallate (CG); (-)-epicatechin gallate (ECG); (-)-gallocatechin gallate (GCG); (-)-epigallocatechin gallate (EGCG).

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1.7. OBJECTIVES OF THE PRESENT STUDY

Tea is a socio-economic crop, which plays a major role in Indian economy. The tea industry in India contributes significantly towards the national and state economy by uplifting the foreign exchange pool and providing employment to a multitude of people. Tea consumption is associated with various health-promoting properties. The antioxidant activity of tea has been reported extensively, and numerous studies have been performed to assess the effects of tea polyphenols on the mutagenicity of carcinogens. The bottlenecks in tea, with regard to extraction and availability of metabolites, like polyphenols and alkaloids, lie in its out- breeding nature and long reproductive cycle that makes one to wait for long periods to obtain the tea bush. Improvement by conventional methods is also restricted because of the same reason. In this respect, tissue culture techniques can play an important role. Apart from shortening the growth cycle, they can also offer varied practical applications. The purpose of this investigation, therefore, was to raise haploids of *Camellia* spp. This will aid future studies related to generation of homozygous diploid lines in a single step that in turn will take care of qualitative and quantitative variation in tea metabolites due to heterozygosity. Apart from this, some very useful recessive mutations, which do not get a chance to express in presence of their dominant alleles in heterozygous, cross-pollinating trees, can be easily expressed in haploid callus and plantlets. These mutations may prove to be beneficial for consistent yield of secondary metabolites, independent of seasonal and geographical variations. Therefore, the present study was undertaken with the following specific objectives and the attempted biotechnological interventions in tea are presented in **scheme 1.5**.

Specific objectives:

- Androgenesis via anther culture.
- Histological and cytological studies of *in vitro* raised cultures.
- Antioxidant activity of in vitro raised androgenic cultures.
- Analysis of haploids for production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophylline.
- Establishment of batch suspension cultures and analysis for production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophylline.

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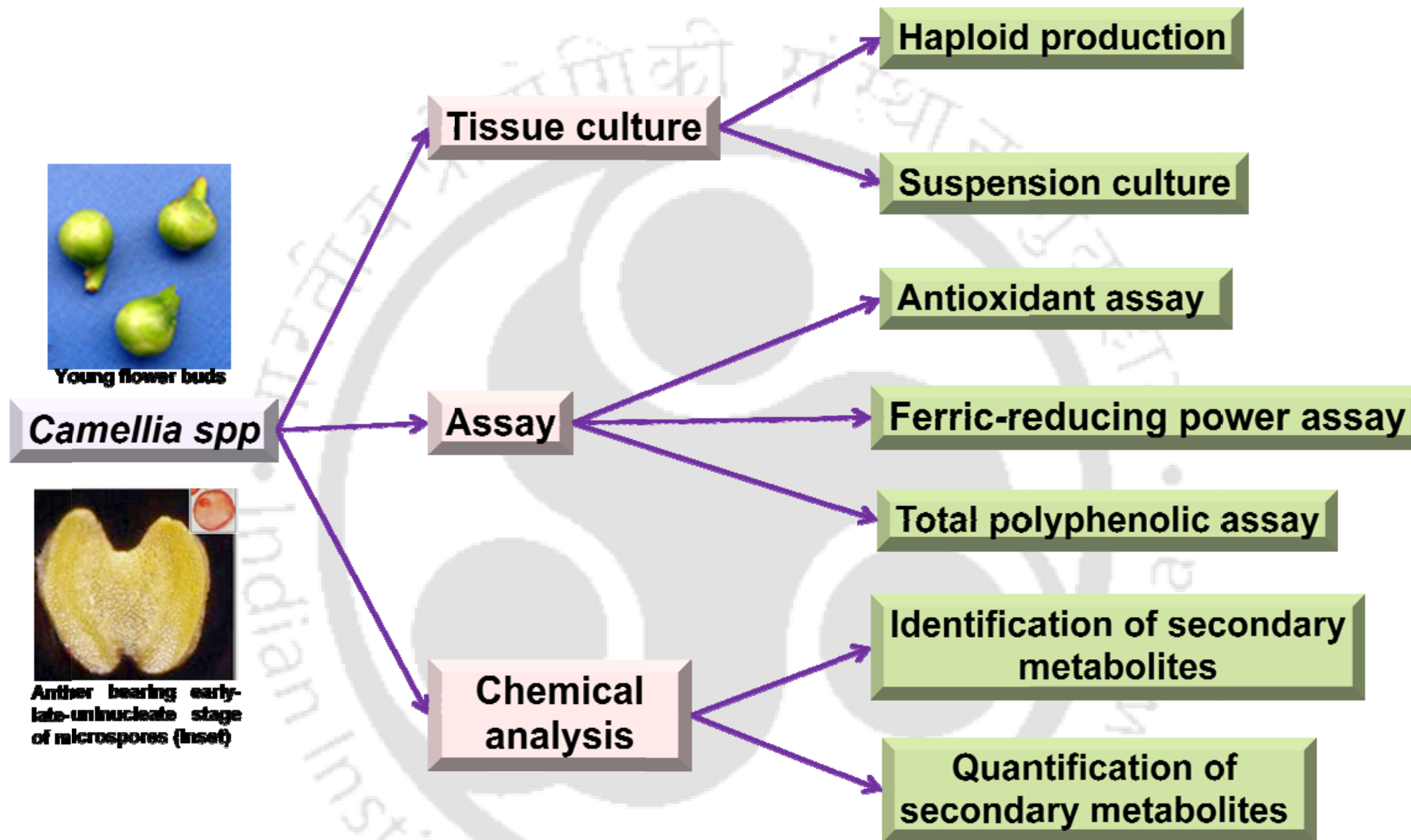

Scheme 1.5: Biotechnological interventions in *Camellia spp.*

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Chapter 2

Materials and methods

Materials used and methodologies followed, in the present study, have been divided into four sections: the first section groups together all methodologies related to tissue culture studies, second section deals with the antioxidant activity. Third and forth sections contain all methodologies related to secondary metabolite production and batch kinetic studies, respectively.

2.1. MATERIALS

Six cultivars from three taxa of tea plants viz. Assam, Cambod and China, were selected for the present study (**Table 2.1**). The experimental plants (**Figure 1 A, B**) are growing in New Botanical Area (NBA), Tocklai Experimental Station, Jorhat, Assam, India (94º12'E and 26º47'N). For identification of cultivars, the classification advocated by Wight (1962) was followed. The morphological characteristics of all the selected clones are presented in **Table 2.2**.

Table 2.1: Selected cultivars from three taxa of tea and their respective flower buds bearing uninucleate stages of microspores

Sl. No.	Cultivar name	Age of plant (in year)	Taxa	Size of bud (in mm)
1.	TV1	10	Assam – China hybrid	3.0
2..	TV18	35	Cambod type	3.5
3..	TV19	35	Cambod type	4.0
4.	TV21	40	Assam type	4.0
5.	317/1	30	China type	3.0
6	14/100/1	30	China type	3.0

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Table 2.2: Morphological characteristics of the selected cultivars

Clones	TV1	TV18	TV19	TV21	317/1	14/100/1
Bush habit	Tree	Tree	Tree	Tree	Shrub	Shrub
Habit	Semi arbour	Semi arbour	Semi arbour	Semi arbour	Semi arbour	Semi arbour
Growth habit		Erect upright	Erect upright	Erect upright	Horizontal spreading	Horizontal spreading
Immature leaf color	Light green	Yellowish green	Yellowish green	Light green	Dark green	Dark green
Mature leaf shape		Lanceolate	Lanceolate	Lanceolate	elliptic	Broadly elliptic
Leaf apex	Acuminate	Acuminate	Acuminate	Acuminate	Obtuse	Obtuse
Leaf margin		Serrulate	Serrulate	Serrulate	Serrulate	Serrulate
Leaf length (cm)	13.9	15.2	17.1	14.7	11.6	10.9
Leaf angle	Erect	Erect	Erect	Erect	Erect	Erect
Leaf surface	Hard, Glabrous, Matt	Thin, Glossy	Thin, Glossy	Thin, Glabrous	Hard, Leathery, Matt	Hard, Leathery, Matt
Flowering Position	Axillary	Axillary	Axillary	Axillary	Axillary	Axillary
Bud Size (cm)	1.6	1.7	1.6	1.6	1.5	1.6
Calyx aestivation	Imbricate	Imbricate	Imbricate	Imbricate	Imbricate	Imbricate
Number of sepals	5	5	5	5	5	5
Petal surface	5	5	5	5	5	5
Attachment of filament to anther	Adaxial	Adaxial	Adaxial	Adaxial	Adaxial	Adaxial
Splitting of style	Tri-fid	Tri-fid	Tri-fid	Tri-fid	Tri-fid	Tri-fid
Stigma	Stalked	Stalked	Stalked	Stalked	Stalked	Stalked
Ovary type	Trilocular	Trilocular	Trilocular	Trilocular	Trilocular	Trilocular
Carpel position	Superior	Superior	Superior	Superior	Superior	Superior
Fruit Shape	Coccate	Coccate	Coccate	Coccate	Coccate	Coccate
Number of seeds / fruit (range)	1-3	1-3	1-3	1-3	1-3	1-3
Seed color	Dark Garnet brown	Dark Garnet brown	Dark Garnet brown	Dark Garnet brown	Dark Garnet brown	Dark Garnet brown

Young, healthy flower buds (**Figure 2 B**), bearing early-to-late uninucleate stage of microspores (**Figure 2 D-F**), were harvested from the first flush of flowering in the morning, between 6.00 to 7.00 AM, during the months from October to December (2007-2010).

The plant growth regulators, Folin-Ciocalteu reagent, DPPH (2,2-diphenyl-2-picrylhydrazyl), Gallic acid and standard compounds, like catechins, caffeine and theophylline, used in the present study, were procured from Sigma-Aldrich, USA. Constituents of MS (Murashige and Skoog, 1962) and N_6 (Chu, 1978) basal media were

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purchased from Merck, India. Glassware and plasticware were obtained from Borosil, India and Tarsons, India, respectively. For ploidy analysis, propidium iodide and RNAase were procured from Sigma, USA.

For chemical analysis, HPLC grade acetonitrile, ethyl acetate, orthophosphoric acid (H_3PO_4), methanol and hexane were purchased from Merck, India. Milli-Q water (Millipore, USA) was used for extraction and HPLC analysis.

2.2. METHODS

2.2.1. In vitro culture

2.2.1.1. Initiation and establishment of anther cultures

The buds from first flush of flowers were used to initiate anther cultures (**Figure 2 A**). The stage of microspore development was checked with acetocarmine squash preparation of anthers. The selected, appropriate size of flower buds (**Table 2.1**), having early-to-late-uninucleate stage of microspores, were surface sterilized with either 0.1 % Mercuric Chloride ($HgCl_2$, Merck, India) or 0.8 % Sodium hypochlorite (NaClO, Merck, India), solution for 7 min, followed by rinsing with sterile distilled water at least thrice. All the steps were performed inside a laminar-air-flow cabinet (Klenzaids, India). Stages of microspores were visualized under light microscope (Nikon, Japan) after staining with acetocarmine (Sigma-Aldrich, USA). Anthers bearing correct stage of microspores were isolated very carefully from flower buds under a stereo-microscope (Nikon, Japan) using pre-sterilized Petriplates, forceps and fine needles. The damaged anthers, if any, were discarded and filament was gently removed. Twenty anthers from single bud (a bud contains 50 anthers) were cultured in 60 mm x 15 mm pre-sterilized, disposable Petriplates (**Figure 2 I**) containing 10 ml of MS basal medium. The basal medium was variously supplemented with different combinations and concentrations of growth regulators like, 2,4-D, BAP, IAA, IBA, Kn, NAA, TDZ, Zeatine, and amino acids like, L-Glutamine and L-Serine. After inoculation of anthers, Petriplates were sealed with parafilm (Pechiney, USA) and cultures were incubated either in light or in dark until callus induction. Best responding medium was tested with varying concentrations of glucose and sucrose at range of 3 %, 6 %, 9 % and 12 %.

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2.2.1.2. Pre-treatment

Temperature pre-treatment for a particular duration seems to have promotary effect for induction of androgenesis. Therefore, entire buds or anthers at culture were given temperature pre-treatments for 0, 5 and 10 days at cold (5°C in dark) or heat (33°C in dark) where continuous light or dark incubation at 25°C served as a control in all the experiments. After temperature pre-treatments, the cultures were transferred to 25±2°C and maintained in dark with 50-60 % relative humidity. For each treatment, 100 anthers were raised and each experiment was repeated at least three times. The cultures were examined periodically and the morphological changes were noted on the basis of visual observations. The results are expressed as percentage responding cultures.

2.2.1.3. Callus multiplication

Only those calli, which were induced from inside the anther locules (microspores) in the induction medium were considered for further experiments. After the passage of two subcultures, each of 6 weeks duration, on the induction medium, then the calli were transferred to diffused light under 16-hour photoperiod irradiance (1000-2000 lux) provided by cool day light fluorescent tubes (Philips TL 40W) at 25±2ºC and 50-60 % relative humidity. After the third subculture of six weeks duration on induction medium, the calli (ca. 0.2 g) were transferred to multiplication medium consisting of different sets of growth regulators. Increasing value of fresh weight (callus growth index (CGI)) was calculated as following equation

$$\mathbf{CGI} = \frac{(\mathbf{CW_f} - \mathbf{CW_i})}{\mathbf{CW_i}}$$

Where, CW_f is final (after 8 weeks) and CW_i is initial (at the time of culture) weight of callus, respectively.

2.2.1.4. Callus regeneration

Since the origin of callus often affects the regeneration, the calli derived from all multiplication media were utilized for regeneration experiments. Regeneration in the calli occurred via embryogenesis or organogenesis. The nodulated calli from multiplication

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medium were transferred to the MS medium consisting of various combinations and concentrations of auxins (2,4-D, NAA), cytokinins (BAP, TDZ, Kinetin), Gibbrellic acid (GA_3), amino acids (L-Glutamine and L-Serine) and additives like, Casein hydrolysate (CH) and Adenine sulphate. MS basal medium served as control.

2.2.1.5. Embryo maturation and germination

For embryo maturation, fully developed embryos were transferred to either MS basal medium or basal medium consisting of 10 times reduced concentrations of the growth regulators (BAP and GA_3) and amino acids (Glutamine and Serine) present in the embryo differentiation medium (regeneration medium). After 6 weeks on maturation medium, the embryos were transferred to germination medium consisting of ½ MS medium (major salts reduced to half strength) supplemented with growth regulators, like BAP, GA_3 and IBA and amino acids, like Glutamine and Serine.

Cultures for callus multiplication, regeneration, embryo maturation and germination were grown in 150x25-mm glass culture tubes (Borosil, India), each containing 20 ml of medium. All the cultures were maintained at 25±2ºC and 50-60 % relative humidity under 16-hour photoperiod irradiance (1000-2000 lux) provided by cool day light fluorescent tubes (Philips TL 40W). Media containing 3% sucrose is used unless mentioned elsewhere. Fifty-four cultures were raised for each treatment, and each experiment was repeated at least three times. Observations were recorded at weekly intervals.

2.2.1.6. Culture media

The anther cultures were raised on MS and N_6 basal media (for composition see **Table 2.3**). For subsequent experiments, only MS basal medium was used. The media were variously supplemented with growth regulators and other adjuvants (**Table 2.4**). Unless mentioned otherwise, all the media contained 3% sucrose and were gelled with 0.8 % agar (HiMedia, India).

Analytical grade (AR) chemicals and Milli-Q water were used for preparation of all stock solutions and culture media. Stock solutions of macronutrients (x 20), micronutrients (x 200), iron (x 200) and vitamins (x 200) were made separately and stored at 4°C till

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further use. Growth regulators stock solutions were prepared at a concentration of $1x10^{-3}$ M and stored in refrigerator at 4°C till further use. Myoinositol and sugar were weighed and directly added to the culture medium at the time of media preparation. Required quantities of the various stock solutions, growth adjuvants and sugar were added to molten agar and final volume was made up with distilled water (Elix, Millipore, USA). After adjusting the pH to 5.8, by using 0.1N HCl or 0.1N NaOH, the medium was dispensed into 150 x 25 mm Borosil rimless glass tubes with 20 ml medium per tube. The culture tubes were plugged with nonabsorbent cotton wrapped in cheesecloth and autoclaved at 15 psi pressure and 121°C for 20 min. For initiation of anther cultures, the media were autoclaved in Erlenmeyer flasks and allowed to cool down to ca. 50°C, before dispensing into 60 mm pre-sterilized Petriplates under aseptic conditions. The thermolabile substances, such as IAA, ABA, GA_3, TDZ, L-Glutamine and L-Serine were filter sterilized using Millipore filters (0.45 µm pore size) and added to the autoclaved medium cooled to 50°C. The medium was then dispensed into glass tubes / Petriplates inside the laminar-air-flow cabinet.

2.2.1.7. Inoculation

All aseptic manipulations of the plant material, as well as inoculations, were made inside the laminar-air-flow cabinet. Before starting the work, the platform and the inner sides of the cabinet and the Stereo-microscope, used for dissecting the flower buds, were swapped with rectified spirit (70% Alcohol) and exposed to UV light for 15-20 min. The instruments used for inoculation (forceps, scalpels and needles etc.), glass-slides and Petriplates, used for dissection and chopping the material at subculture, were wrapped in aluminum foil and autoclaved. During inoculation, at regular intervals, the instruments were dipped in 90% ethanol, flamed and cooled before use. At the time of raising fresh cultures or subcultures, the rim of the glass tube was flamed, the plug was removed and after planting the material on the medium the plug was replaced in quick succession.

2.2.1.8. Culture conditions

The cultures were generally incubated at 25±2°C and 50-60 % relative humidity. Depending on the experiment, the cultures were maintained in either diffuse light (1000-

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2000 lux) with 16 hour photoperiod, provided by cool day light fluorescent tubes (Philips TL 40W) or in continuous darkness. To study the effect of temperature on callus induction, the anther cultures were incubated at 5°C and 33°C for different periods before being transferred to 25°C.

Table 2.3: Constituents of MS (Murashige and Skoog, 1962) and N_6 (Chu, 1978) basal media. Concentrations are expressed in mg/l.

Medium components	MS	N_6
Major constituents		
NH_4NO_3	1650	-
$(NH_4)_2SO_4$	-	463
KNO_3	1900	2830
$CaCl_2.2H_2O$	440	166
$MgSO_4.7H_2O$	370	185
KH_2PO_4	170	400
Minor constituents		
KI	0.83	0.8
H_3BO_3	6.2	1.6
$MnSO_4.4H_2O$	22.3	4.4
$ZnSO_4.7H_2O$	8.6	1.5
$Na_2MoO_4.2H_2O$	0.25	-
$CuSO_4.5H_2O$	0.025	-
$CoCl_2.6H_2O$	0.025	-
Organic constituents		
Myoinositol	100	-
Nicotinic acid	0.5	0.5
Pyridoxine.HCl	0.5	0.5
Thiamine.HCl	0.1	1
Glycine	2	2
Iron constituents		
$FeSO_4.7H_2O$	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3
Sucrose	30,000	30,000
Agar	8,000	8,000
pH	5.8	5.8

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Table 2.4: Growth regulators, amino acids and other compounds used as additives to basal media.

Additives	Manufacturer
Auxins	
2,4-D	Sigma, USA
NAA	Sigma, USA
IAA	Sigma, USA
IBA	Sigma, USA
Cytokinins	
BAP	Sigma, USA
Kinetin	Sigma, USA
Zeatin	Sigma, USA
TDZ	Sigma, USA
2-iP	Sigma, USA
Aminoacids	
L-Glutamine	Sigma, USA
L-Serine	Sigma, USA
Other compounds	
GA_3	Sigma, USA
ABA	Sigma, USA
Casein hydrolysate	HiMedia, India
Glucose	Merck, India
Sucrose	Merck, India

2.2.1.9. Observation of cultures

Unless mentioned otherwise, eighteen cultures were raised for each treatment and all the experiments were repeated at least three times. The cultures were observed periodically, and the morphological changes were recorded at weekly intervals or whenever necessary. Final observations were taken after 8 weeks. Standard error of the mean was calculated and is indicated by ± sign. In primary cultures of anthers, the number of explants showing callus induction was recorded. The degree of callusing was expressed in terms of diameter of callus. Regeneration in the subcultures of callus is expressed as percent response.

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2.2.1.10. Histological studies

For histological studies, the materials, like regenerating calli and embryos, were sampled, and wax sections were cut to trace their origin and developing vascular strands in the tissue. Materials were fixed in FAA (5:5:90 v/v/v Formaldehyde: Acetic acid: 70 % Ethanol) for 48 hours and then preserved in 70 % alcohol. The material was passed through the tertiary-butyl-alcohol series for dehydration, infiltrated with paraffin wax (melting point 60ºC, Merck, Germany) and, finally, embedded in pure paraffin wax. The paraffin blocks were mounted on wooden stubs, and 8-10 µm-thick sections were cut using a Manual rotary microtome (Leica, Germany) fitted with a steel knife. The sections were mounted on microslides, dewaxed and double stained with safranin (1 %) and astra-blue (1 %) and finally examined under the microscope (Nikon, Japan).

2.2.1.11. Scanning electron microscopy

The anther-derived nodulated calli from regeneration media were fixed in 2.5 % glutaraldehyde and dehydrated through a graded alcohol series (30 %, 50 %, 70 %, 90 % and pure alcohol) and, thereafter, kept in desiccators for drying. After drying, the samples were sputter-coated with gold and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

2.2.1.12. Ploidy analysis

The ploidy levels of the in vitro regenerants were determined by cytological squash preparation which was further confirmed by flow cytometry analysis.

2.2.1.12A. Squash preparation

For cytological analysis, in vitro grown root-tips of haploid plants and shoot-tips from field grown parent plant (control) were fixed in 0.02 % 8-Hydroxyquinoline for 4 hours at 4ºC around 10.00 am, and, thereafter, washed thoroughly, transferred and stored in modified Carnoy's fixative (7:3:1:1v/v/v/v Absolute alcohol: Choloroform: Methanol: Glacial acetic acid) under refrigeration for 48 hour. The fixed material was placed in a mixture of nine drops of 1 % aceto-orcein and one drop of 1N HCl in a watch glass and

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heated gently. After cooling, the material was placed in a drop of fresh stain on a glass slide, and a cover slip was placed over it. The slide was warmed gently and the material was squashed. The number of chromosomes was counted under 100X objective of Nikon 80i microscope.

2.2.1.12B. Flow cytometry analysis

The ploidy levels of regenerants were also determined by using leaves of in vitro derived plantlets in FACs Calibur cytometer (Becton-Dickinson, USA) with an argon laser (15mV) at 488 nm encompassing an emission range of greater than 590 nm. The nuclear suspension from fresh material was prepared by chopping the tissues with scalpel in cold nuclear isolating buffer such as woody plant buffer (with some modification) (Loureiro et al., 2007). Constituents of modified woody plant buffer was prepared by mixing 0.2 M Tris HCl, 4mM $MgCl_2.6H_2O$, 2.5 mM EDTA $Na_2.2H_2O$, 86mM NaCl, 10 mM Sodium Metabisulfite, 1.5 % Triton-X-100 and 2 % PVP-10. The pH was adjusted to 7.5. The solution was filtered through 0.22 µm PVDF membrane filter (Pall Corporation, Mumbai, India) and stored at 4ºC. The 50 mg fresh callus and leaf (as an external standard) sample were chopped in modified woody plant buffer, and then filtered with 30.0 µm nylon membrane (Millipore, USA). The filtered materials were treated with 50 µg/ml of RNAse (Sigma, St. Louis, USA) and simultaneously stained with 50 µg/ml concentration of Propidium iodide (Sigma, St. Louis, USA). Using instrument gain (photomultiplier voltage and amplitude gain), the position of peak G_1 nuclei of the reference sample i.e. control was established on channel 200 on a 1024 scale following which the instrument settings were kept constant and the unknown samples were analysed under the same parameters.

2.2.2. Antioxidant activity

2.2.2.1. Selection of tea cultivar and cell lines

The anther derived cultures of the three cultivars, TV19, TV21 and 317/1, one each from the three taxa, Cambod, Assam and China, respectively, were selected for antioxidant activity assay. Out of the three cultivars, in vitro embryogenesis was observed only in TV19 and TV21. Therefore, the growing calli (from the multiplication medium) and

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fresh, young leaves of the parent plants (control) from all the three cultivars and in vitro embryos of TV19 and TV21 cultivars, were employed for antioxidant activity assay. The cultures from the multiplication and the embryogenic media (**Table 2.5**) were harvested after 8 weeks, washed with distilled water and filtered under vacuum. Thereafter, washed samples and parent plant leaves (control) were dried in an oven at 35°C ± 2°C until the constant weight was achieved.

Table 2.5: In vitro cultures of respective cultivars taken for extraction.

Cultivar	Botanical name	Callus proliferation media constituents	Embryogenic media constituents
TV19	*Camellia assamica* ssp. *lasiocalyx* (Planch MS)	MS (3% Sucrose) + NAA (5 µM) + BAP (10µM)	MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)
TV21	*Camellia assamica* ssp. *assamica* (Masters)	MS (3% Sucrose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)
317/1	*Camellia sinensis* (L.) O. Kuntze	MS (3% Sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM)	-

2.2.2.2. Extraction procedure

For extraction, the dried powdered samples, (weighing 10 g dried weight), were soaked for 12 hours in 400 ml of either hot-water or separately in different analytical grade organic solvents, like methanol, ethyl acetate and hexane. Water soaked samples were then boiled at 80ºC for 20 min in round bottom flask (Borosil, India) covered with aluminum foil, on magnetic stirrer (Ika, China) and then cooled to room temperature. Both, water and solvent based extracts were individually filtered through Whatman filter paper no.-1 (GE Healthcare, England) and filtrate was centrifuged at 10,000 rpm for 10 min (Sigma, USA). While the water based resulting supernatant was lyophilized in freeze dryer (Chaist (Model alfa 1-4), Osterode am Harz, Germany), the organic solvent based supernatants were evaporated to dryness in a rotary evaporator at 40°C (Buchi Rotavapor R-200, Japan). The samples were stored at 4ºC for further studies. The percentage yield of extracts was calculated by using formula:

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$$\textbf{Extraction yield (\%)} = \frac{\textbf{Weight of dried extract (in g) X 100}}{\textbf{Weight of dried callus or leaves (in g)}}$$

2.2.2.3. Total phenolic content

Total phenolic constituents of extracts were determined by following the methods adopted by Singh et al. (2013) and Hazarika and Chaturvedi (2013) involving Folin–Ciocalteu reagent and gallic acid standard (Sigma, USA). Solutions of each extract amounting to 20 µl were taken in individual test tubes. To this solution, 1.58 ml water and 100 µl Folin Ciocalteau reagent (2N; Sigma, USA) were added. After 3 min, 300 µl of 20 % Na_2CO_3 (Merck, India) was added. The reaction mixture was incubated at 40ºC for 30 minutes and the absorbencies were measured at 765 nm, using a spectrophotometer (Carry 100). The concentration of total phenolics was expressed as gallic acid equivalents (GAE) (mg/g dry weight). The same method was repeated for the standard gallic acid solutions, and a standard curve obtained. All the samples were analysed in triplicate and total phenolic contents as gallic acid equivalents was reported.

2.2.2.4. Determination of antioxidant activity

2.2.2.4A. DPPH free-radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity of calli, embryogenic cultures and leaves from parent plants (control) was evaluated by following the method used by Leong and Shui (2002) and Chan et al. (2007) with minor modifications. Stock solution of DPPH (Sigma, USA) was prepared at concentration of 5.9 mg/100 ml methanol. Ascorbic acid, Catechin, Epicatechin, Epigallocatechin gallate, Gallic acid and Vanillic acid (all purchased from Sigma, USA) were used as reference standards and their stock solutions (1 mg/ml) were also prepared in Methanol. The stock solutions (1 mg/ml) of the crude extracts were prepared in their respective solvents. In separate test-tubes, working solutions of each crude extracts and reference standards were prepared at different dilutions (concentration range of 1 µg/ml -180 µg/ml), amounting to a total of 1 ml. To these dilutions, 2 ml of DPPH solution was added. After being mixed gently it was left to stand for 30 min at room temperature, the absorbance of each mixture

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was measured at 517 nm with spectrophotometer (Carry 100, Netherlands). The Radical scavenging activity was expressed as the percentage (I %) inhibition of free-radical by the tested samples and was calculated by using following formula:

$$\textbf{Percentage inhibition (I \%)} = \frac{(A_{blank} - A_{sample})\ X\ 100}{A_{blank}}$$

Where A_{blank} is the absorbance value of the control reaction containing 1 ml of respective solvents (water, methanol, ethyl acetate and hexane), separately, with 2 ml of DPPH without the extract and A_{sample} is the absorbance value of the each tested sample. The antioxidant activity of each sample was expressed as IC_{50} (concentration in μg/ml required to inhibit DPPH radical formation by 50 %) and was calculated from the dose inhibition curve. Lower the IC_{50} value, higher is the antioxidant activity. The best results were also expressed as AEAC (ascorbic acid equivalent antioxidant activity) in mg/100g. Calculation of AEAC was done by following formula:

$$\textbf{AEAC (mg AA/100g)} = \frac{IC_{50\ (ascorbate)}\ X\ 100,000}{IC_{50\ (sample)}}$$

Where $IC_{50\ (ascorbate)}$ is IC_{50} value of ascorbic acid and $IC_{50\ (sample)}$ is IC_{50} value of sample.

2.2.2.4B. Ferric-reducing antioxidant power

For assessing ferric-reducing antioxidant power (FRAP), the methods were adapted from Chu et al. (2000) and Chan et al. (2007) with minor modifications. FRAP assay was done only for those samples which have higher capacity for free-radical scavenging activity. From the stock solution (1 mg/ml) of three tested samples, calli, embryogenic cultures and leaves from parent plants (control), 1 ml was taken and added to 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1 %, w/v). After proper mixing, the mixture was incubated at 50°C for 20 min. Then 10 % (w/v) trichloroacetic acid solution (2.5 ml) was added, to stop the reaction. The mixture was separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To every diluted aliquot, 500 µl of 0.1 % ferric chloride solution (w/v) was added. After 30 min, absorbance was measured in spectrophotometer (Carry 100, Netherlands) at 700 nm. FRAP of extracts was expressed as mg GAE/g. Standard curve was generated by using Gallic acid for assaying reducing

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power. The calibration equation for Gallic acid was y = 15.91x (R^2 = 0.9974), where y is the absorbance and x is the Gallic acid concentration in mg/ml

2.2.3. Secondary metabolite production

2.2.3.1. Preparation of sample solution

The in vitro callus from the cultivars TV19, TV21 and 317/1 and embryos of the cultivars TV19 and TV21, grown on various media (**Table 2.5**), were taken for screening of metabolites. The leaves from the field grown parent plants of the three cultivars served as controls. As hot water extracts possess high phenolic contents with significant antioxidant activity, therefore, it is considered for analysis of secondary metabolite production. The details of hot water extraction procedure and sample preparation is described in section 2.2.2.2.

2.2.3.2. Preparation of standard solutions

Stock solutions of standard (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline were prepared, individually, in Milli Q water at a concentration of 5 mg/ml and these solutions were stored at 4°C. The quantification was performed using 5 levels of external standards, obtained by serial dilutions of stock solutions, at a concentration range of 25 µg/ml to 500 µg/ml. Each concentration of standards was filtered through 0.22 µm nylon membrane filter (Millipore, USA) before HPLC analysis and run at least thrice to check the repeatability and precision of results. Both external and internal standards (spiking) were employed for confirmation of the compounds.

2.2.3.3. High performance liquid chromatography (HPLC)

Identification and quantification of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline was carried out on Varian Prostar HPLC system (Varian, USA) equipped with a binary pump, UV detector and a 20 µl injection loop. Hypersil BDS RP-C_{18} column (Thermo, USA) of dimensions 250 x 4.6 mm with 5 µm particle size was used. For the analysis of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate and caffeine, acetonitrile:ethyl acetate:0.05 % H_3PO_4 (12 : 2 : 86 v/v) was used as the mobile phase at a flow rate of 0.5 ml/min and the eluted samples

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were detected at 280 nm wavelength. While for analysis of theophylline, acetonitrile:water (10:90) (v/v) was used as the mobile phase, at a flow rate of 1 ml/min and the eluted samples were detected at 273 nm wavelength. The identification of all five compounds was done by comparing their retention times with those of authentic standards. The crude and standard samples were filtered through 0.22 μm nylon membrane filters (Millipore, USA), prior to analysis, and aliquots of 20 μl of clean solution were injected into the HPLC system. System suitability tests were performed by checking linearity, precision and recovery of compounds in the developed assay. Each of the five peaks, corresponding to the five compounds, were isolated from the crude extracts and used for mass spectroscopic analysis.

2.2.3.4. Linearity, precision and recovery of compounds

Linearity of developed method was checked by running the standard compounds at five different concentrations. A calibration curve was generated by plotting concentration against peak area on Microsoft office excel 2007. The standard equation obtained from the curve, was used for quantification of all the compounds in the unknown samples. The correlation coefficients (R^2) were also generated in the same software by fitting the linear trend lines to the standard curves obtained for each of the five compounds.

Precision of developed assay was evaluated by running same concentration of standard compounds atleast three times on the same day (intraday) and thrice at one day interval (interday). The values were calculated in terms of relative standard deviation (RSD).

$$\textbf{RSD} = (\textbf{SD}/\textbf{average}) \times 100\ \%$$

The recovery experiments for all the five compounds were performed by adding known amount of C, EC, EGCG, CAF and T standards to the extracting sample, which were then extracted in a similar manner as mentioned in the section 2.2.2.2 The recovery percentage was calculated as:

$$\textbf{Recovery (\%)} = (A - B / C) \times 100$$

Where, *A* is the quantity of C, EC, EGCG, CAF and T in the spiked powder, *B* is the quantity of C, EC, EGCG, CAF and T in the powder without adding standards and *C* is the quantity of added C, EC, EGCG, CAF and T.

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2.2.3.5. Mass Spectroscopy (MS) analysis

MS detection was carried out on Waters quadrapole-Tof premier mass spectrometer with micro channel plate detector (Waters, USA) and was operated in the positive ion mode, with collision energy of 5 V. Voltage on ESI probe tip was 3 to 3.5 kV in positive mode. The MS data were obtained in full scan mode (mass range 100–1000 amu). A comparison of mass spectra of HPLC eluted five standard compounds obtained from Sigma-Aldrich, with those of the individual samples isolated from HPLC, confirmed the presence of all the compounds.

2.2.4. Batch kinetics of cell suspension cultures

2.2.4.1. Selection of elite cell line

Among the three cultivars, TV21 yielded maximum amount of metabolite production. Therefore, for establishment of cell suspension cultures, a significantly high metabolite producing callus line of only TV21 cultivar was selected. After several passages of subcultures, the calli turned soft and friable on responding semi-solid medium, MS + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). The friable calli, thus obtained, were utilized to establish cell suspension cultures of TV21.

2.2.4.2. Establishment of cell suspension cultures

The cell suspension cultures were initiated in Erlenmeyer flasks of 250 ml capacity, containing 50 ml of liquid medium. To established the cultures, 100 mg of fresh, friable calli, obtained on semi-solid medium, were inoculated initially in four media combinations, viz. MS + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l), MS + BAP (22 µM) + IBA (10 µM), MS + 2,4-D (10 µM) + BAP (5 µM) and MS + 2,4-D (3 µM) + TDZ (18 µM). The cultures were incubated in an orbital shaker under shaking conditions at 120 rpm and continuously maintained in dark at 25 ± 2ºC. Further studies to observe the effect of pH, sucrose and agitation speed were conducted only on the best medium supporting maximum cell proliferation.

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2.2.4.2A. pH of medium

Generally, the pH of the culture medium has critical role on the cell biomass growth. Therefore, to assess the effect of pH, cells were inoculated into media adjusted at pH range of 5.4 to 6.2. The pH of the medium was set before autoclaving the medium.

2.2.4.2B. Sucrose concentration

Sucrose has been widely employed as a principal carbon source for cultivation of plant cell cultures. In general, it has been observed that the rate of cell growth is directly proportional to the concentration of sucrose in the medium. Therefore, the effect of sucrose concentration (1 % – 7 %) was also tested on cell biomass growth. The cells were inoculated into the medium in a way that each 250 ml Erlenmeyer flasks containing 50 ml of the medium had 100 mg of the cells.

2.2.4.2C. Agitation speed

Effect of agitation speed was evaluated on fresh and dry weight of cells and their viability, at the end of each passage. Callus cells weighing approximately 100 mg were harvested from the best medium, at the end of growth period (at 21st day) and were re-inoculated in 50 ml of the fresh medium of the same composition. The cultures were incubated in shaking conditions at 90, 120 and 150 rpm, under darkness, for a period of 30 days and their fresh and dry weights were recorded. The viability of cells under each condition was checked with 1 % fluorescein diacetate (FDA) solution.

Further multiplication and maintenance of cell suspension cultures, and batch kinetic studies were performed only on the best medium, under optimized parameters, which favoured maximum cell biomass growth.

2.2.4.3. Batch kinetics studies

To determine the specific growth rate, cells were harvested from optimized liquid medium at an interval of every three days. The cells were washed and dried by following the same procedure as mentioned in the section 2.2.2.1. The pH of the cell suspension cultures was monitored after every three days. Phosphate consumption was calculated by the standard calibration curve made from dihydrogen sodium phosphate (NaH_2PO_4); to

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0.5 ml of standard or sample solution, 4 ml of reagent [Acetone (CH_3COCH_3), Sulphuric acid (H_2SO_4) 2.5 M and Ammonium molybdate tetrahydrate $(NH_4)_2MoO_4.4H_2O$) 10 mM, mixed in the ratio of 2:1:1] was added. After thoroughly mixing the solutions, 0.4 ml of 1M citric acid was added and absorbance was taken at 355 nm. Similarly, for nitrate estimation, standard curve was made from 0.01 N stock solution of Potassium nitrate (KNO_3), preserved in chloroform. After acidification of samples with hydrochloric acid, absorbance was recorded at 275 nm in a UV visible spectrophotometer (Cary 100, Netherlands). All estimations and results are average readings obtained from three flasks. Results are represented as mean ± standard deviation (SD). Specific growth rate (μ) was calculated by: $\mu = \ln (MT_2 - MT_1) / T_2 - T_1;\ T_2 > T_1$

where, MT_2 and MT_1 are biomasses at different time points (T_1 and T_2) respectively (Srivastava et al., 2011).

2.2.5. Statistical analysis

For initiation of anther cultures, 20 anthers were placed in one Petridish with a total of 100 anthers per treatment and each experiment was repeated thrice. Data were collected as number of responding anthers relative to total number of anthers cultured per treatment. Callus induction responses and regeneration in the subcultured callus was expressed as percent response. The investigated parameters were analysed using analysis of variance (ANOVA) and significance was determined at $p<0.05$. The data was analysed statistically using SPSS 16.0 software version and significant differences among the mean values were compared using Duncan's multiple range test. .

For antioxidant activity, all experiments were done in triplicates including controls. Results are expressed as mean ± SD. For secondary metabolite estimation from callus and embryo cultures, observations are an average of three separate analysis. For batch kinetics studies, all experiments were done in triplicate to check the reproducibility of the results. Results are represented as mean ± SD.

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Chapter 3

Results

***Camellia* sp. or** Tea (Family: Theaceae) is the most important non-alcoholic beverage drink in the world, which plays an important socio-economic role in earning foreign exchange of developing countries, like India. The plant is highly heterozygous and heterogeneous, therefore, the production of haploid plants would help to generate pure homozygous lines to improve the commercial value of tea. The present investigation is an attempt to develop an efficient and reproducible protocol for in vitro production of haploids in anther cultures of tea and to study the effect of various stress treatments on embryo induction and plant regeneration. Additionally, an attempt was made to evaluate the androgenic lines for antioxidant activity and metabolite production. The outcomes of the experiments have been divided into four sections: the first section presents results related to tissue culture studies, the second section groups together the results on antioxidant activity. The third and fourth sections report results on biochemical analysis and batch kinetic studies.

3.1. ANTHER CULTURE

Androgenesis in anther cultures of tea involved four steps:

(1) Callus induction: The anthers were cultured at uninucleate stage of microspores on the induction medium to induce calli.

(2) Callus multiplication: The individual calli, obtained from anthers, was grown on proliferation medium to support sustained growth of callus.

(3) Regeneration from anther callus: Calli from multiplication medium were subcultured on a regeneration medium to obtain embryogenesis.

(4) Embryo maturation and germination: Distinct embryos from regeneration medium were subjected to various treatments to obtain bipolar germination.

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3.1.1 Callus induction

For establishment of cultures and androgenic callus induction, anthers bearing early-to-late uninucleate stages of microspore were used. Depending on the cultivar, the bud size that yielded anthers with uninucleate microspores vary from 3.0 to 4.0 mm in diameter (**Table 3.1, Figure 2 D-F**). The anthers in these selected flower buds were whitish yellow in color. The size of the buds was measured perpendicularly from the tip of the sepal to the base of the calyx. The smaller bud size contain tetrad stage of microspores (**Figure 2 G&H**) and bigger size of buds bear bi-nucleate stage of microspores. The appropriate sized flower buds were surface sterilized with either 0.1 % $HgCl_2$ or 0.8 % NaClO solution for 7 min. Surface sterilization with NaClO (0.8 %) for 7 min yielded >93 % aseptic cultures while with 0.1 % $HgCl_2$ for 7 min gave only around 86 % aseptic cultures (**Graph 3.1**). Therefore, for further experiments, 0.8 % NaClO for 7 min was considered for surface sterilization of flower buds.

Table 3.1: Bud size bearing different stages of microspores in the selected cultivar.

Sl. No.	Cultivar name	Bud size with tetrad stage of microspores (in mm)	Bud size with uninucleate stage of microspores (in mm)	Bud size with binucleate stage of microspores (in mm)
1.	TV1	2.5	3.0	3.5
2.	TV18	3.0	3.5	4.0
3.	TV19	3.5	4.0	4.5
4.	TV21	3.5	4.0	4.5
5.	317/1	2.5	3.0	3.5
6.	14/100/1	2.5	3.0	3.5

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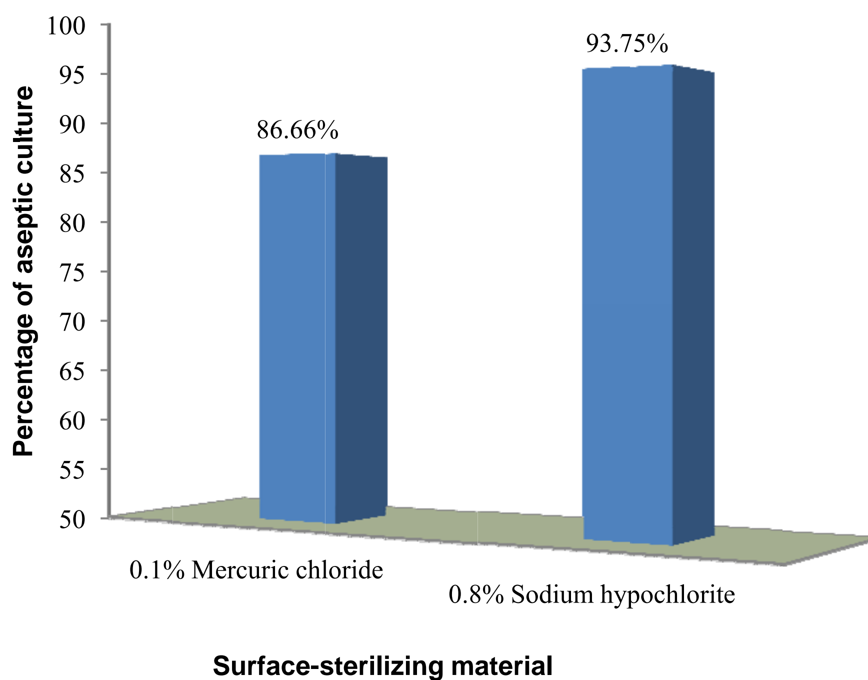

Graph 3.1: Percentage of aseptic culture establishment after surface sterilization with 0.1 % $HgCl_2$ or 0.8 % NaClO solution.

The anthers with uninucleate microspores, freshly released from the tetrads are the ideal material to induce androgenic calli as their wall is thin and it is easy to divert their gametophytic mode of development towards sporophytic pathway. On several treatments, the anthers callused and the distinct patterns of callusing were seen on the responding medium. The anther walls burst open and were pulled apart due to pressure of callusing microspores, further shiny, white, transparent mass of calli emerged from within the anther sacs along with some granulated callus from the peripheral anther walls **(Figure 3 A-I)**. The calli developed from the anther walls were excluded from further experiments. For subculture and multiplication, only those calli were considered which originated from microspores. Within 2 weeks of culture initiation, the anthers got swelled and enlarged to almost doubl of their original size **(Figure 3 A, B)** and within 6 weeks complete longitudinal furrow on anther walls appeared (**Figure 3 C**). After 7 weeks, anther sacs burst open with small calli inside the locules (**Figure 3 D**) which later on released as shiny, white, transparent callus from within the anther locules along with minute callusing from the peripheral anther walls (**Figure 3 E**) after 8 weeks. After 10 weeks,

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distinct, white shiny, profuse callus was seen from each anther locules (**Figure 3 F-I**). After 12 weeks of culture, entire anthers were covered with cream and brown growing callus (**Figure 3 J**). Therefore, after 10 weeks, the calli originating from inside the anther locules were subcultured to fresh medium of the same composition and maintained in dark.

Several factors were tested to improve callus induction from microspores in anther cultures:

i. Effect of growth regulators

No response from anthers was observed on MS and N_6 basal media. Out of various growth regulator combinations tested, the combined presence of at least one auxin and one cytokinin favored callus induction. The responded media for all cultivars are presented in **Table 3.2**. Out of all the four responded media, the combined presence of one cytokinin and two auxins, BAP (5 µM) + 2,4-D (1 µM) + NAA (1 µM) and 60 g/l Sucrose as carbon source (MS3 Medium) was observed to be most significant ($p<0.05$) for maximum percentage of callus induction in anther cultures of most of the cultivars, like TV1, TV18, TV19 and 317/1. On this medium, percentage callus induction was 66.0±4.1 TV1 (**Table 3.3**), 71.0±4.1 in TV18 (**Table 3.4**), 91.0±4.1 TV19 (**Table 3.5**) and 63.0±5.7 in 317/1 (**Table 3.7**). On the other hand, the cultivar TV21 showed significantly high callus induction response (96±1.9 %) MS2 medium consisting of MS (with 6 % Glucose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) (**Table 3.6**). While for the cultivar 14/100/1, maximum percentage of callus induction (78±5.7 %) was observed on MS4 medium consisting of MS (with 4 % sucrose) + 2,4-D (3 µM) + TDZ (18 µM) (**Table 3.8**). Irrespective of cultivars, continuous dark incubation of anther cultures at 25±2ºC was more than four times favorable for callus induction compared to those maintained continuously in diffused light at 25±2ºC (**Table 3.3 to 3.8**).

Table 3.2: Media constituents and their concentrations used for callus induction in anther cultures of tea.

Constituents	MS1	MS2	MS3	MS4
Basal medium	MS	MS	MS	MS
Sucrose (g/l)	30	-	60	40
Glucose (g/l)	-	60	-	-
2,4-D (µM)	5	5	1	3
NAA (µM)	-	-	1	-
BAP (µM)	-	-	5	-
Kn (µM)	5	5	-	-
TDZ (µM)	-	-	-	18
L-Glutamine (g/l)	800	800	-	-
L-Serine (g/l)	200	200	-	-

ii. Effect of carbon source

MS medium normally contains 3 % Sucrose. However, in the previous experiments on effect of growth regulators, the media contained higher concentrations of carbon source (Sucrose or Glucose) gave significant results on callus induction (**Table 3.9**). Therefore, to study the effect of type and concentration of carbon source and to improve the callus induction responses, various concentrations of Sucrose and Glucose were tested with the best medium of each cultivar. The results are presented in **Table 3.9**. In general, the concentrations of carbon source above 9 % were found to be inhibitory. Sucrose at its optimum concentration of 6 % was observed to be significantly better ($p<0.05$) for callus induction in majority of the cultivars, like TV1, TV18, TV19, 317/1 and 14/100/1. However, in case of TV21, Glucose at 6 % served to be the best carbon source with significant ($p<0.05$) amount of callus induction

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Table 3.3: Effect of temperature pre-treatments on percent callus induction in anthers of TV1 cultivar cultured on the responding media. Growth Period: 10 weeks

Media	Total anthers cultured	% Anthers responded at 25°C dark incubation (Control)	% Anthers responded at 5°C dark incubation for 5 days	% Anthers responded at 33°C dark incubation for 5 days	% Anthers responded at 25°C light incubation (Control)
MS1	100	40.0±5.0[b]	19.0±9.1[cd]	10.0±7.9[ef]	6.0±6.5[fg]
MS2	100	0.0±0.0[g]	0.0±0.0[g]	0.0±0.0[g]	0.0±0.0[g]
MS3	100	66.0±4.1[a]	39.0±6.5[b]	12.0±7.5[ef]	15.0±7.9[de]
MS4	100	24.0±4.1[c]	6.0±6.5[fg]	6.0±6.5[fg]	2.0±2.7[g]

Values are mean ± standard deviation
Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test

Table 3.4: Effect of temperature pre-treatments on percent callus induction in anthers of TV18 cultivar cultured on the responding media. Growth Period: 10 weeks

Media	Total anthers cultured	% Anthers responded at 25°C dark incubation (Control)	% Anthers responded at 5°C dark incubation for 5 days	% Anthers responded at 33°C dark incubation for 5 days	% Anthers responded at 25°C light incubation (Control)
MS1	100	50.0±5.0[b]	28.0±5.7[c]	9.0±6.5[e]	8.0±5.7[e]
MS2	100	0.0±0.0[e]	0.0±0.0[e]	0.0±0.0[f]	0.0±0.0[f]
MS3	100	71.0±4.1[a]	50.0±5.0[b]	22.0±5.7[cd]	18.0±9.0[d]
MS4	100	45.0±6.5[b]	21.0±4.1[d]	10.0±7.9[e]	11.0±4.1[e]

Values are mean ± standard deviation
Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test

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Table 3.5: Effect of temperature pre-treatments on percent callus induction in anthers of TV19 cultivar cultured on the responding media. Growth Period: 10 weeks

Media	Total anthers cultured	% Anthers responded at 25°C dark incubation (Control)	% Anthers responded at 5°C dark incubation for 5 days	% Anthers responded at 33°C dark incubation for 5 days	% Anthers responded at 25°C light incubation (Control)
MS1	100	52.0±2.7[c]	29.0±4.1[e]	17.0±5.7[g]	6.0±4.1[h]
MS2	100	41.0±4.1[d]	21.0±4.1[fg]	4.0±4.1[h]	4.0±6.5[h]
MS3	100	91.0±4.1[a]	69.0±4.1[b]	25.0±3.5[ef]	21.0±4.1[fg]
MS4	100	69.0±4.1[b]	40.0±5[d]	19.0±4.1[fg]	18.0±5.7[g]

Values are mean ± standard deviation

Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test

Two-way ANOVA to study the effect of different media and temperature pre-treatments on anther cultures of TV19 cultivar

SUMMARY	Count	Sum	Average	Variance
MS1	4	104	26	388.6667
MS2	4	70	17.5	309.6667
MS3	4	206	51.5	1166.3330
MS4	4	146	36.5	572.3333
25°C dark	4	253	63.25	474.9167
5°C dark for 5 days	4	159	39.75	440.9167
33°C dark for 5 days	4	65	16.25	78.2500
25°C in light	4	49	12.25	72.2500

Source of Variation	SS	Df	MS	F	P-value	F crit
Medium	2574.75	3	858.25	12.37365	**0.001519**	3.862548
Pre-treatment	6686.75	3	2228.917	32.13496	**0.000039**	3.862548
Error	624.25	9	69.36111			
Total	9885.75	15				

Table 3.6: Effect of temperature pre-treatments on percent callus induction in anthers of TV21 cultivar cultured on the responding media. Growth Period: 10 weeks

Media	Total anthers cultured	% Anthers responded at 25ºC dark incubation (Control)	% Anthers responded at 5ºC dark incubation for 5 days	% Anthers responded at 33ºC dark incubation for 5 days	% Anthers responded at 25ºC light incubation (Control)
MS1	100	67.0±1.2[c]	51.0±4.1[d]	21.0±4.1[f]	7.0±2.5[ij]
MS2	100	96.0±1.9[a]	77.0±2.7[b]	36.0±4.1[e]	24.0±4.1[f]
MS3	100	40.0±3.5[e]	18.0±5.7[fg]	12.0±10.4[gh]	5.0±5[ij]
MS4	100	64.0±5.4[c]	37.0±5.7[e]	13.0±9.7[gh]	4.0±4.1[j]

Values are mean ± standard deviation

Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test

Two-way ANOVA to study the effect of different media and temperature pre-treatments on anther cultures of TV21 cultivar

SUMMARY	Count	Sum	Average	Variance
MS1	4	146	36.50	750.3333
MS2	4	233	58.25	1148.2500
MS3	4	75	18.75	228.9167
MS4	4	118	29.50	723.0000
25ºC dark	4	267	66.75	526.2500
5ºC dark for 5 days	4	183	45.75	616.9167
33ºC dark for 5 days	4	82	20.50	123.0000
25ºC in light	4	40	10.00	88.66667

Source of Variation	SS	df	MS	F	P-value	F crit
Medium	3339.5	3	1113.167	13.81862	**0.001022**	3.862548
Pre-treatment	7826.5	3	2608.833	32.38552	**0.000038**	3.862548
Error	725.0	9	80.55556			
Total	11891.0	15				

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Table 3.7: Effect of temperature pre-treatments on percent callus induction in anthers of 317/1 cultivar cultured on the responding media. Growth Period: 10 weeks

Media	Total anthers cultured	% Anthers responded at 25ºC dark incubation (Control)	% Anthers responded at 5ºC dark incubation for 5 days	% Anthers responded at 33ºC dark incubation for 5 days	% Anthers responded at 25ºC light incubation (Control)
MS1	100	0.0 ± 0.0^{h}	0.0 ± 0.0^{h}	0.0 ± 0.0^{h}	0.0 ± 0.0^{h}
MS2	100	35.0 ± 7.0^{c}	22.0 ± 5.7^{d}	11.0 ± 4.1^{fg}	6.0 ± 4.1^{gh}
MS3	100	63.0 ± 5.7^{a}	46.0 ± 6.5^{b}	22.0 ± 7.5^{d}	14.0 ± 7.4^{ef}
MS4	100	39.0 ± 4.1^{c}	19.0 ± 4.1^{de}	8.0 ± 5.7^{fg}	6.0 ± 4.1^{gh}

Values are mean ± standard deviation

Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test

Table 3.8: Effect of temperature pre-treatments on percent callus induction in anthers of 14/100/1 cultivar cultured on the responding media. Growth Period: 10 weeks

Media	Total anthers cultured	% Anthers responded at 25ºC dark incubation (Control)	% Anthers responded at 5ºC dark incubation for 5 days	% Anthers responded at 33ºC dark incubation for 5 days	% Anthers responded at 25ºC light incubation (Control)
MS1	100	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
MS2	100	62.0 ± 8.6^{b}	23.0 ± 5.7^{d}	11.0 ± 4.1^{e}	15.0 ± 11.7^{e}
MS3	100	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
MS4	100	78.0 ± 5.7^{a}	46.0 ± 6.5^{c}	15.0 ± 7.9^{e}	17.0 ± 4.4^{de}

Values are mean ± standard deviation

Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test

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Table 3.9: Effect of carbon source (Glucose and Sucrose) and its concentration on callus induction from anthers cultured on the best responding media of respective tea cultivars. Growth Period: 10 weeks.

Percent Concentration	Percent Callus induction from anther culture					
	TV1	**TV18**	**TV19**	**TV21**	**317/1**	**14/100/1**
Sucrose						
3	49±3[b]	56±2[b]	68±3[b]	68±4[c]	53±2[b]	75±4[a]
6	66±4[a]	71±4[a]	91±4[a]	61±2[d]	63±6[a]	79±6[a]
9	51±5[b]	45±1[c]	52±5[c]	51±5[e]	42±5[d]	61±2[b]
12	29±1[d]	36±3[d]	30±4[d]	34±3[f]	26±2[f]	38±3[d]
Glucose						
3	42±2[c]	46±2[c]	48±4[c]	80±3[b]	48±3[c]	51±2[c]
6	46±3[b]	53±3[b]	67±3[b]	96±2[a]	44±1[cd]	58±3[b]
9	23±1[d]	37±1[d]	21±5[e]	63±5[cd]	32±2[e]	43±1[d]
12	16±2[e]	14±1[e]	13±3[f]	24±4[g]	19±1[g]	21±1[e]

Value ±SD are mean of three replicate
Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range test for each cultivar separately.

iii. Effect of temperature pre-treatments on callus induction

In many species, application of low or high temperature pre-treatments to entire buds or anthers, for a particular duration, is known to be promotory for induction of androgenesis. Therefore, the entire buds or anthers of tea were exposed to low (5°C in dark) and high (33°C in dark) temperatures for 0 and 5 days, to study their effect on callus induction. Light or dark incubation at 25ºC served as control in all the experiments. Among control, 25ºC dark incubation was better than 25ºC continuous light incubation cultures for callus induction. Temperature pre-treatments to entire buds did not show any response but it had pronounced effect on enhanced androgenic callus induction when anthers were pre-treated at low or high temperatures before being shifted to 25°C dark incubation. Best response was observed in control at 25°C temperature with dark incubation, followed by cold pre-treatment (5ºC for 5 days in dark) and heat pre-treatment (33ºC for 5 days in dark) (**Table 3.3 to 3.8**). Although dark incubation of anther cultures at 25ºC promoted highest percentage of callusing, the callus originated mostly from walls. The calli were

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induced from inside the burst open anther locules in maximum percentage of cultures, only, when they were given a cold pre-treatment at 5ºC for 5 days (**Table 3.10**).

The highest callus induction responses were observed in the two cultivars, TV19 and TV21. Therefore, two-way ANOVA was carried out in these two cultivars only, to study the effect of media interaction and temperature pre-treatments. Though both the two factors, media and temperature pre-treatments, are significant ($p\text{-value}<0.05$) in these two cultivars, but temperature pre-treatments are more significant than responded media (**Table 3.5 and 3.6**).

3.1.2. Callus multiplication and maintenance

For further callus multiplication, the calli (measuring ca. 5 mm x 5 mm) were subcultured twice at 6 weeks interval and maintained in dark. The calli grew well as white hard callus without any sign of regeneration (**Figure 4 A-C**). On third subculture in the same medium, the calli were transferred to diffused light conditions at 25 ± 2ºC where green areas developed in the white, hard and compact callus (**Figure 4 D**). From now on the cultures were maintained in light at 25ºC ± 2ºC. Overall multiplication rate was very slow on the induction medium. Therefore, to obtain profusely growing calli, a range of growth regulator combinations were tested. Multiplication of calli was measured in terms of fresh cell biomass increase. Highest rate of proliferation (CGI more than 2) was observed in TV21 and TV19 cultivars of tea.

In case of TV21 cultivar, significant amount of callus proliferation was observed when concentration of carbon source in the induction medium was reduced from 6 % to 3 %. On MS (3 % Sucrose) + 2,4-D (5 μM) + Kn (5 μM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) medium, the rate of callus proliferation CGI was 2.35±0.05, followed by MS (3 % Glucose) + 2,4-D (5 μM) + Kn (5 μM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) with 1.98 CGI, MS (3 % Sucrose) + 2,4-D (3 μM) + TDZ (18 μM) with 1.59 CGI and MS (3 % Sucrose) + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM) 1.20 CGI, in a single growth cycle of 8 weeks **Table 3.11**. Nature of the callus on the best proliferation medium, MS (with Sucrose) + 2,4-D + Kn + L-Glutamine + L-Serine, was hard, green, fresh, compact and fast growing (**Figure 4 E, F**).

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Table 3.10: Percentage of anther cultures showing callusing from inside the anther locules.

Cultivar	Media	Total anthers cultured	% Callus induction from inside the anther locules at 25ºC dark (Control)	% Callus induction from inside the anther locules at 5ºC dark for 5 days	% Callus induction from inside the anther locules at 33ºC dark for 5 days
TV1					
	MS1	100	14±1	8±1	1±1
	MS2	100	0±0	0±0	0±0
	MS3	100	19±2	19±1	1±0
	MS4	100	6±1	2±0	0±0
TV18					
	MS1	100	10±2	13±3	0±0
	MS2	100	0±0	0±0	0±0
	MS3	100	21±3	23±3	2±1
	MS4	100	7±2	9±2	1±0
TV19					
	MS1	100	21±2	18±1	2±0
	MS2	100	18±1	13±2	1±0
	MS3	100	35±2	41±4	3±1
	MS4	100	24±3	24±2	3±1
TV21					
	MS1	100	28±2	31±2	11±1
	MS2	100	47±6	60±5	18±2
	MS3	100	10±2	11±1	4±1
	MS4	100	17±3	21±2	3±2
317/1					
	MS1	100	0±0	0±0	0±0
	MS2	100	10±1	8±1	1±1
	MS3	100	20±2	22±3	2±1
	MS4	100	9±1	8±0	0
14/100/1					
	MS1	100	0±0	0±0	0±0
	MS2	100	6±1	5±1	1±0
	MS3	100	0±0	0±0	0±0
	MS4	100	8±2	14±3	3±1

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In the cultivar TV19, maximum callus proliferation in terms of fresh cell biomass increase i.e. CGI (2.21) was observed on MS (3 % Sucrose) + NAA (5 µM) + BAP (10 µM) medium, followed by MS (3 % Sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) with 1.85 callus growth index, MS (3 % Sucrose) + 2,4-D (3 µM) + TDZ (18 µM) with 0.81callus growth index and MS (3 % Sucrose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) with 0.26 callus growth index, in a single growth cycle of 8 weeks. The nature of the callus, on the best proliferation medium, MS + NAA + BAP, was hard, bright green, fresh and fast growing (**Figure 4 G, H**). On this medium, TV18 cultivar also showed highest cell biomass increase (CGI is 1.28). In case of TV1 cultivar, maximum multiplication of calli (CGI is 1.61) was seen on MS (3 % Sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) medium. While in case of 317/1 and 14/100/1, maximum cell biomass increase of 1.52 and 1.64 CGI, respectively, was obtained on MS (3 % Sucrose) + 2,4-D (3 µM) + TDZ (18 µM) medium. These results confirmed that reduced concentration of carbon source at 3 % was the best for callus multiplication. In general, sucrose proved to be the better carbon source than glucose.

In case of TV 19 and TV 21 cultivars, calli were initially green, fresh, fast growing and friable at the periphery of callus, on the multiplication medium. However, after 8 weeks of incubation in the multiplication medium, the texture of the calli changed and the entire callus turned as hard, compact, nodulated, green callus with bright green areas (**Figure 4 F, H**). Histological sections of the nodulated callus showed the occurrence of meristemoids within the calli (**Figure 5 A**). Various shapes and size of nodules were seen on the surface of calli (**Figure 5 B, C**).

3.1.3. Regeneration from anther callus

Since the origin of callus often affects the frequency of regeneration, the calli from various multiplication media (**Table 3.11**) were utilized for regeneration experiments.

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Table 3.11: Callus growth index (CGI) of cultivars on various multiplication media. Growth Period: 8 weeks

Sl. No.	Callus proliferation media	TV1	TV18	TV19	TV21	317/1	14/100/1
1	MS (3 % Sucrose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	0.19±0.01[c]	0.07±0.02[d]	0.26 ± 0.02[d]	2.35±0.05[a]	0.59±0.01[c]	0.37±0.02[b]
2	MS (3 % Glucose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	-	-	-	1.98±0.06 [b]	0.37±0.02[d]	0.41±0.023[b]
3	MS (3 % Sucrose) + 2,4-D (3 µM) + TDZ (18 µM)	0.95±0.05[b]	0.65±0.03[c]	0.81 ±0.03[c]	1.59±0.10 [c]	1.52±0.05[a]	1.64±0.02[a]
4	MS (3 % Sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM)	1.61±0.05[a]	0.97±0.04[b]	1.85 ±0.05[b]	1.20±0.03[d]	0.62±0.04[b]	0.58±0.03[b]
5	MS (3 % Sucrose) + NAA (5 µM) + BAP (10 µM)	1.03±0.04[b]	1.28±0.05[a]	2.21 ± 0.07[a]	-	-	0.05±0.00[c]

Values are mean ± standard deviation

Among individual cultivar, same letter after the values are not significantly different ($p<0.05$) according to Duncan's multiple range test.

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Table 3.12: Effect of various growth regulators and their combinations on regeneration from calli of TV 19 and TV 21 cultivars. Growth period: 10 weeks.

Sl. No.	Regeneration Treatments	TV19 cultivar		TV21 cultivar	
		Observations	**% cultures showing regeneration**	**Observations**	**% cultures showing regeneration**
1	Control (MS basal medium)	Nil	Nil	Nil	Nil
2	BAP (10 µM)	Bright green, hard and compact calli	Nil	Bright green, hard and compact calli	Nil
3	Kn (10 µM)	Light green, compact calli	Nil	Light green, compact calli	Nil
4	2-iP (10 µM)	Light green, compact calli	Nil	Nil	Nil
5	TDZ (10 µM)	Dark green, granular, friable calli	Nil	Dark green, granular, friable calli	Nil
6	L-Glutamine (800 mg/l) + L-Serine(200 mg/l)	Nil	Nil	Nil	Nil
7	2,4-D(3 µM) + TDZ(18 µM)	**Moderately hard, bright green callus with massive growth showing differentiation of shoot-buds**	**4 (5)***	Bright green, granular, friable calli	Nil
8	2,4-D (5 µM) + Kn (5µM)	Fresh, dark green, granular, friable calli	Nil	Fresh, dark green, granular, friable calli	Nil
9	2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine(200 mg/l)	Fresh, dark green, granular, friable calli	Nil	**Fresh green, friable callus with green patches**	Nil
10	NAA(5 µM) + BAP(10 µM)	**Dark green, nodulated callus with green patches (Figure 4 H)**	Nil	**Dark green, nodulated callus with green patches (Figure 4 F)**	Nil
11	BAP (10 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	Dark green, granular, friable calli	Nil	Dark green, granular, friable calli	Nil
12	BAP (10 µM) + GA_3 (1µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	Dark green, nodulated, friable calli	Nil	Dark green, nodulated, friable calli	Nil
13	BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	**Dark green, nodulated callus with numerous asynchronous embryos**	**75 (18)***	**Dark green, nodulated callus with numerous asynchronous embryos**	**85 (20)***
14	BAP (10 µM) + GA_3 (6 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l)	Bright green, granular, hard calli	Nil	Bright green, granular, hard calli	Nil
15	BAP (10 µM) + GA_3 (1µM) + CH (250 mg/l)	Light green, compact calli	Nil	Nil	Nil
16	BAP (10 µM) + IBA (1 µM)	Nil	Nil	Green callus with brown spots	Nil
17	BAP (2.5 µM) + IAA(5 µM) + CH(500 mg/l)	Light green, dry calli	Nil	Light green, dry calli	Nil
18	Zeatin (10 µM) + Adenine sulphate(20 mg/l) + CH (10 mg/l)	Green callus with brown spot	Nil	Green callus with brown spot	Nil

*Values in parentheses represent average number of embryos/shoot buds developed per explants.

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Numerous growth regulator combinations were tested (**Table 3.12**) to obtain regeneration from calli. MS basal medium was kept as control. Except TV19 and TV21 cultivars, other cultivars did not show any sign of regeneration on any of the tested media. Both, TV19 and TV21 cultivars did not show regeneration responses on MS basal medium or basal medium supported with either auxin or cytokinin. However, a combination of one auxin and one cytokinin favored shoot-bud differentiation in small percentage of cultures of TV 19. On MS + 2,4-D (3 μM) + TDZ (18 μM) medium, bright green, hard, massively growing calli of TV19, showed differentiation of tiny shoot-buds (5 shoot buds/culture) only in 4 % of the cultures, after 4 weeks of culture initiation. Histology of the regenerating calli showed the presence of distinct shoot-tip with two leaf-primordia (**Figures 7 A**). Apparently, these shoot-buds did not grow further.

Regeneration via embryogenesis was achieved only when the nodulated calli from callus multiplication media, MS + NAA (5 μM) + BAP (10 μM) and MS + 2,4-D (5 μM) + Kn (5 μM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l), respectively, of the cultivars TV 19 and TV 21, were transferred to the regeneration medium consisted of MS + BAP (10 μM) + GA_3 (3 μM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). Asynchronous embryogenesis was observed on regeneration medium after two subcultures, each of 6 weeks duration. An average of 18 embryos per culture were developed, in a single passage, in 75 % of the cultures of TV19 (**Figures 6 A, B**), while in TV21 cultivar, 85 % of the cultures showed embryogenesis with an average of 20 embryos per culture (**Figure 6 C, D**). These embryos remained attached to the callus in the form of clusters. All the stages of embryo development, globular, heart, torpedo and dicotyledonous, were observed in single culture. Globular shape, heart-shape and early torpedo shape embryos were fresh, shiny, white or cream in color while late torpedo and dicotyledonous embryos were green and fresh (**Figure 6 A, B, D**).

In histological sections, distinct stages of embryos at various stages of development were also observed, like globular (**Figure 7 B**), heart-shape (**Figure 7 C),** torpedo-shape (**Figure 7 D, E**) and early dicot (**Figure 7 F**) and late dicot stages (**Figure 7 G**). These embryos, on maturity, developed complete vascular strand properly connected from radicular to the plumular end, as revealed from the histological sections (**Figure 7 H, I**). SEM images also confirmed first the occurrence of the nodulated calli in the

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multiplication medium of TV 19 cultivar (**Figure 8 A**) and TV 21 (**Figure 8 B, C**) which later on showed asynchronous embryogenesis in the regeneration medium. Later, these nodules developed into distinct globular and heart-shaped embryos (**Figure 8 D, E**).

3.1.4. Embryo maturation, germination and plantlets development

i. Morphology of anther derived embryos and embryo maturation

The embryos, differentiated on MS + BAP (10 μM) + GA_3 (3 μM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) medium, were morphologically very distinct and showed various developmental patterns:

I. Globular embryos: which were, initially, whitish, transparent, shiny and fresh. During the development process, these embryos turned yellow, bright and fresh (**Figure 9 A**) and follow the normal maturation pattern, showing all sequential stages of embryo development, a heart-shape (**Figure 9 B**), early torpedo stage (**Figure 9 C**), late torpedo stage (**Figure 9 D**) and finally complete dicotyledonous embryo (**Figure 9 E**). These embryos later matured when transferred to 10 times reduced concentration of embryo differentiation medium, MS + BAP (1 μM) + GA_3 (0.3 μM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l) medium by developing bipolar structures with distinct radicular and plumular regions (**Figure 9 F**) and gave rise to complete plantlets.

II. Seed-like embryos (**Figure 9 G**) were whitish yellow structures resembling that of mature zygotic embryos. These structures turn green at maturity. Usually two cotyledons were formed with distinct, well developed, root system (**Figure 9 H**). These embryos germinated normally and gave rise to complete plantlet (**Figure 9 I**).

III. Some of the embryos showed only monopolar germination by giving rise to either root systems at the radicular end (**Figure 10 A**) or shoot systems at the plumular end (**Figure 10 B,C**). There may be single shoot (**Figure 10 D**).at the plumular region or multiple shoots developed (**Figure 10 E**).

IV. Occasionally, abnormal development of embryos were observed in the cultures and these embryos did not show any sign of germination:

A. embryos having abnormally thick plumular region with well defined radical end (**Figure 11 Ai-iii**).

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B. embryos with no cotyledons at the plumular region but with normal developed radicular end (**Figure 11 B**).

C. embryo with completely closed, green, rounded, fused cotyledons at the plumular end and well developed radicular end (**Figure 11 C**).

D. embryo with multiple, green color, fused cotyledons forming cup-shaped structure (**Figure 11 D**).

E. Flower-shape, abnormal embryo with multiple, green color, fused cotyledons (**Figure 11 E**)

V. Very frequently, embryos showed secondary embryogenesis, if, left in the cultures for longer duration of more than 8 weeks (**Figure 11 F**).

ii. Embryo germination and complete plantlets development

On maturation medium, MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), embryos matured by developing complete bipolar structures after 8 weeks (**Figure 12 A and 13 A-F**). Complete plantlets were developed only from those embryos which were present in clusters. Matured bipolar embryos were germinated only when they were transferred to ½MS (major salts reduced to half strength) medium supplemented with BAP (10 µM), GA_3 (0.5 µM), IBA (1 µM), L-Glutamine (80 mg/l) and L-Serine (20 mg/l) (**Figure 12 B, C and 13 G, H**) and gave rise to complete plantlets within 2½ months in TV19 cultivar (**Figure 12 D**) as well as in TV21 cultivar (**Figure 13 I,J**) cultivar. Later, germination medium also served as the shoot multiplication medium.

3.1.5. Ploidy analysis

Ploidy analysis of in vitro raised plantlets was carried out by two methods – **i.** cytological squash preparation of root-tips of haploid plants and shoot-tips of field grown parent plants (control), and **ii.** flow cytometry analysis by using leaves from in vitro raised plantlets as well as parent plantlets.

i. Cytological analysis

Cytological analysis of shoot-tips of field grown parent plants (control) of both the cultivars revealed the diploid number of chromosomes as 2n=2x=30, TV 19 and TV 21

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(**Figures 14 A, 15 A**). While root-tips of in vitro developed plantlets of TV 19 and TV 21 cultivars revealed that majority of cells were in haploid state with the chromosome number as $2n=x=15$ (**Figures 14 B, 15 B**). In case of TV21 cultivar, few plants (25 %) showed the aneuploid number of chromosomes $2n=2x-4=11$ (**Figure 15 C**).

ii. Flow cytometry analysis

The linear fluorescence intensity histograms of relative nuclear DNA content of leaves from field grown parent plant (control) and in vitro derived plantlets showed distinct G0/G1 peaks with coefficients of variation (CV) less than 5.0 % for leaves from field grown plant, less than 4.1 % for leaves from in vitro derived plantlets. **Figures 14 C and 15 D** shows representative histograms from leaves of field grown parent plant (control) of TV 19 and TV 21 cultivars, respectively, where single peak was observed at channel position 200. Leaves from in vitro grown plantlets of TV 19 and TV 21 cultivars showed single peak at channel position 100 (**Figure 14 D, 15 E**). With this it was concluded that in vitro grown cultures have maintained their haploid status.

3.2. ANTIOXIDANT ACTIVITY

This section reveals the antioxidant properties of androgenic lines of the three taxa of Tea- Assam, Cambod and China type. The androgenic haploid embryos and calli, developed from the three taxa are evaluated to (1) investigate the best extraction procedure using various solvents (2) estimate the total phenolic contents, and (3) check antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) methods. Until before no attempts were made on these aspects on in vitro cell lines of the three taxa.

3.2.1. Yield of extracts

The in vitro calli and embryos of the three taxa, developed on various media (*see* **Table 2.5**), were subjected to extraction with hot water and various organic solvents, methanol, ethyl acetate and hexane. The percentage yield of extracts (**Table 3.13**) was calculated by using the formula as described in material and methods. Of the three cultivars, TV19 (Cambod type), TV21 (Assam Type) and 317/1 (China type), the overall highest yield of

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extracts was obtained with the cultivar TV21. The yields of extracts from different solvents were obtained in the order: hot water > methanol > ethyl acetate > hexane. In general, the percent yield of extracts were maximum from young leaves of parent plants (control), followed by embryos and then calli. In TV21, extraction yield varied from 3.2 % to 29.2 % for parent plant leaf extracts and from 2.5 % to 23.9 % for embryos and 2.1 % to 21.8 % for calli.

Table 3.13: Extraction yield with different solvents from androgenic lines and parent plant leaves of TV21, TV19 and 317/1 cultivars.

Cultivars	Solvents	Extraction yield from Leaves (Control) (%)	Extraction yield from embryos (%)	Extraction yield from calli (%)
TV19				
	Hot water	26.8 ± 0.2^{b}	21.1 ± 1.2^{ab}	20.3 ± 0.9^{a}
	Methanol	22.9 ± 0.5^{c}	17.2 ± 1.2^{c}	15.6 ± 0.7^{bc}
	Ethyl acetate	10.6 ± 0.4^{ef}	8.7 ± 0.7^{d}	7.6 ± 0.8^{d}
	Hexane	3.4 ± 0.2^{g}	2.7 ± 0.8^{e}	2.5 ± 0.3^{e}
TV21				
	Hot water	29.2 ± 1.2^{a}	23.9 ± 1.3^{a}	22.8 ± 1.5^{a}
	Methanol	25.1 ± 0.9^{b}	19.8 ± 1.1^{bc}	17.6 ± 0.9^{b}
	Ethyl acetate	11.8 ± 0.3^{e}	9.3 ± 0.6^{d}	7.5 ± 0.6^{d}
	Hexane	3.2 ± 0.2^{g}	2.5 ± 0.2^{e}	2.1 ± 0.3^{e}
317/1				
	Hot water	19.7 ± 1.5^{d}	-	15.4 ± 1.5^{bc}
	Methanol	18.2 ± 0.8^{d}	-	13.5 ± 0.9^{c}
	Ethyl acetate	8.6 ± 0.5^{d}	-	7.8 ± 0.8^{d}
	Hexane	2.8 ± 0.3^{g}	-	1.8 ± 0.3^{e}

Values are mean ± standard deviation
Same letter after the value in each column are not significantly different ($p<0.05$) according to Duncan's multiple range test.

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3.2.2. Total phenolic content of androgenic lines

The total phenolic contents in various sample extracts, hot water, methanol, ethyl acetate and hexane, was determined through a linear standard curve (y = 0.002x+0.074; R^2 = 0.986). The results on total phenolic contents of the three taxa, TV19, TV21 and 317/1 are presented in **Graph 3.2**. In general, TV21 extracts have more phenolic contents followed by TV19 and 317/1. The total phenolic contents were obtained in order of young leaves from parent plants > embryos > calli. Hot water extracts had been observed to be the most suitable solvents in all the taxa for accumulation of phenolics (**Graph 3.2**). Embryo extracts being the best among the androgenic lines and contained 47.20 ± 2.5 mg GAE/g dry weight and 43.12 ± 2.21 mg GAE/g dry weight of phenolics in hot water extracts of TV21 and TV19, respectively.

3.2.3. Antioxidant activity of androgenic cultures

3.2.3.1. DPPH radical scavenging activity of various extracts

The percent scavenging activity of various solvent extracts, obtained from control and androgenic calli and embryos of TV19, TV21 and androgenic calli of 317/1, was calculated from the equation mentioned in Material & Methods Section. These inhibition values (I %) are represented in **Graph 3.3**. After analysis of pattern, it was observed that the slope of DPPH inhibition curve was greater in extracts prepared from leaves of parent plant (control) (**Graph 3.3 A, D**) followed by embryos (**Graph 3.3 B, E**) and then calli (**Graph 3.3 C, F**) of TV19 and TV21 cultivars. The same is true with the cultivar 317/1 where DPPH inhibition curve was greater in extracts prepared from leaves of parent plant (control) (**Graph 3.3 G**) than the androgenic calli (**Graph 3.3 H**). Solvent wise DPPH inhibition pattern in the three cultivar was seen in the order of hot-water > methanol > ethyl acetate > hexane for TV19 (**Graph 3.3 A, B, C**), TV21 (**Graph 3.3 D, E, F**) and 317/1 (**Graph 3.3 G, H**).

IC_{50} values of reference standards and each extracts were calculated from the linear equations of the DPPH inhibition curves and are represented in **Table 3.14** and **Table 3.15**, respectively. Lower the IC_{50} values, higher is the antioxidant activities of the extracts. Of the three cultivars, TV21 showed higher radical scavenging activities than TV19 while 317/1 showed the least. The IC_{50} values were in the order of young leaves

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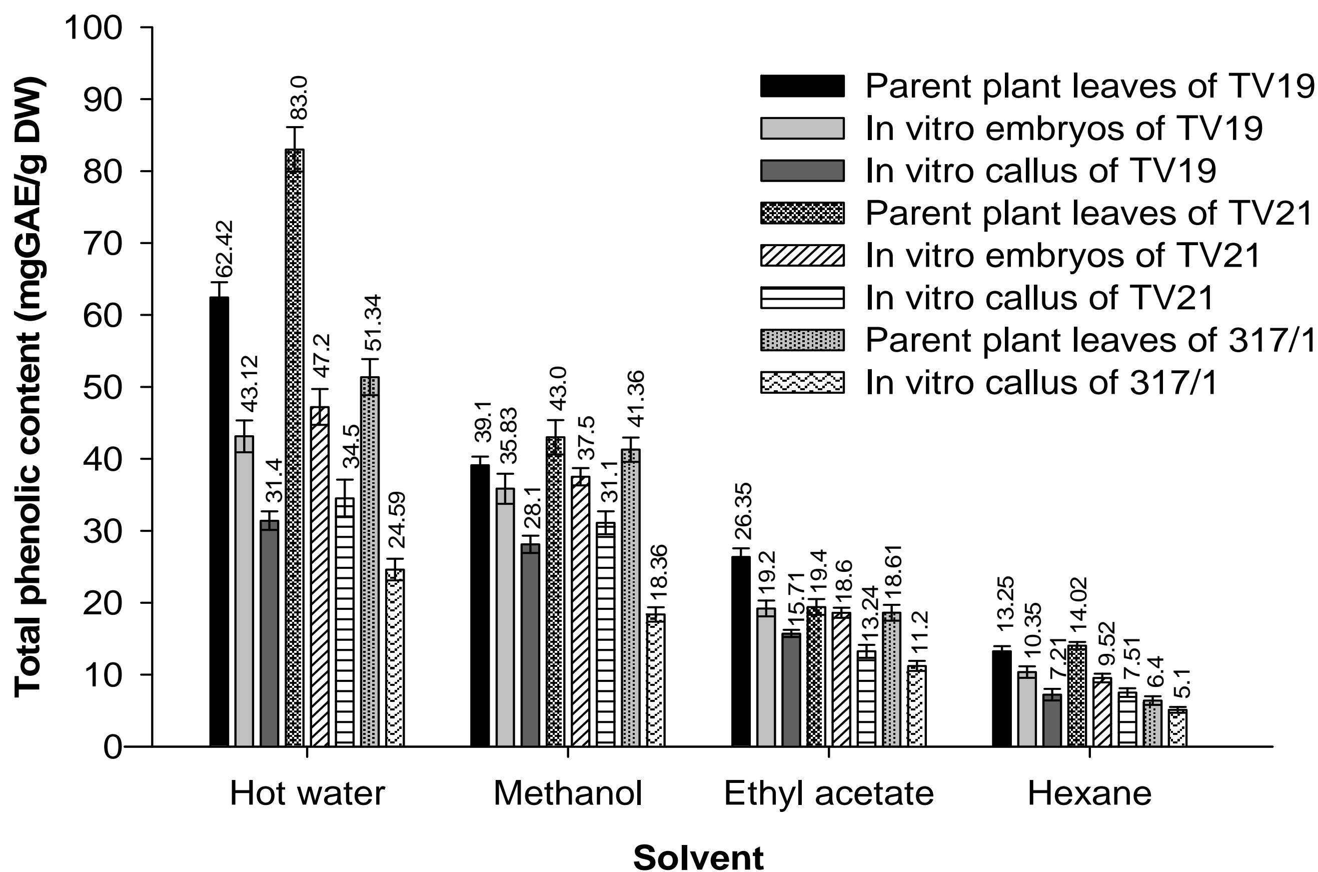

Graph 3.2: Total phenolic content in androgenic lines of tea. Control: leaves from field grown parent plants of TV19, TV21 and 317/1 cultivars.

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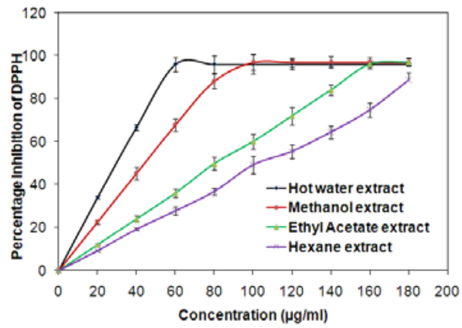

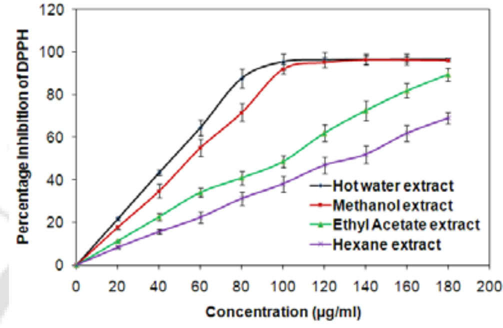

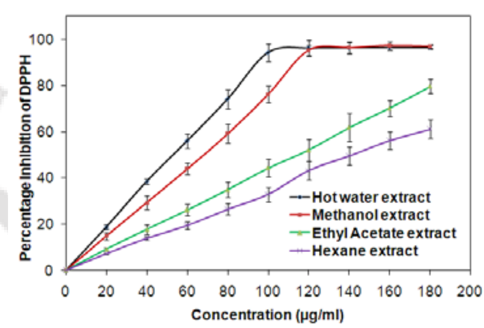

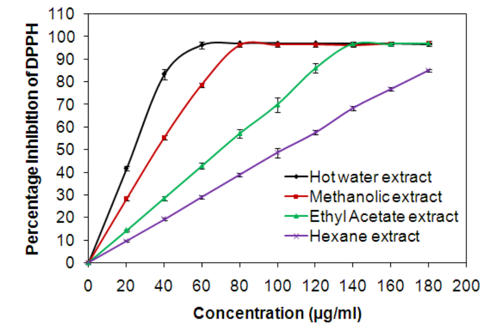

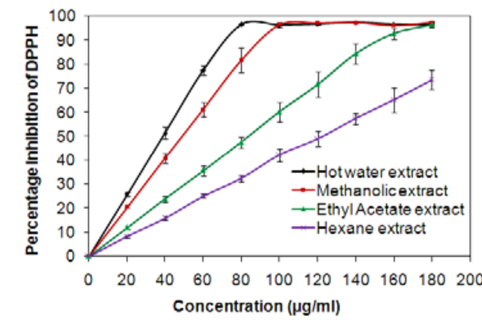

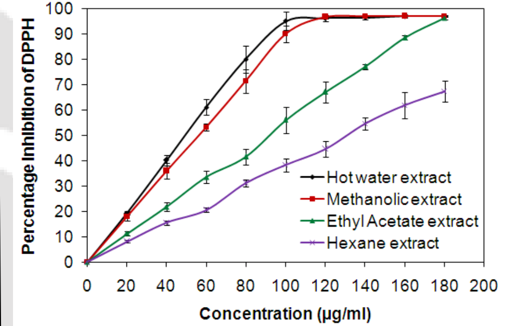

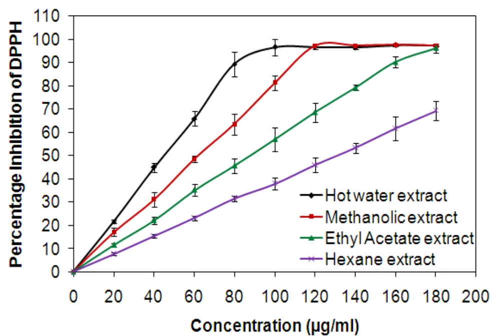

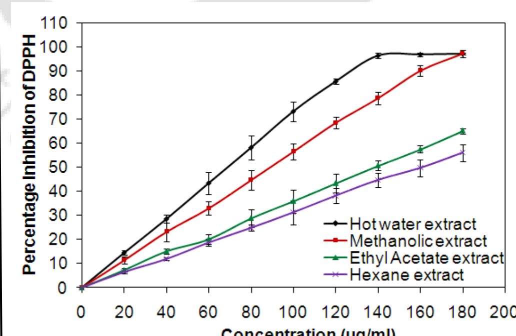

Graph 3.3: DPPH inhibition pattern in various extracts of tea
A: Extract from young leaves of parent plant of TV19
B: Extracts from androgenic embryos of TV19
C: Extracts from androgenic calli of TV19
D: Extract from young leaves of parent plant of TV21
E: Extracts from androgenic embryos of TV21
F: Extracts from androgenic calli of TV21
G: Extract from young leaves of parent plant of 317/1
H: Extracts from androgenic calli of 317/1

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from parent plant < embryos < calli. Hot water extracts has significantly very high antioxidant activity followed by methanol, ethyl acetate and hexane (**Table 3.15**). The hot water extracts of TV21 leaves (control), embryos and calli exhibited DPPH inhibition with IC_{50} values as 24.41±1.12 µg/ml, 38.61±1.92 µg/ml and 47.0±2.82 µg/ml, respectively.

In order to understand the free radical scavenging capacities of sample extracts, their activities were compared with the relative activities of standard antioxidant compounds. To perform comparative studies, ascorbic acid, catechin, epicatechin, epigallocatechin gallate, gallic acid and vanillic acid were used as reference standards and their IC_{50} values are detailed in **Table 3.14**. Of these, epigallocatechin gallate showed excellent radical scavenging activity with IC_{50} value of 2.14±0.24 µg/ml which is 11.41-fold, 18.04-fold and 21.97-fold higher than the antioxidant activity of leaves (control), embryos and calli hot water extracts of TV21, respectively. While, vanillic acid showed very low radical scavenging activity with IC_{50} values as 4060 µg/ml which is 166.32-fold, 105.15-fold and 86.38-fold lower than the leaf (control), embryos and calli hot water extracts of TV21. Ascorbic acid equivalent antioxidant activity (AEAC) for hot water extracts of all the samples were given in **Graph 3.4** and pattern of these values are similar to that of IC_{50} values of each sample.

Table 3.14: Free radical scavenging activity (IC_{50} value) of reference standards

Sl. No.	Samples	IC_{50} (µg/ml)
1.	Ascorbic acid	4.20±0.41
2.	Catechin	3.24±0.53
3.	Epicatechin	4.13±0.39
4.	Epigallocatechin gallate	2.14±0.24
5.	Gallic acid	4.78 ±0.38
6.	Vanillic acid	4060.00 ±60

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Table 3.15: Free radical scavenging activity (IC_{50} value) of various solvents extracts of androgenic lines and parent plant leaves of TV19, TV21, and 317/1 cultivar

Cultivars	Solvents	Parent plant leaves (control) (Concentration in µg/ml)	Haploid embryos (Concentration in µg/ml)	Haploid calli (Concentration in µg/ml)
TV19				
	Hot water	29.98±1.34^{b}	46.21±2.21^{d}	53.37±2.15^{ef}
	Methanol	44.21±1.82^{d}	56.18±2.42^{f}	68.25±3.12^{h}
	Ethyl acetate	83.21±3.33^{i}	98.13±3.64^{l}	110.72±4.12^{n}
	Hexane	108.31±5.41^{mn}	120.01±3.41^{o}	140.41±5.41^{q}
TV21				
	Hot water	24.41±1.12^{a}	38.61±1.92^{c}	47.0±2.82^{d}
	Methanol	36.16±2.15^{c}	48.82±2.11^{de}	56.45±3.31^{f}
	Ethyl acetate	70.21±3.94^{h}	84.16±3.12^{ij}	91.25±4.25^{k}
	Hexane	104.81±4.21^{m}	121.10±4.6^{o}	131.45±4.77^{p}
317/1				
	Hot water	45.51±2.22^{d}	-	69.18±2.41^{h}
	Methanol	62.15±1.5^{g}	-	89.91±2.72^{jk}
	Ethyl acetate	87.69±3.54^{ijk}	-	140.60±4.64^{q}
	Hexane	131.06±4.45^{p}	-	160.25±4.0^{r}

Values are mean ± standard deviation
Same letter after the value in each column are not significantly different ($p<0.05$) according to Duncan's multiple range test.

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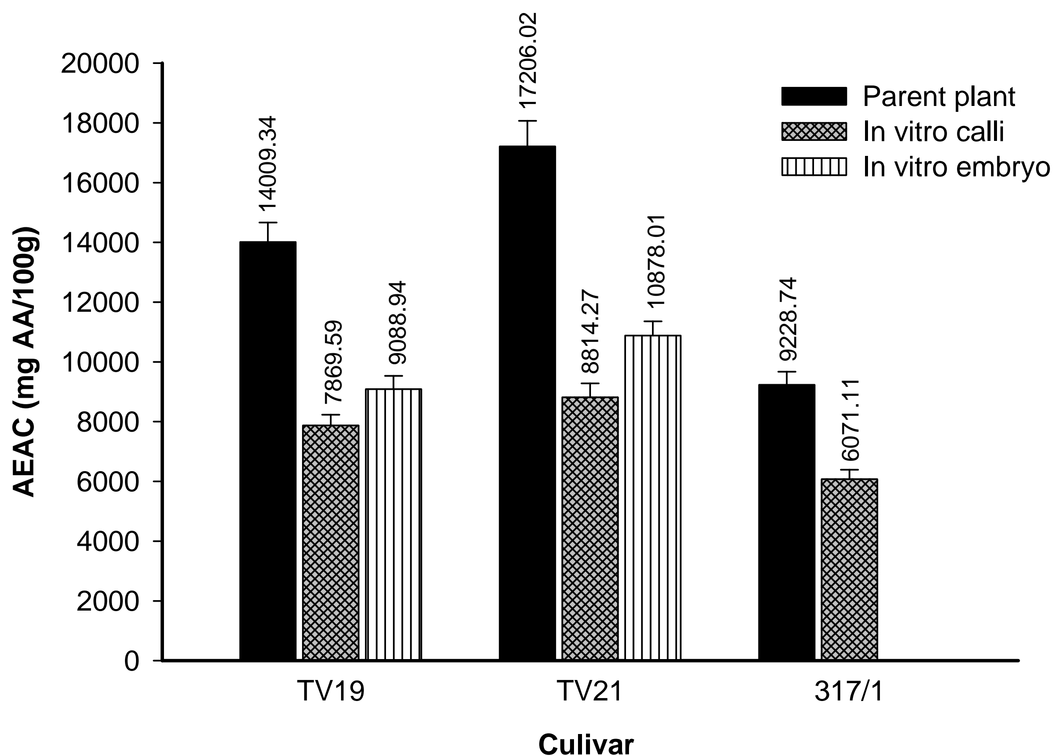

Graph 3.4: Ascorbic acid equivalent antioxidant activity (AEAC) values of hot-water extracts of androgenic cultures and parent plant leaves of TV19, TV21 and 317/1 cultivars.

3.2.3.2. Ferric-reducing antioxidant power (FRAP) of cultures

In the previous section, as the hot water extracts yielded overall maximum antioxidant activity, therefore, ferric-reducing antioxidant power (FRAP) analysis was done with hot water extracts only. The FRAP measures the capability of compounds to act as an electron donor while DPPH measures their capability to act as hydrogen donor. In FRAP assays, all hot water extracts of TV21 showed higher antioxidant activity than TV 19 while the least activity was observed in 317/1 cultivar (**Graph 3.5**). The obtained FRAP of TV 21 were 51.61 mg GAE/g for parent plant leaf (control), 35.79 mg GAE/g for embryos and 26.31 mg GAE/g for androgenic calli. The FRAP values in TV19 were 43.96 mg GAE/g for parent plant leaf (control), 26.19 mg GAE/g for embryos and 24.86 mg GAE/g for androgenic calli. The FRAP values in 317/1 were 27.89 mg GAE/g for parent plant leaf (control) and 19.21 mg GAE/g for androgenic calli.

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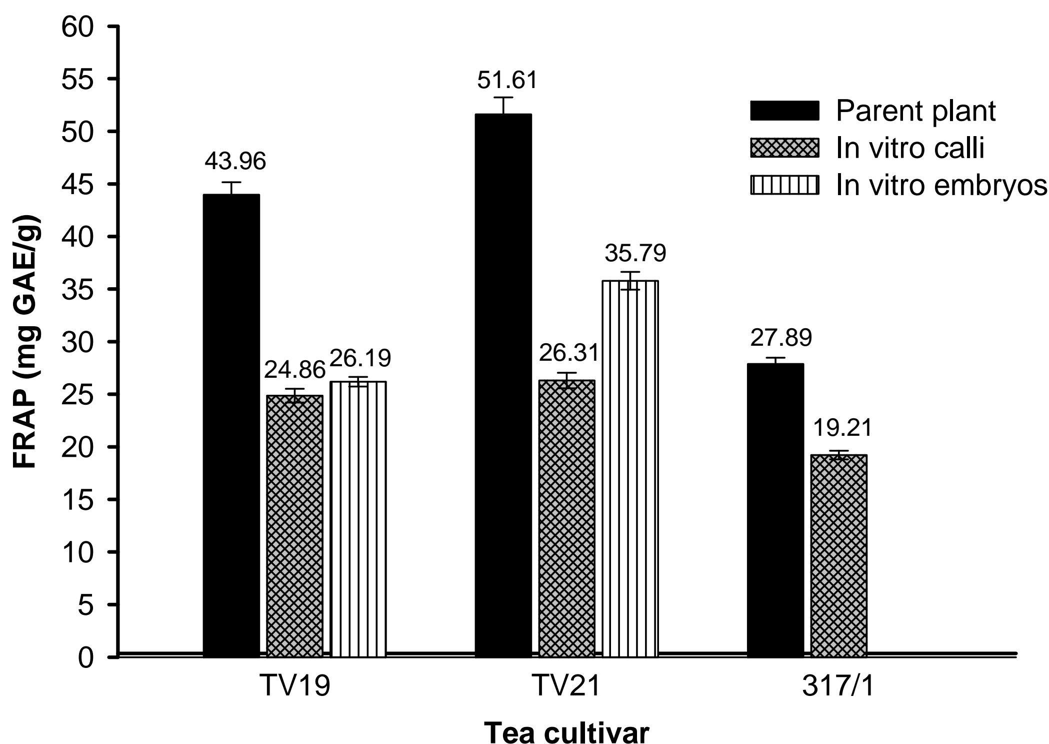

Graph 3.5: Ferric-reducing antioxidant (FRAP) power assay of hot water extracts of androgenic cultures and leaves from field grown plants (control) of TV19, TV21 and 317/1 cultivar.

3.3. SCREENING OF ANDROGENIC CULTURES FOR SECONDARY METABOLITE PRODUCTION

As maximum extraction yield and antioxidant activity were obtained with hot-water extracts, therefore, it was further utilized for the screening of secondary metabolites. Hot water extracts from in vitro callus cultures of the cultivars TV19, TV21 and 317/1 and embryos of the cultivars TV19 and TV21 were tested for the accumulation of metabolites. Leaves from the field grown parent plants of the three cultivars served as controls.

3.3.1. Detection and estimation of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline

Screening of (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), caffeine (CAF) and theophylline (T) wasdone by high performance liquid

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chromatography (HPLC) by following the protocol as described in the section 2.2.3 of materials & methods.

3.3.1.1. Standard curve analysis of all concerned standard compounds

With the protocol adopted, authentic standards, C, EC, EGCG, CAF and T, were eluted at 12.29±0.31, 13.9±0.32, 20.9±0.36, 8.3±0.3 and 6.19±0.35 min respectively (**Figure 16 A- E**). Calibration curve for C, EC, EGCG, CAF and T standards showed good linearity with high reproducibility and accuracy at all tested concentrations (0.025 mg/ml to 1 mg/ml). As shown in **Table 3.16**, regression analysis of the calibration curve data points showed excellent correlation coefficient (R^2).

(a) Method optimization for separation of compounds

The detection wavelength of 280 nm was selected by checking the absorption maxima of the C, EC, EGCG and CAF standards, dissolved in water, by a UV-Visible spectrophotometer (Cary, USA). For T standard compound, 273 nm was found as absorption maxima. In a preliminary study, methanol was used as one of the mobile phases in HPLC. As the absorption of methanol was higher, high interference was observed especially at 280 nm. Moreover, as catechins (C & EC) are structural isomers, their separation needed a low absorbing solvent that doesn't interfere with the resolution process. Hence, for separation of all catechins, acidic water with acetonitrile and ethyl acetate were chosen for the purpose. Acetonitrile:ethyl acetate:0.05 % H_3PO_4 (12 : 2 : 86 v/v) was found to be appropriate as the mobile phase for satisfactory separation of the these four components, at a flow rate of 0.5 ml/min. The chromatograms of C, EC, EGCG and CAF standards obtained by this method, are presented in **Figure 16 A-D**. A fairly short acquisition time of less than 25 min was adequate for good separation of all the four compounds. For theophylline separation, acetonitrile:water (10:90) (v/v) was used as the mobile phase at a flow rate of 1 ml/min which did not interfere with the resolution process. The chromatogram of T obtained by this method is represented in **Figure 16 E** which shows that less than 20 min was adequate for its separation.

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(b) Linearity and precision

Calibration curves for all the five standards showed good linearity at tested concentrations (0.025 mg/ml to 1 mg/ml) with correlation coefficients (R^2) of 0.997, 0.996, 0.991, 0.999 and 0.973 for C, EC, EGCG, CAF and T, respectively. The equation generated from the curve (**Table 3.16**) by external standard method were used to calculate the amount of compounds present in the crude sample. Presence of compounds in the crude extracts were reconfirmed with the use of internal standards by co-injecting all concerned compounds together with the extract in HPLC (**Figure 17 G, H; 18 G, H; and 19 E, F**). A very distinct and clear separation of five compounds can be observed. The precision of the developed method, as already mentioned in chromatographic conditions of materials and methods section, was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard sample, at same concentration, was analyzed at least three times within the same day and the RSD values obtained were 1.3 %, 0.9 %, 0.5 %, 1.2 % and 0.3 % for C, EC, EGCG, CAF and T respectively. Similarly, for inter-day variability, same concentration of the five standards was run at least twice at one day interval and the RSD values came out to be 1.1 %, 1.2 %, 0.8 %, 1.4 %, and 0.2 % for C, EC, EGCG, CAF and T, respectively (**Table 3.17**).

(c) Recovery studies

From the standard equations, the amounts of C, EC, EGCG, CAF and T in the cell extracts were calculated. The recovery experiments for all the five compounds were performed by adding known amount of C, EC, EGCG, CAF and T standards to the cells, which were extracted in a similar manner as described in materials and methods section. The percentage recoveries for all five compounds were observed to be above 91.6 % in all cases (**Table 3.17**).

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Table 3.16: Standard curves and retention times of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline

Parameters	(+)-Catechin	(-)-Epicatechin	(-)-Epigallocatechin gallate	Caffeine	Theophylline
Retention time (min)	12.29±0.31	13.90±0.32	20.90±0.36	8.3±0.30	6.19±0.35
Standard equation	y=0.347x+7.994	y=3.444x+6.085	y=561.7x-14.31	y=0.482x+4.085	y=0.902x
R^2	0.997	0.996	0.991	0.999	0.973

Table 3.17: Precision and recovery percentages of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline.

Sl. No.	Compound	RSD (%)		Recovery (%)		
		Intra-day	Inter-day	TV19	TV21	317/1
1	Caffeine	1.2	1.4	93.2±1.3	93.0±1.9	92.9±1.1
2	(+)-Catechin	1.3	1.1	91.6±1.1	92.3±2.1	91.6±1.3
3	(-)-Epicatechin	0.9	1.2	95.2±1.2	94.5±1.8	93.4±1.5
4	(-)-Epigallocatechin gallate	0.50	0.8	96.1±1.1	95.3±1.6	94.9±1.4
5	Theophylline	0.3	0.2	96.3±.9	97.2±.8	95.3±1.2

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3.3.1.2. Identification and quantification of compounds by HPLC

From the standard equations obtained, the amounts of C, EC, EGCG, CAF and T in various callus and embryo samples were calculated and listed in **Table 3.18**. The fragmentation pattern was similar in both standard and sample compounds, where almost all the fragments present in the standard were present in the sample as well (**Figure 17 to 19**). Presence of all the compounds was confirmed in all the tested in vitro lines. It was observed that embryogenic cultures obtained on MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) medium, possessed highest content of all the compounds compared to that of dedifferentiated cultures (callus) in both TV19 and TV21 cultivars. In general, hot water extracts from leaves field grown parent plant (control) contained highest amount of metabolites compared to in vitro androgenic cultures of 317/1, TV19 and TV21 cultivars **Table 3.18**. While concentration of (+)-catechin was significantly high in TV21 followed by 317/1 and TV19 cultivars; (+)-catechin content in embryos of TV19 and callus of 317/1 fall at the same significant level ($p<0.05$). The concentration of (-)-epicatechin was significantly high in TV21 followed by TV19 and least in 317/1 cultivar. The amount of (-)-epigallocatechin gallate content was also significantly high in TV21 followed by TV19 and 317/1 cultivars, though in vitro calli of 317/1 contained comparatively higher EGCG than TV19 calli. Caffeine content was higher in TV19 cultivar followed by TV21 and 317/1. Theophylline content was highest in in vitro callus cultures of 317/1 followed by TV19 and TV21, while embryogenic cultures and leaves from parent plants of TV21 consisted of highest amount of theophylline **Table 3.18**.

3.3.1.3. Analysis of metabolites by mass spectra

The fraction of crude extract, eluted from HPLC, was collected at respective retention time, analysed by mass spectrometry and the fragment characteristics were compared with that of HPLC eluted standards of respective compounds, procured from Sigma, Aldrich. In the present study, positive mode electrospray ionization (+ESI) conditions were applied for all the five compounds. Spectra were obtained in full scan mode. For caffeine base peak of m/z 195 was obtained due to the addition of hydrogen ion in positive mode

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Table 3.18: (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophylline content in in vitro and in vivo sample of TV21, TV19 and 317/1.

Cultivar	Sample	(+)-Catechin concentration (mg/g DW)	(-)-Epicatechin concentration (mg/g DW)	(-)-Epigallocatechin gallate concentration (mg/g DW)	Caffeine (mg/g DW)	Theophylline (mg/g DW)
TV19						
	In vitro callus	1.6±0.1[f]	5.3±0.2[c]	65.1±0.9[f]	21.1±0.4[f]	1.1±0.1[de]
	In vitro embryos	2.1±0.2[ef]	8.4±0.4[b]	83.8±1.2[d]	28.1±0.5[bc]	1.6±0.1[cd]
	In vivo leaf	4.8±0.2[d]	12.5±0.3[a]	112.8±1.0[b]	31.4±0.8[a]	2.3±0.2[ab]
TV21						
	In vitro callus	2.6±0.1[e]	5.4±0.2[c]	75.1±1.4[e]	19.2±0.4[f]	0.9±0.1[e]
	In vitro embryos	7.5±0.2[b]	8.5±0.3[b]	94.5±1.5[c]	26.4±0.6c[d]	2.2±.0.3[abc]
	In vivo leaf	11.6±0.2[a]	12.8±0.4[a]	148.3±2.6[a]	29.6±1.2[ab]	2.8±0.2[a]
317/1						
	In vitro callus	2.1±0.1[ef]	5.1±0.2[c]	68.4±2.1[f]	23.6±0.5[e]	1.2±0.1[de]
	In vivo leaf	6.4±0.1[c]	9.42±0.3[b]	81.6±2.9[d]	24.8±0.6[e]	1.9±0.2[bc]

Values are mean ± standard deviation
Same letter after the value in each column are not significantly different ($p<0.05$) according to Duncan's multiple range test.

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electrospray ionization [MH^+], m/z 217 formed due to formation of sodium adduct [MH^++Na^+] in HPLC eluted crude sample as well as in standard (**Figure 20 A,B**). For (+)-catechin m/z fragments in standard compound and HPLC eluted crude fraction are shown in **Figure 20 C, D**, where the base peak of m/z 291 was obtained due to the addition of hydrogen ion in positive mode electrospray ionization [MH^+] and fragments peaks are m/z 139 and m/z 183 due to fragmentation of compound. Similarly in (-)-epicatechin, base peak of m/z 291 was seen due to due to the addition of hydrogen ion [MH^+] and it's fragment peak was seen as m/z 139 **Figure 21 A, B.** Mass spectra of (-)-epigallocatechin gallate HPLC eluted standard and crude samples are shown in **Figure 21 C, D,** base peak is m/z 459 of [MH^+] and two fragment characteristic peaks are m/z 139 and m/z 289, thus, confirming presence of EGCG; m/z 497 formed due to formation of potassium adduct [MH^++K^+] in HPLC eluted crude samples as well as in standards (**Figure 21 C, D**). Mass spectra of HPLC eluted standard and crude samples of theophylline are presented in **Figure 22 A, B** where base peak is m/z 181 due to addition of hydrogen ion in positive mode electrospray ionization [MH^+] and m/z 200 is due to adduct with water [MH^++H_2O].

3.4. KINETICS OF CELL SUSPENSION CULTURE

3.4.1. Establishment of cell suspension culture

The cell suspension culture is a simple system to study growth and production kinetics that can help to evaluate and implement optimal conditions for the production of a number of high value medicinal compounds in good quantities. Previous sections revealed that androgenic calli obtained from TV21 cultivar showed comparatively faster rate of callus proliferation with high yield of extracts. These calli possess high phenolic content with maximum antioxidant activity. As overall metabolite production was also higher in these calli, therefore, cell suspension cultures were established to perform growth kinetic studies. Androgenic friable, fresh and fast growing calli of TV21 grown on MS + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) were utilized to raise cell suspension cultures. Several media combinations were tested to obtain fine suspension of cells with high biomass accumulation.

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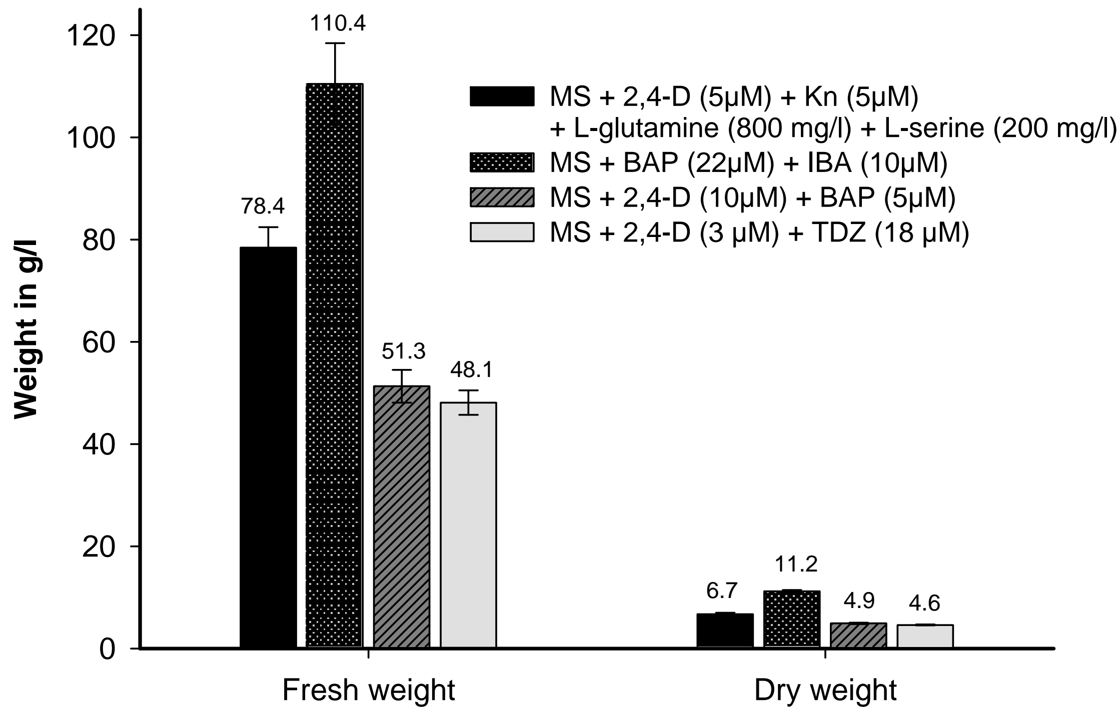
Of the several media combinations tried, cell suspension cultures were established on four media, MS + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l), MS + BAP (22 µM) + IBA (10 µM), MS + 2,4-D (10 µM) + BAP (5 µM) and MS + 2,4-D (3 µM) + TDZ (18 µM) medium, but maximum biomass accumulation was obtained with the medium, MS + BAP (22 µM) + IBA (10 µM), on 27th day (**Graph 3.6**).



Graph 3.6: Effect of media combinations on biomass accumulation in cell suspension cultures of TV21 cultivar

3.4.1.1. Effect of sucrose concentration

As MS + 2,4-D (10 µM) + BAP (5 µM) medium supported maximum cell biomass growth, it was further tested at various sucrose concentrations ranging from 1 % to 7 % to know the effect of sucrose on rate of cell proliferation. Results are presented in **Graph 3.7**. On 27th day, sucrose at 3 % concentration supported maximum cell biomass growth (110.4 ± 8.0 g/l FW and 11.2 ± 0.24 g/l DW) followed by 5 % sucrose (91.7±6.7 g/l FW and 9.7±0.2 g/l DW), 7 % sucrose (76.3±5.1 g/l FW and 6.5±0.18 g/l DW) and least cell biomass in 1 % sucrose concentration.

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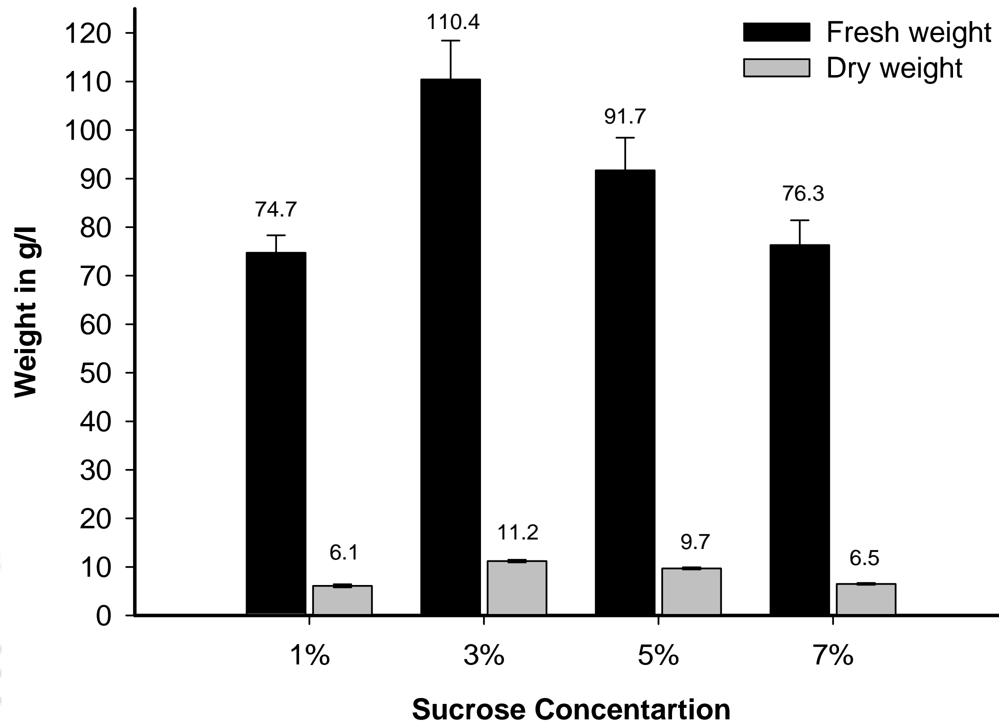

Graph 3.7: Effect of sucrose concentration on cell biomass accumulation in suspension cultures of TV21 cultivar.

3.4.1.2. Effect of pH

As the medium pH plays a critical role on the cell biomass growth, therefore, to assess its effect, cells were inoculated into the best medium at pH range of 5.4 to 6.2. Results are shown in **Graph 3.8**. The pH of 5.8 was observed to be the best for maximum cell biomass growth (110.4±8.0 g/l FW and 11.2±0.24 g/l DW), at 27th day.

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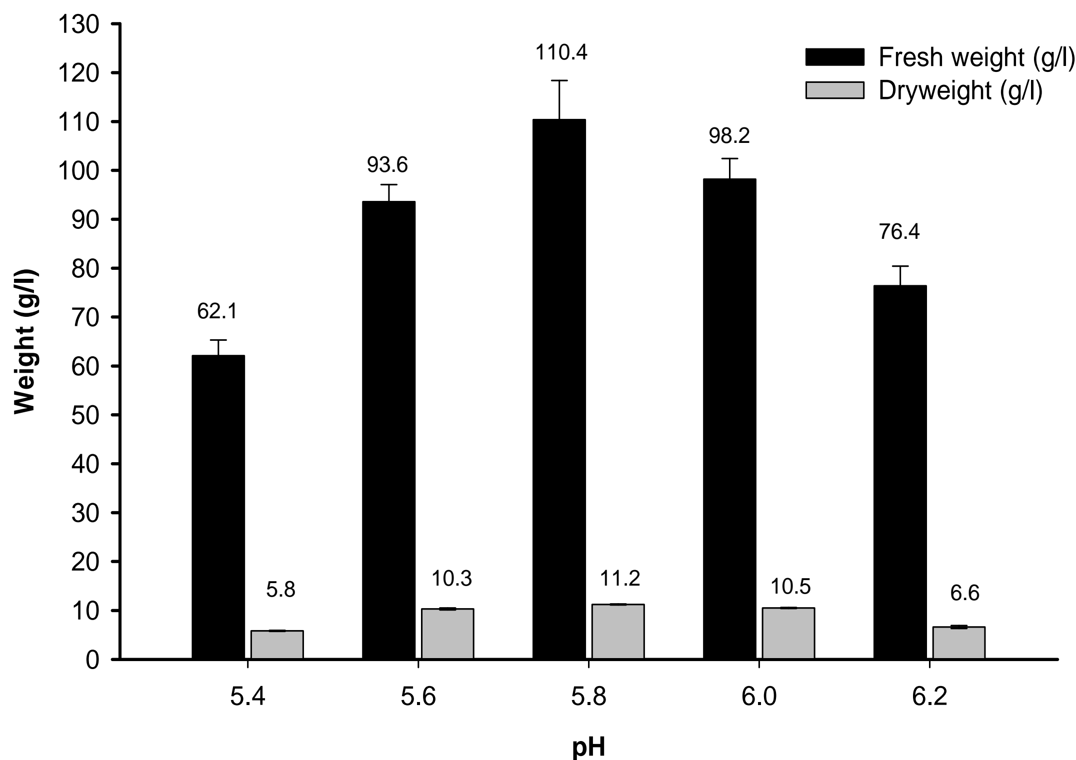

Graph 3.8: Effect of pH on cell biomass growth in cell suspension cultures of TV21 cultivar.

3.4.1.3. Effect of agitation speed on cell biomass increase and cell viability

Speed of agitation directly affected the growth and viability of cells in suspension cultures due to aeration and shearing effect. To observe this, cells were grown at various agitation speeds of 90, 120 and 150 rpm. Speed of agitation on growth and viability of cells in shake flask suspension cultures were assessed at 27th day. The maximum fresh weight (110.4±8.0 g/l) **(Graph 3.9)** was observed at 120 rpm.

The viability profile of tea cells at various agitation speeds is shown in **Figure 23 A-G**. Similar to other plant species, tea cells were also found to be highly sensitivity to increase in agitation speed. The maximum cell viability (**Figure 23 C-F)** was observed at 120 rpm. At the other two rpm, the biomass and viability profile was highly unsatisfactory. At higher speed (150 rpm), the cells died due to rupturing and shear effect (**Figure 23 G**). At lower agitation (90 rpm), the cells died due to aggregation and clumping (**Figure 23 A, B**); only the cells at the outermost layer of the aggregate were alive and fluorescent green

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when stained with fluorescein diacetate (FDA). FDA is a cell permeant dye. Within the living cells, the molecule is cleaved by esterase activity to fluorescein which is unable to pass through the cell membrane of live cells while it leaches out from the dead cells. Hence, only the live, intact cells take up the stain and fluoresce green.

3.4.2. Kinetics of cell growth and nutrient uptake

The kinetic profile of cell growth calculated by fresh and dry cell weight is shown in **Graph 3.10**. With the formula mentioned in **section 2.2.4.3,** the specific growth rate (μ) of the suspended cells was found to be 0.1267 day^{-1}. It was observed that the cultures remained in the lag phase till the 3rd day. Biomass increased till the 24th day following which the stationary phase started (**Graph 3.10**). Typical profiles of the consumption of substrates (phosphate, nitrate and sucrose) during cell suspension culture are shown in **Graph 3.11.** The pH of the medium underwent variation during different stages of culture. It was observed that after showing a slight decrease in the value, the pH dropped sharply between 9-24 days and then slightly increased after 27 days (**Graph 3.11**). The drop of pH may be primarily due to the uptake of NH_4^+ ions which in turn resulted in decreased pH because of liberation of H^+ ions; pH tends to increase if NO_3^- is utilized faster than NH_4^+. The increase in the pH after 27th day may be attributed to the release of intracellular substances into the medium. Uptake of phosphate was slightly slower than nitrate. Nitrate and phosphate were not completely utilized up to 27th day. After 27th day nitrate and phosphate concentrations were slightly increased and remain constant until 30th day. Increased concentration of nitrate and phosphate after 27th day might be due to death and lysis of cells under stress conditions. This stress may be due to the complete utilization of sucrose at 27th day. Sucrose utilization occurred at a relatively faster rate and it was consumed completely from the medium by the cells at 27th day of culture. Therefore, it may be speculated that complete utilization of sucrose from the medium resulted in the onset of decline phase of cell growth in suspension culture and this was the major limiting nutrient for cell growth.

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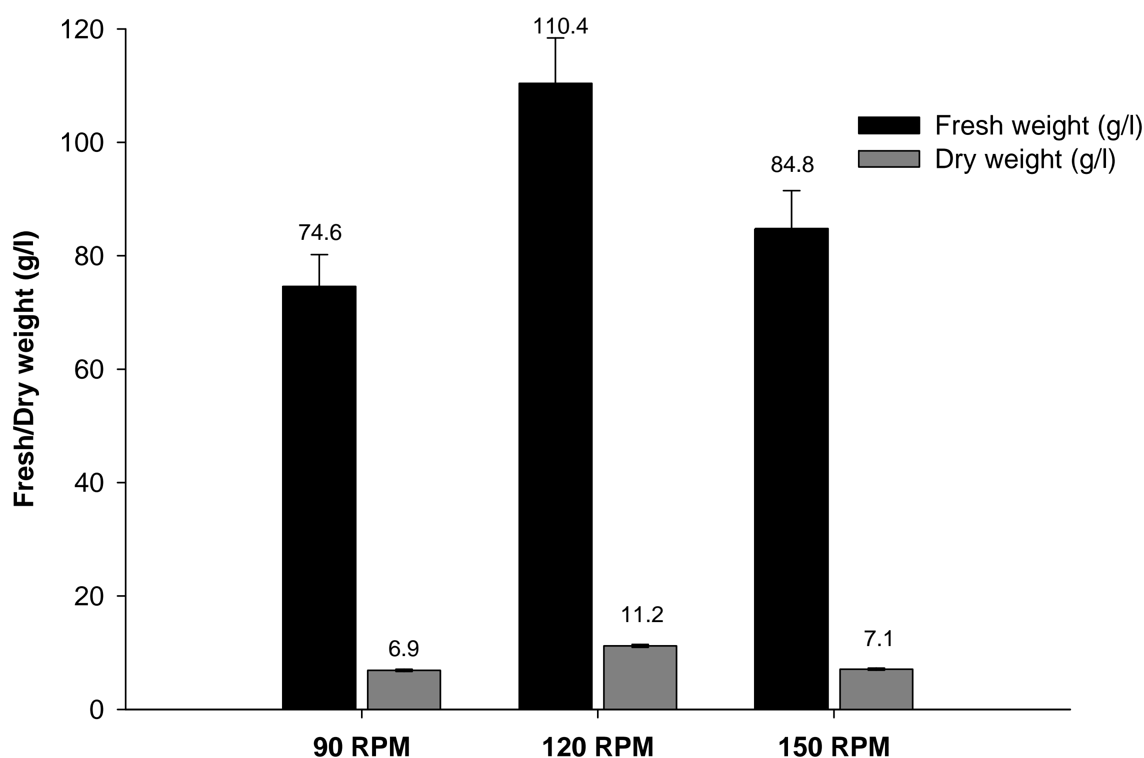
3.4.3. Chromatographic detection of secondary metabolites

The concomitant synthesis of all the concerned secondary metabolites in the cell suspension was found to be growth associated and showed an increase with the increase in cell biomass (**Graph 3.12**). In an extensive study, in this section, the cells harvested at every third day from liquid suspension cultures were extracted and analysed by HPLC. Till 3rd day there was no production of metabolites and 3rd day onwards accumulation of compounds was observed. It was revealed that the synthesis of these metabolites is associated with the log phase of cultures and continues until the stationary phase upto 27th day and afterwards it declined. In general, in cell suspension cultures, the production of metabolites was higher than the callus cultures, however, it is lesser than that produced in embryo cultures.



Graph 3.9: Effect of rpm on cell biomass accumulation in suspension cultures of TV21 cultivar.

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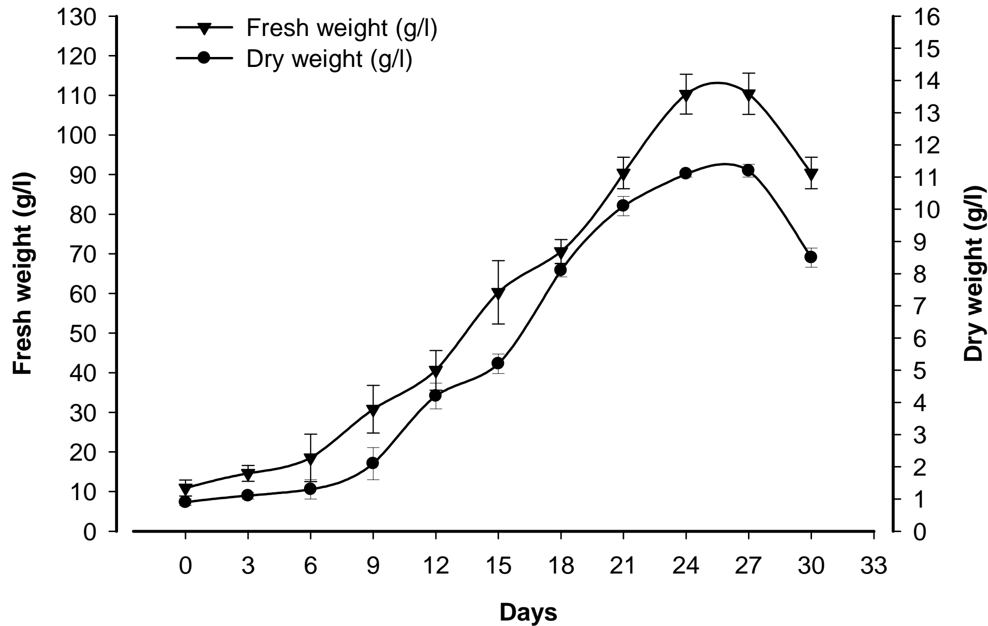
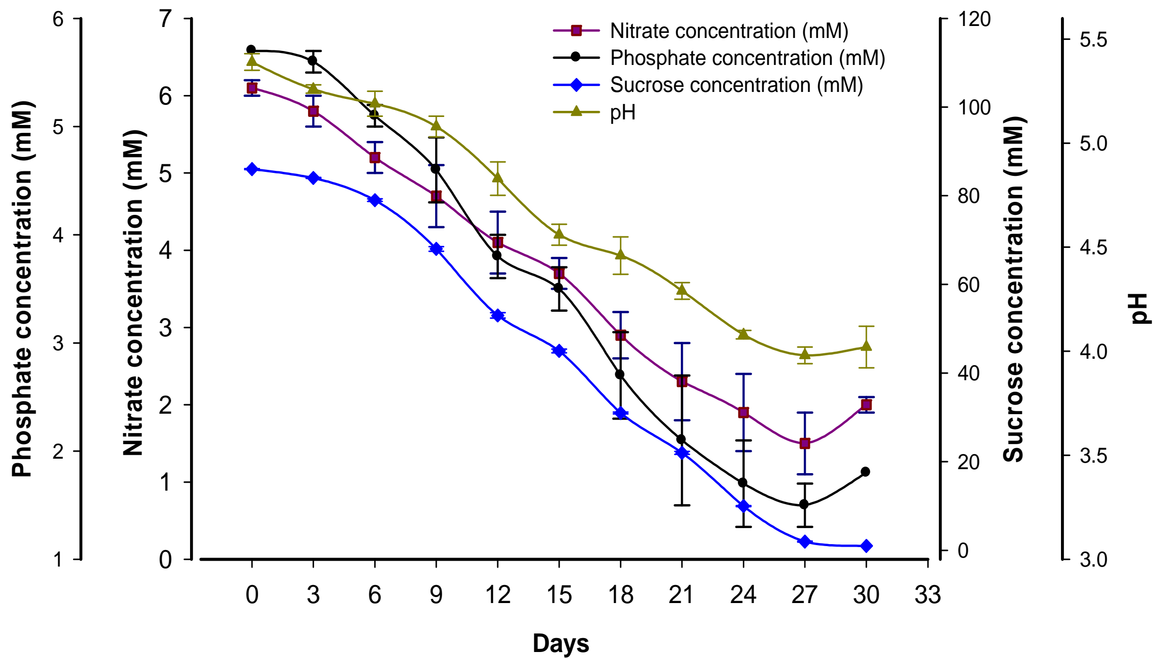


Graph 3.10: Kinetics of cell growth in cell suspension cultures of TV21 cultivar.



Graph 3.11: Kinetics of nutrient uptake and pH in cell suspension cultures of TV21 cultivar.

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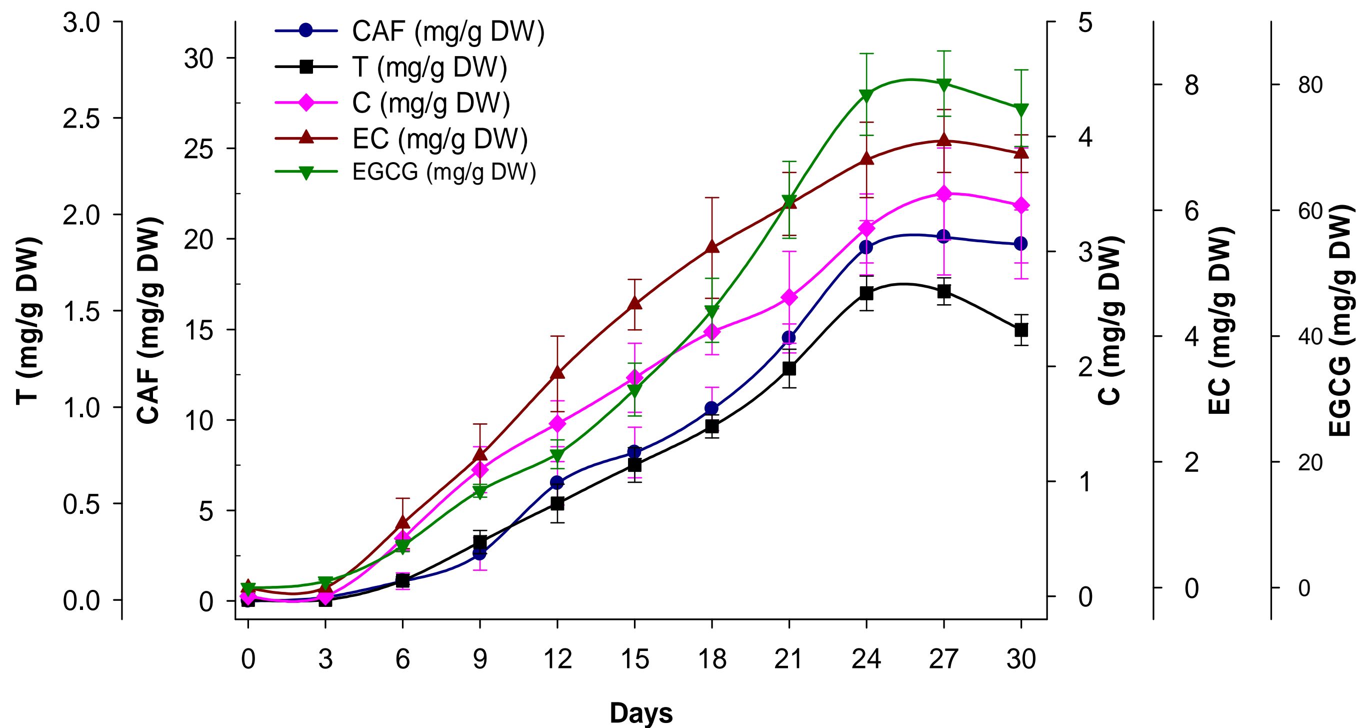

Graph 3.12: Kinetics of secondary metabolites production in cell suspension culture of TV21 cultivar; (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), caffeine (CAF) and theophylline (T).

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Chapter 4

Discussion

Tea, a product made up from leaf and bud of the plant *Camellia sinensis*, is the second most widely consumed beverage in the world, well ahead of coffee, beer, wine and carbonated soft drinks (Rietveld and Wiseman, 2003; Cabrera et al., 2006; Bansal et al., 2012). Chinese mythology teaches that in the year 2737 B.C. emperor Shen nung discovered tea. Before the Tang dynasty (618 to 906 A.D.), it was probably considered as a medicinal plant but later become popular as beverage crop (Harbowy and Balentine, 1997). It is an important socio-economic crop that has a significant role to play towards the economy of India. India ranks first as a producer, consumer and exporter of tea regardless of occupying only around 16.4% of the total tea growing areas of the world (Mondal et al., 2004). Tea consumption is associated with various health-promoting properties. Several studies over the past decade have demonstrated that tea is more than a mere stimulant and may owe its special medicinal properties due to its high polyphenol content (Astill et al., 2001). Polyphenols account for about 25–35% of the total dry weight of fresh tea leaves; two-thirds of the polyphenol content is contributed by catechins (Saravanan et al., 2005). The antioxidant activity of tea has been studied extensively, and numerous studies have been performed to assess the effects of tea polyphenols on the mutagenicity of carcinogens (Weisburger, 1996).

The cultivated tea majorly comprises of three major taxa: Assam type (*C. assamica* ssp. *assamica* (Masters), Cambod type (*C. assamica* ssp. *lasiocalyx* (Planch MS)) and China type (*Camellia sinensis* (L.) O. Kuntze) (Sharma et al., 2010). The six major forms of tea, green tea, black tea, oolong tea, white tea, pu'erh tea, and red tea, differ in terms of their production methods and chemical composition (Sharangi, 2009). Tea plants prefer a warm and humid climate with plenty of rainfall, diffused light, weak acidic and well-drained soil (Wan et al., 2008). Conventionally, tea plants are propagated by seeds and vegetative cuttings though the method is very lengthy and time consuming. It takes about

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three years to a tea plant ready for harvesting the leaves and 4 to 12 years for it to bear seeds (Barua, 2008). Further, the widespread heterozygosity and absence of pure lines of tea plants due to extremely outbreeding nature and long generation cycle has made it difficult to perform selection and genetic studies. Due to the same reasons, it is difficult to produce homozygous pure lines by conventional breeding programmes which involves repeated selfing for several generations (Srivastava and Chaturvedi, 2008). Moreover, the wild populations are circumvented with the problems of low rate of fruit set, poor seed yield/ germination/ viability, and in most cases the clonal uniformity cannot be maintained through seed propagated plants.

In such situations, there is an urgent need for the conservation of elite germplasms. In this context, the advantages of plant tissue culture have been widely recognized. Tremendous progress in this technique has been observed in the last few decades (Kato, 1989; Viettez et al., 1992; Dood, 1994; Viettez, 1995; Das, 2001; Mondal et al., 2004; Hazarika et al., 2013). The plant tissue culture comprises of a set of techniques and the choice of technique depends on the results that one wants to retrieve. In plants, where molecule of interest is localized in the seeds, heterozygosity is not a desirable trait. Therefore, production of pure lines would be advantageous. By means of in vitro technique, haploid and doubled-haploid plants can be regenerated. This process presents diverse advantages in both basic and applied research (Touraev et al. 2001; Maluszynski et al. 2003; Dunwell, 2010), the chief one being related to plant breeding. A doubled-haploid plant genetically a pure line (100% homozygous), and can be obtained in just one in vitro generation. Androgenesis is an indispensable tool for producing doubled haploids. Androgenesis can be obtained by anther culture or microspore culture. The development of callus from somatic tissues of anther walls can be avoided by culture of isolated microspores. However, there are not much successful reports on microspore culture in woody plants (Chaturvedi et al., 2003). Ever since the production of haploids through anther culture of tea, pioneered by Katsuo (1969), Okano and Fuchinone (1970), several attempts were made to produce haploid tea plants via androgenesis. However, success remained up to the mere development of micro calli (Mondal et al., 2004; Hazarika et al., 2013).

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The present report has evaluated various factors to induce androgenesis in anthers cultured at early-to-late uninucleate stage of microspores. The cultures responded by callusing of microspores preceded by minute callusing from anther walls. In subsequent subcultures, only microspore calli were used to achieve regeneration from them. Complete plantlets were obtained via embryogenesis in calli. The haploids attained in the present study may serve as precursors to achieve homozygous diploid lines for uniform quality and quantity of metabolite production. Further, an attempt was made, in the present study, to investigate the androgenic lines of the three taxa of Tea- Assam, Cambod and China type, (1) to evaluate the extraction yield using different solvents, hot-water, methanol, ethyl acetate and hexane, (2) to estimate total phenolic contents, (3) to check antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) methods, (4) screening of catechins like, (+)-catechin, (-)-epicatechin and (-)-epigallocatechin gallate, and alkaloids like, caffeine and theophylline. Additionally, cell suspension cultures were established from the best metabolite producing lines for growth kinetic studies. Until now no attempts were made on these aspects on in vitro cell lines of the three taxa.

4.1. TISSUE CULTURE STUDIES

The plant life cycle proceeds via alternation of generations of sporophytes and gametophytes where sporophyte is the dominant life form of higher plants with 2n genomic constitution. It is formed as a result of fertilization of male and female gametes, each of which contributes a set of chromosomes. “Androgenesis”, by definition, is the switching of pollen from gametophytic mode of development, usually manifested in pollen-tube growth, to the formation of a sporophyte, which is haploid. Therefore, haploids are sporophytic plants carrying gametophytic chromosome number of their parents (Germanà, 2011a; Kasha and Maluszynsky, 2003). The first natural sporophytic haploid plant was observed in 1921 by Bergner in a weed species *Datura stramonium* L. and reported by Blakeslee et al. (1922). This was followed by *Nicotiana tabacum* (Clausen and Mann 1924), *Triticum aestivum* (Gains and Aase, 1926) and subsequently, in several other plant species (Kimber and Riley, 1963). About 40 years after the identification of the first natural haploid, Guha and Maheshwari (1964) discovered an

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efficient technique of in vitro haploid production from immature anthers of *Datura innoxia* of the family solanaceous. This method of androgenic haploid production was quickly attempted in many species to hasten the breeding programme in several economically important plants. In vitro androgenesis via anther/microspore culture is the most preferred techniques for obtaining haploids (Germanà, 2011a; Srivastava and Chaturvedi, 2008) but, in vitro gynogenesis via ovary-ovule culture can prove to be a complementary technique in species where anther culture is inaccessible or less productive (Hazarika et al., 2013). The production of haploids via in vitro gynogenesis is more tedious, less efficient in comparison to androgenesis (Mishra and Chaturvedi, 2012). Since then the haploid production via androgenesis by anther and isolated microspore cultures has been reported in more than 250 plant species, belonging to 100 genera and 40 families. The number and extent of studies on the induction of androgenesis in trees, however, are limited and results obtained to date are nowhere near those of model crops, such as *Brassica* and *Nicotiana*. The major problem encountered is the extreme recalcitrance of tree species in culture. The most effective and popular technique to obtain haploids is by in vitro anther or microspore culture. Anther culture has been successfully applied to several plant species to produce haploids; its single biggest advantage is its simplicity. The large number of anther cultures can conquer the limitations of low per-anther yield. However, the presence of extraneous tissues (eg. the anther wall) makes this a messy system for cell biology and other precise studies (Forster et al. 2007).

Isolated microspore culture in contrast to anther culture, has several important advantages (Bonga et al., 1997; Germanà, 2011b; Radojevic et al., 2002). Proliferation of diploid sporophytic tissues can be avoided from the anther wall. More importantly, homogeneous population of microspores at the developmental stage, most appropriate for androgenesis can be obtained and studied using cell biology, microbiology and functional genomic techniques (Touraev et al., 2001). Additionally, as the development of microspores is independent of the sporophytic tissues, the media components and culture treatments have direct access to the microspores. Thus, under optimal conditions, more microspores can be induced to form sporophyte. Inspite of these positive attributes, microspore culture cannot yet compete with anther culture for doubled haploid production, especially in tree

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species, but might be used to understand the mechanism on gametophytic to sporophytic transition in pollen, for transformation and in vitro selection systems (Kasha 1989; Tuvesson and Ohland 1992).

In this regard, Pelletier and Ilami (1972) introduced the concept of "Wall Factor", according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen. Factors that affect androgenesis have been described elaborately by Atanassov et al. (1995) and Bhojwani and Razdan (1996). It has been observed that physiological status of the donor plant, genotype, environment, developmental stage of microspores at culture, type and concentration carbon source, culture conditions and media, play a pivotal role in induction of androgenesis (Mishra and Chaturvedi, 2012; Hazarik et al., 2013). Anthers from the first flush of flowers are generally more responsive (Shtereva et al. 1998; Srivastava and Chaturvedi, 2011).

Present study describes androgenesis in tea, growing at New Botanical Area (NBA), Tocklai Experimental Station, Jorhat, Assam, India. Interactions between the genotype and prevalent environment of any given area greatly influence the morphometric parameters as well as nutritional requirements of trees in natural as well as in in vitro conditions (Bhojwani and Razdan 1996; Pattnaik et al. 2006). This is the first report on production of haploid tea plants in TV21 cultivar of Assam type and TV19 cultivar of Cambod type. However, the cultivar 317/1 of Cambod variety, though responded for androgenic callus induction, but regeneration could not be obtained.

It has been claimed that androgenic induction is only possible with immature anthers, containing immature pollens at early-to-late uninucleate stages of development i.e. just before or just after pollen mitosis. The stage of microspore development at which the anthers are cultured is more important (Mishra and Chaturvedi, 2012). During this phase of development, the microspores are non-committal in their developmental potential, as most of the sporophyte-specific gene products are eliminated from the cytoplasm before meiosis (Porter et al., 1984), and the gametophyte specific genes are generally transcribed only after first pollen mitosis (Scott et al., 1991). After the first mitosis, the cytoplasm gets inhabited with gametophytic information and it gradually becomes irreversibly programmed, to form the male gametophyte (Mascarenhas, 1971). The switch between the two modes can be amplified by different abiotic stresses (Barret et al., 2004), for

example heat in rapeseed (Lichter, 1982) and cold in corn (Brettel et al., 1981. Stress, in the form of pre-treatments, can be applied to various explants, such as intact flowers and isolated anthers or microspores at culture. In the present study, the anthers were cultured at early-to-late uninucleate stages of microspores. Flower bud size can be used as an indicator of picking the correct anthers at right stage of microspore development (Pedroso and Pais, 1993; Chaturvedi et al., 2003; Srivastava and Chaturvedi, 2011). In the present study, flower buds of selected experimental clones are 3mm to 4mm diameter (**Table 3.1**) which contained anthers at uninucleate stage of microspores.

The medium salt composition has been noticed to have negligible effect on the frequency of initial divisions, a moderate effect on plating efficiency, but have a dramatic effect on embryogenesis and plant regeneration (Mordhorst & Lorz 1993). In the present study, the two basal media, MS and N_6 did not show any response on androgenesis. However, androgenic calli were induced only when MS basal medium was supplemented with plant growth regulator and/or amino acids. Several pre-treatments enhanced the embryogenic ability of microspores (Atanassov et al., 1995). The type and concentration of plant growth regulators in media have been recognized as one of the most critical factors in the development of plantlets in anther cultures (Reynolds, 1987; Tuvesson et al., 1989; Ball et al., 1993; Chaturvedi et al. 2003). In the present study, it was observed that the combined presence of either two auxins or two cytokinins did not show significant effect. However the combined presence of atleast one auxin (2,4-D) and one cytokinin (Kinetin) was shown to have synergistic effect when added together in the medium in presence of additives like, L-Glutamine and L-Serine. Mixture of auxin and cytokinin (*see* in **Table 3.2**), was suitable for initial haploid callus induction and selective division from microspores. Maximum (96%) callus induction was observed in case of TV21 cultivar on MS medium with 6% glucose and supplemented with 2,4-D (5 µM), Kn (5 µM), L-Glutamine (800mg/l) and L-Serine (200 mg/l). Pedreso and Pais (1993) also received callusing in anther cultures of *C. japonica* on MS (with 2% Sucrose) + 2,4-D (4.5 µM) + Kn (4.5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). Likewise, in the present study, the cultivars, TV1, TV18, TV19 and 317/1, showed maximum callus induction in anther cultures on MS medium when supplemented with one cytokinin and two auxins viz. BAP (5 µM) + 2,4-D (1 µM) + NAA (1 µM) and carbon source as 60 g/L sucrose

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(MS3 medium). And in 14/100/1 cultivar, maximum percentage (78.0±5.7 %) of callus induction from anther culture was observed on MS (4% sucrose) + 2,4-D (3 μM) + TDZ (18 μM). The exact requirements of growth regulator combinations were genotype dependent. Auxins in combination with cytokinins, mainly support the callus growth, organogenesis and regulation of direction of morphogenesis (George et al., 2008). In the existing reports on tea anther culture, the responded medium consisted of 2,4-D as the main auxin which evoked callusing followed by rhizogenesis but not adventitious shoot-bud development (Nakamura, 1984 and 1985; Sarwar, 1985; wang et al., 1994). During auxin signaling, a very short and effective pathway involves an auxin receptor, located in the nucleus. When auxin moves into the nucleus, it facilitates the recognition and degradation of repressors of auxin-regulated gene expression. Once the repressors are degraded, transcription of early auxin response genes can occur directly and leads to auxin responses. An additional protein, the auxin binding protein 1 (ABP1) contribute in auxin responses at the plasma membrane, to mediate auxin regulated cell division and cell elongation, and lately, to modulate regulation of genes for early auxin responses, supporting its presumed function as an extracellular receptor of auxins (Tromas and Perrot-Rechenmann, 2010). There are three main, early auxin response gene families which are the Small Auxin Up RNA (SAURs) genes, the GH3s and the Auxin/IAA inducible genes (AUX/IAAs) (Chapman and Estelle, 2009). Highly conserved short-lived small transcripts are coded by SAURs genes (McClure and Guilfoyle, 1987). Though its functions are not clear, it is proposed to act as calmodulin-binding proteins (Yang and Poovaiah, 2000). Gene *gh3*s of early auxin response gene code for conjugating enzymes, a class of which acts as feedback regulators by reducing free auxin levels (Staswick et al., 2005). In the *Arabidopsis* genome, most of the genes of AUX/IAA gene family are upregulated by auxin (Abel and Theologis, 1996; Hagen, 2001; Overvoorde et al., 2005). They encode short-lived transcriptional repressors of the auxin response genes, contributing to maintain their low expression in the absence of auxin and to exert a negative feedback on the expression of auxin response genes after auxin stimulus. AUX/IAA repressors are critical for transient and brief auxin mediated responses as discussed in (Benjamins and Scheres, 2008).

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The high percentage of sugar (5% to 9%) during induction phase is critical as it tends to suppress the divisions of somatic cells, thereby, selectively promoting microspore callusing/ embryogenesis (Clapgham 1973; Wang et al. 1974; Sopory et al. 1978; Chaturvedi et al., 2003; Srivastava and Chaturvedi, 2011). The later stages grow better at lower sugar concentrations (2% to 4%) (Sopory, 1979). Sucrose is the most preferred carbon source in tissue culture. The favorable effect of sucrose was also seen in the present study. This could be because the sucrose is easily hydrolyzed by cell wall bound invertase into more efficiently utilizable form of sugars, glucose and fructose, which are incorporated into the cells. Cells exhibit a preference for glucose whereas fructose is utilized as a substrate only after glucose is depleted from the medium (Kretzschmar et al., 2007). Thus, the presence of sucrose facilitated the constant availability of utilizable forms of carbohydrates, in the present study, throughout the long culture period of 8 weeks in the callus induction medium. Other advantages of sucrose may be attributed to its contribution in increasing the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, production of high amount of energy (Bonga and Voan Aderkas, 1992; Singh and Chaturvedi, 2010) which may be the cause to induce divisions from microspores. In the present study also, it was observed that higher percentage of sugar concentration (6 %) is favorable for callusing from microspores. Sucrose at its optimum concentration of 6 % was observed to be significantly better ($p<0.05$) for callus induction in majority of the cultivars, like TV1, TV18, TV19, 317/1 and 14/100/1. while, in case of TV21, Glucose at 6% served to be the best carbon source with significant ($p<0.05$) amount of callus induction. Similarly, in Neem, high percentage of cultures showed callusing from microspores in anther cultures in presence of 9 % sucrose Chaturvedi et al. (2003) or 12 % sucrose Srivastava and Chaturvedi (2011).

In many tree species, temperature pre-treatments had promotary effect on androgenesis. In *Peltophorum*, proliferation of haploid calli, embryos and plantlets from anthers, cultured at mid-late uninucleate stages of microspores, was observed after pre-treating the flower buds at 14°C for 8 days (Rao and De, 1987). In *Populus*, anthers at uninucleate stage of microspores, taken from cold pre-treated flower buds (at 4°C for 4 days), produced globular calli after 4-8 weeks of dark incubation at 20°C on MS + 2.4-D (2.26 μM) + Kn (0.46 μM) (Stoehr and Zsuffa, 1990; Kiss et al., 2001). It is found that

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combined pre-treatments of cold temperature and activated charcoal was very favorable to induce embryogenesis in *M. pumila* (Hidano et al., 1995). Jain et al. (1996) who evaluated the effect of various physical and chemical pre-treatments on induction of haploid plants in anther cultures of *Morus alba*, revealed that cold pre-treatment to flower buds at 4°C for 24 h, increased the percentage of anther cultures showing callus induction from microspores (9.20 %). Germanà and Chiancone (2003) published an improved and detailed protocol for haploid induction through anther cultures of *C. clementina* Hort. Ex Tan. cv Nules by evaluating a number of factors that affect androgenesis. They found that temperature pre-treatment to flower buds at 4°C and 25°C, for 14 days, were very favorable to induce embryogenic callus and embryoids in anther cultures. Rimberia et al. (2005) examined the positive effect of high temperature pre-treatment to anthers on induction of embryos from microspores in *Carica papaya*. Whole anther pre-treatment results in changes in cell wall properties of anther tissues (Wang et al., 2000). It is, generally associated with rapid loss of chlorophyll from the middle layer, degeneration of tapetal cells and finally damaged innermost layer of locules. Cold treatment kills weak or non-viable anthers and microspores, while arresting many of the viable microspores in the first mitotic stage due to blockage of starch production. Microspore and tapetal development then become asynchronous, influencing the switch from the gametophytic to the sporophytic phase (Pickering and Devaux, 1992; Foroughi-Wehr and Wenzel, 1993). Heat pre-treatment is linked with the appearance of small heat-shock proteins (smHSP) before induction of androgenesis (Pechan, 1991; Smykal and Pechan, 2000). Generally, larger the temperature differences between the donor plant growth conditions and in vitro culture conditions, stronger are the smHSP signal. However, no smHSP is produced below 25°C.

In the present study, the entire buds or anthers of tea were exposed to low (5°C in dark) and high (33°C in dark) temperatures for 0 and 5 days, to study their effect on callus induction. Light or dark incubation at 25ºC served as controls. Among controls, 25ºC dark incubation was better than 25ºC continuous light incubation of cultures for callus induction. Temperature pre-treatments to entire buds did not show any response but it had pronounced effect on enhanced androgenic callus induction when anthers were pretreated at low or high temperatures before being shifted to 25°C dark incubation. Best response

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was observed in control at 25°C temperature with dark incubation, followed by cold pretreatment (5ºC for 5 days in dark) and heat pretreatment (33ºC for 5 days in dark). Although dark incubation of anther cultures at 25ºC promoted highest percentage of callusing but the callus originated mostly from walls. The calli were induced from inside the burst open anther locules in maximum percentage of cultures, only, when they were given a cold pre-treatment at 5ºC for 5 days before transferring to 25^0C dark incubation.. Similarly in case of anther culture of Neem, dark incubation was observed to be the best for androgenesis (Chaturvedi et al., 2003; Srivastava and Chaturvedi, 2011). An specific amino acid may be needed to induce a given physiological response in plant tissue culture (Dodds and Roberts, 1982). In case of TV19, anther wall burst open longitudinally and calli were originated from microspores on MS (6 % Sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM). These calli were later maintained and proliferated on MS + NAA (5 µM) + BAP (10 µM) where nodulation of calli was observed. While in TV21 cultivar, the best medium for callus induction was MS (6 % Glucose) supplemented with 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). The callus prolifration rate enhanced in subsequent subcultures when 6 % Glucose was replaced with 3 % Sucrose in the same medium composition (maintenance medium).

Regeneration in callus cultures may occur either by organogenesis or embryogenesis (Bhojwani and Razdan, 1996). The plant growth regulators and stress play a chief role in mediating the signal transduction cascade leading to the reprogramming of gene expression. Due to the reason, a sequence of cell divisions occurred that induced either unorganized callus growth or polarized growth leading to embryogenesis (Dudits et al., 1995; Quiroz-Figueroa et al., 2006). An additional class of stress (auxin starvation) can also effectively induce embryogenesis (Smith and Krikorian, 1989; Lee et al., 2001). The embryogenic inductions consisted of the termination of a current gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression program. One acceptable mechanism for down regulation of current gene expression is DNA methylation, which is influenced by auxin (Lo Schiavo et al., 1989). In the present study, in both the cultivars, TV 19 and TV21, regeneration via embryogenesis was observed on MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/L) + L-Serine (200 mg/L) after three months of transfer of callus where the nodules turned into distinct embryos at

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various developmental stages. The absence of auxin in the medium results in starvation and which in turn had induced embryogenesis.

Presence of gibberellic acid (GA_3) in the medium inhibited embryogenesis in carrot (Tokuji and Kuriyama, 2003), citrus (Kochba et al., 1978) and geranium (Hutchinson et al., 1997). However, in some plant systems, exogenous GA_3 stimulated embryogenesis, such as in chickpea immature cotyledon cultures (Hita et al., 1997) and in *Medicago sativa* petiole derived tissue cultures (Ruduś et al., 2002). Mondal et al. (2002) observed that in tea, embryos without GA_3 treatment failed to germinate but remained fresh while those treated with GA_3 underwent bleaching and necrosis at all concentration. It was explained that GA_3 brings about *de novo* synthesis of α-amylase and hydrolase for the metabolism of starch reserves and normal embryo germination (Bewley and Black, 1985; Mondal et al., 2002). Das et al. (1995) observed that the combination of L-Glutamic acid and GA_3 in *Hardwickia binata*, had greatly improved the frequency of normal embryo differentiation. A number of genetic and environmental factors have been known to affect embryogenesis in anther culture. Genotype has been reported to be a key factor (Chen and Dribnenki 2002; Perrin et al. 2004).

In *C. japonica* L. two distinct types of embryos were identified, seed-like and bud-like embryos. Seed-like embryos had large cotyledons while bud-like embryos had bipolar structures. Both type of embryos had capacity to develop into whole plantlets (Vietez, Borciela, 1990). In *C. sinensis* var T 78 (Darjeeling tea clone), four distict types of somatic embryos were identified from immature cotyledons like, seed-like, globular, cup shaped and monopolar embryos with either root or shoot. Out of them only globular and few seed-like embryos induced to develop into whole plantlets (Jha et al., 1992). Same types of embryo morphotypes and their development pattern were observed in the present study where globular and seed-like embryos gave rise to complete plantlets. Occassionally, abnormal development of embryos were also observed in the cultures, like, embryos with abnormally thick plumular, with no cotyledons, with fused cotyledons, with multiple cotyledons and flower-shape embryos with multiple cotyledons. None of these embryos show any sign of germination. These types of abnormalities are commonly observed in embryogenesis of both monocotyledonous and dicotyledonous species (Vasil and Vasil, 1982; Barwale et al., 1986; Popescu, 1996).

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Several methods are available to determin of ploidy level of plants, like chromosome counts, flowcytometry and the evaluation of morphological or anatomical parameters. Chromosome counting for determination of ploidy level of plant is the most concrete methods because it determines the exact chromosome numbers (Dolezel et al., 2007). However, chromosome counting method is very laborious, involving plant specific enzymatic (cellulose, pectinase etc.) or chemical (Hydroxyquinoline, Colchicines etc.) treatments. Accordingly, only a limited number of cells can be analyzed for counting chromosome (Bohanec, 2003). Furthermore, at the time of counting, many small chromosomes error may occur. Flow cytometry is another alternative technique for plant ploidy analysis (Eeckhaut et al. 2006; Dolezel et al., 2007). The technique involves isolation of nuclei from cells by bead beating (Roberts, 2007) or by razor blade chopping (Galbraith et al., 1983), followed by DNA staining by fluorescence dye and finally, analysis through a flow-cytometer. This machine aligns the nuclei by hydrodynamic focusing, uses a light source to illuminate the stained nuclei, and detects the fluorescence emitted. The amount of fluorescence in every nucleus is correlated to its DNA content. Samples can be run in bulk and tens of thousands of cells can be counted in a single run. The tea chromosomes are small in size (karyotype ranges from 1.28 μ to 3.44 μ) and tend to clump together due to 'stickiness'. Tea is diploid (2n=30) and basic chromosome number as n=15 (Bezbarua, 1971). In the present study, flowcytometry of calli and regenerants has confirmed their haploid status. Cytological analysis of shoot-tips of field grown parent plants (control) of the three cultivars revealed the diploid number of chromosomes as 2n=2x=30, in TV19, TV21 and 317/1. While root-tips of in vitro developed androgenic plantlets of TV19 and TV21 cultivars and calli of 317/1 cultivar revealed that a majority of cells were in haploid state with the chromosome number as 2n=x=15. The developed haploids will be sterile due to uneven segregation of chromosomes during meiosis. To make them fertile, it is necessary to diploidize them by mutagenic treatments, like colchicines or oryzalin.

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4.2. ANTIOXIDANT ACTIVITY

The prospective health benefits associated with tea consumption are attributed to several active ingredients particularly, polyphenols, which contribute to antioxidative properties

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of tea (Wiseman et al., 1997; Zhang and Shen, 1997; Hajimahmoodi, 2008). This has attracted significant attention, both in the scientific and consumer communities for its multiple health benefits for a variety of disorders ranging from cancer, cardiovascular, diabetes, and neurodegerative diseases, like Parkinson and Alzheimer's to the weight loss. The antioxidant property is because of their efficient free-radical scavenging activity which is due to their one-electron reduction potential, an ability to play as hydrogen or electron donors (Higdon and Frei, 2003; Chan et al., 2007). If reduction potential is less it means less energy is required for hydrogen or electron donation and this indicates higher antioxidant activity. To a great extent, agriculture and pharmaceutical industries rely on the harvest of these polyphenols from "wild" heterozygous plants; therefore, potential for variability in agronomic performance and active ingredients is high. All the three taxa of tea, Assam, China and Cambod, are highly cross-pollinating and intercrossable without any reproduction barrier. The extensive cross breeding results in heterogeneous tea population and these genetic differences are well reflected in biochemical composition of leaves which are principally used for tea production. Considering the benefits, constraints and limitations experienced, it is pertinent to analyse genetically pure androgenic lines for the production of total phenolic contents and their antioxidant activity. In the present study, the total phenolic constituents of various extracts were determined by following the methods adopted by Singh et al. (2013) and Hazarika and Chaturvedi (2013) involving Folin–Ciocalteu reagent and gallic acid standard. The antioxidant activity was performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) methods. DPPH describes the ability of a compounds to act as hydrogen donor while FRAP acts as an electron donor (Chan et al., 2007). DPPH is a purple-colored stable free radical, which is reduced to α,α-diphenyl-β-picrylhydrazine (yellow colored) by accepting an electron or hydrogen radical from an antioxidant (Soares et al., 1997; Hossain and Shah, 2011; Singh et al., 2013). Until now no attempts were made on these aspects on in vitro cell lines of the three taxa, Assam, Cambod and China. Moreso, androgenic organized cultures were not generated due to recalcitrant nature of the plant. Although in limited earlier reports, haploid microcalli were obtained but it could not be proliferated further in subsequent subcultures. In the present study, one cultivar from

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each taxa: Assam (TV21 cultivar), Cambod (TV19 cultivar) and China (317/1cultivar) type of tea were analysed for both total phenolic contents and antioxidant activity.

Antioxidant activity of tea is affected by many natural polyphenols, like catechins, oxyaromatic acids, tannins, flavonols, thearubigins, theaflavins, etc. (Hajimahmoodi, 2008). Flavonoid (Catechin) with an O-dihydroxy or trihydroxy B-ring and gallate ester moiety at the 3-position of catechins (ECG and EGCG) have very high antioxidant activities (Sang et al., 2002). In the present study, in vitro calli from all the three taxa, TV19 (Cambod type), TV21 (Assam Type) and 317/1 (China type), and embryos of TV19 and TV21 were subjected to extraction with hot water and various organic solvents, methanol, ethyl acetate and hexane. The overall highest yield of extracts was obtained with the cultivar TV21. The yields of extracts from different solvents were obtained in the order: hot water > methanol > ethyl acetate > hexane. In general, the percent yield of extracts were maximum from young leaves of parent plants (control), followed by embryos and then calli. Since IC_{50} value is a measure of DPPH inhibitory concentration, a lower IC_{50} value reflects greater antioxidant activity of the sample. Ascorbic acid, catechin, epicatechin, epigallocatechin gallate, gallic acid and vanillic acid were used as reference standards in the current study. Epigallocatechin gallate showed excellent radical scavenging activity among them, with IC_{50} value of 2.14±0.24 µg/ml and it has 11.41-fold, 18.04-fold and 21.97-fold higher than the antioxidant activity of leaves (control), embryos and calli, respectively, of TV21. While, vanillic acid showed very low radical scavenging activity with IC_{50} values as 4060 µg/ml which is 166.32-fold, 105.15-fold and 86.38-fold lower than the leaves (control), embryos and calli hot water extracts of TV21. It was generally assumed that dedifferentiated cultures proved to be less active as antioxidants (Grzegorczyk et al., 2007). This is in agreement with the present study, where embryos of both the cultivar, TV19 and TV21 showed promising antioxidant activity compared to dedifferentiated calli. Matkowski (2008) discussed that a close association existed between the expression of secondary metabolites and morphological and cellular differentiation in cultures.

Several studies have reported that phenolic contents in plants are associated with their antioxidant activities, probably due to their redox properties, that allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Chang et al., 2001). In

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the present study, it was observed that TV21 has higher total phenolic contents and antioxidant activity compared to TV19 while the least activity was observed in 317/1 cultivar and they were obtained in the order of young leaves from parent plants > embryos > calli. These results confirmed that there was a positive linear correlation between antioxidant activity and total phenolic content, which suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the in vitro androgenic haploid cell lines. These results were consistent with the findings of other research groups who reported such positive correlation between total phenolic content and antioxidant activity in several other plant species (Zheng and Wang, 2001; Cai et al., 2004). To date, this is the first scientific evidence that androgenic lines of Cambod type (TV19), Assam type (TV21) and China type (317/1) of tea possess significant phenolic content with potential antioxidant activity. It can be a better source of effective natural antioxidants. The productivity of which can be further increased by media optimization and elicitor.

4.3. SECONDARY METABOLITES

Secondary metabolite production from plant tissue culture has emerged as a promising and feasible option attracting the attention of scientists worldwide. This technique has been considered as an attractive alternative method for bioactive metabolite production. The range of compounds produced by plant tissue cultures can differ significantly, both quantitatively and qualitatively, from that found in the field grown plants (Verpoorte et al., 1998). The entire exercise becomes obligatory if we aim at metabolites from an out-breeding tree species, due to the variability inflicted upon by heterozygosity in the genus. However, as is the case with the metabolite content under in situ conditions, in context to variability, the same limitation is posed if cultures are established from seeds or other somatic tissues. Haploid lines can fix the genetic variability as they are the raw materials for production of doubled haploid pure lines. With this they can also help in conserving the elite germplasm. Apart from androgenesis, the focal point of this report lies in the assessment of haploid (n) cell lines and their derivatives as source of important catechins and alkaloids. Such kind of investigation has been carried out for the first time in Tea. Till date, the available reports on metabolite production, have utilized cultures from different somatic parts (2n) like leaves.

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The catechins (flavan-3-ol) have the basic structure of C6–C3–C6 with two aromatic rings and several hydroxyl groups (**Scheme 1.4**). The catechins are classifieds into two groups; free catechins and esterified catechins. The free catechins are catechin, gallocatechin, epicatechin (EC), epigallocatechin (EGC), whereas the esterified catechins are EGCG, epicatechin gallate (ECG), gallocatechin gallate (GCG), and catechin gallate (CG). The esterified catechins contribute substantial astringency and a bitter taste, whereas the free catechins are much less astringent and have a slightly sweet taste (Hara, 2001; Vuong et al., 2010). Among the catechins, EGCG, EGC, ECG, and EC are the four major catechins found in green tea; EGCG is normally present at the highest concentration, followed by EGC, ECG, and EC (Chu and Juneja, 1997; Vuong et al., 2010). The catechins are synthesised in the leaves of the tea plants through the acetic-malonic acid and shikimic–cinamic acid metabolic pathways. Chalcone and gallic acid are produced from the shikimic acid pathway from which these different catechins are produced (Chu and Juneja, 1997). There is some variation in the content of the individual catechins in fresh tea leaf; the composition depends on the location of cultivation, variety, nutrition of the tea plant, time of year, and the type of leaves (coarse or young leaves). A typical catechin profile in an extract from green tea leaves comprises 10–15 % EGCG, 6–10 % EGC, 2–3 % ECG, and 2 % EC (Shi et al., 2009; Vuong et al., 2010; Bansal et al., 2012). The normal epistructure of catechins is found to be unstable where ECs tend to epimerize to non-epistructured catechins at temperatures above 80ºC (Chen et al., 1998). In addition, the catechins have been found to be epimerize faster in tap water than in purified water (Wang and Helliwell, 2000). However, the catechins are shown to epimerize at a much slower rate in solutions with a pH of less than 6. In the present study, androgenic culture of the three taxa, Assam, Cambod and China type (one cultivar from each taxa) were analyzed to check the contents of EC and C from free catechin, and EGCG from esterified catechin. Tea is also a good source of methylxanthines, in the form of caffeine, theobromine and theophylline (Hardowy and Balentine, 1997; Dufresne and Farnworth, 2001). Theophylline, a similar di-methylxanthine, has been reported in trace quantities in tea leaves (Sanderson, 1972; Hardowy and Balentine, 1997). Present study had also analysed the production of caffeine and theophylline in androgenic lines.

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There are several methods for identifying and quantifying the tea catechins (Dalluge and Nelson, 2000). These methods can be employed for determining the concentration, the yield, and purity of catechins in final products. The identification and quantification of catechins as well as caffeine have been largely facilitated by chromatographic techniques, such as HPLC and CE (capillary elecrophoresis) using various detectors such as UV, electrochemical, and MS detectors, to analyse the individual catechins (Fernández et al, 2000; Pelillo et al., 2002; Gotti, 2011). Alternatively, near-infrared reflectance spectroscopy, high-speed countercurrent chromatography, TLC, and GC have also been used to identify and quantify the catechins (Dalluge and Nelson, 2000). Curently, HPLC (High-performance liquid chromatography) is the most helpful approach to the routine analysis of non-volatile tea constituents (Finger et al., 1992). Liquid chromatography was initially used for the preparative isolation of flavanols from green tea (unfermented tea leaves). Initially flavanols were extracted from tea leaves with boiling water and aqueous acetone and separated by Sephadex LH-20 columns (Finger et al., 1992). Hoefler and Coggon (1976) introduced HPLC with reverse phases for identification of five catechins (C, EC, EGCG, EGC and ECG). A reverse-phase C_{18} column is now generally employed for HPLC and the mobile phases generally contain aqueous methanol or acetonitrile solution with the addition of acids, such as orthophosphoric, formic, or acetic acid and column conditioners, such as tetrahydrofuran (Finger et al., 1992; Wang et al., 2000). In the present study, C_{18} reverse-phase column (250 mm X 4.6 mm i.d.; 5 μm particle diameter) was used for the analysis of all coumpounds. For catechins and caffeine analysis, acetonitrile:ethyl acetate:0.05 % H_3PO_4 (12 : 2 : 86 v/v) was used as mobile phase, at a flow rate of 0.5 ml/min and the eluted samples were detected at 280 nm wavelength. For theophylline, acetonitrile:water (10:90) (v/v) was used as the mobile phase, at a flow rate of 1 ml/min and the eluted samples were detected at 273 nm wavelength. Calibration curves for all the five standards showed good linearity at tested concentrations (0.025 mg/ml to 1 mg/ml) with correlation coefficients (R^2) of 0.997, 0.996, 0.991, 0.999 and 0.973 for C, EC, EGCG, CAF and T, respectively. Precision of the HPLC method (% RSD) was evaluated through the determination of repeatability and intermediary precision. The intra-day and inter-day precision were estimated from the triplicate injections of all the standards which showed satisfactory response for all

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compound with % RSD values of all the compounds in the range of 0.2 % to 1.4 %. This indicates that method is both repeatable and reproducible. The recovery experiments for all the five compounds were performed by adding known amount of C, EC, EGCG, CAF and T standards to the cells. The percentage recoveries for all five compounds were observed to be above 91.6 % in all cases. Orthophosphoric acid as mobile phase was also used by Wang et al. (2000) for analysis of catechin and caffeine.They also analysed catechins and caffeine by isocratic elution with C_{18} reverse phase column, methanol/water/H_3PO_4 (20/79.9/0.1) as mobile phase with flow rate of 1.0 ml/min. Peng et al. (2008), analysed phenolic compounds, purine alkaloids and theanine in *Camellia* species by HPLC consisting of RP-Amide C_{16} guard column with two-gradient elution system. Thy used ortho-phosphoric acid (85 %) and water (0.05:99.95, v/v) as mobile phase A and acetonitrile (ACN) as mobile phase B. Saito et al. (2007) observed that addition of ethyl acetate at low pH aqueous mobile phase improved the determination and efficient resolution between the peaks of CAF and EGC.

The most abundant catechins in tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC) and (+)-catechin (C) (Liang et al., 2006; Wei et al., 2011). Among them, EGCG is generally the most abundant component, while the contents of EC and C are relatively low (Wei et al., 2011). Recently, catechin and its components have been widely utilized in studying the diversity of tea germplasm (Magoma et al., 2000; Gulati et al., 2009). Catechins are considered to be synthesised through phenylpropanoid and flavonoid biosynthetic pathway. The formation of dihydroquercetin and dihydromyricetin, which are the precursors of dihydroxylated catechins (EC and ECG) and trihydroxylated catechins (EGC and EGCG), respectively, is genetically controlled (Gerats and Martin, 1992). It was found that total catechins of tea leaves increased with the exposure time to sunlight, suggesting that catechin biosynthesis is also environmentally dependent (Mariya et al., 2003). Up to now, little systematic investigation has been carried out on the genetic and environmental effects on catechins, especially the effects of climatic factors during young leaf formation, such as temperature, relative humidity, precipitation and sunlight exposure time, etc (Wei et al., 2011). As climatic factors are generally considered as important contributors to environmental effect on production of catechins in tea (Wei et

al., 2011).The average EGCG contents of the varieties were 121.7 ± 2.4 mg g^{-1} (12.2 %) for Assam, 112.6 ± 2.9 mg g^{-1} (11.2 %) for Cambod and 86.2 ± 1.3 mg g^{-1} (8.6 %) for China (Sabhapondit et al., 2012). In the present study, in in vitro cultures (both calli and embryos) as well as in parent plants, EGCG content was high in assam type followed by cambod type of tea while least amount was present in china type; same pattern were also seen in case in EC. Saito et al. (2007) determined the catechins and caffeine in green tea of *Camellia sinensis* var. *assamica* and found 2.46 ± 0.032 % caffeine, 0.42 ± 0.007 % C, 1.06 ± 0.022 % EC, and 5.18 ± 0.069 % EGCG. In the present study, Assam type (TV21) of tea showed maximum amount of catechins and theophylline content in embryogenic cultures with 0.75 % C, 0.85 % EC, 9.4 % EGCG and 0.22 % T compared to the embryogenic cultures of Cambod type (TV19) of tea. But caffeine content was higher in TV19 cultivar compared to TV21. However, leaves from parent plants (control) of the respective cultivars contained highest amount of metabolites compared to those of any in vitro lines.

Further in the present study, the fractions of crude extract, eluted from HPLC, were collected at respective retention time, analysed by mass spectrometry and the fragment characteristics were compared with that of HPLC eluted standards of respective compounds. Positive mode electrospray ionization (+ESI) conditions were applied for all the five compounds. Spectra were obtained in full scan mode. The fragmentation patterns of the fractions eluted by HPLC were similar to those of their respective standards, which further confirm the presence of C, EC, EGCG, CAF and T in the haploid cell lines of tea. In the positive ion detection mode fragment 139 m/z is the most preferable for catechins in the absence of gallate. This important fragment is formed after cleavage of two bonds in the ring C and can originate from: (i) phenolic ring A of all eight catechins or (ii) phenolic ring B of galocatechins (Spáčil et al., 2010). In present study also in positive ESI mass spectra of C, EC and EGCG showed m/z 139 characteristic fragment which is due to a retro Diels-Alder (RDA) fragmentation of the nonvariable portion of the catechin ring structure (Zeeb et al., 2000). The pathway for this fragmentation was shown in **Scheme 4.1**. For EGCG, EC and C similar type of fragmentation patter was also observed by Zeeb et al. (2000). For EGCG characteristic peaks of m/z 139, m/z 289 were also reported by Zeeb et al. (2000) and Spáčil et al. (2010).

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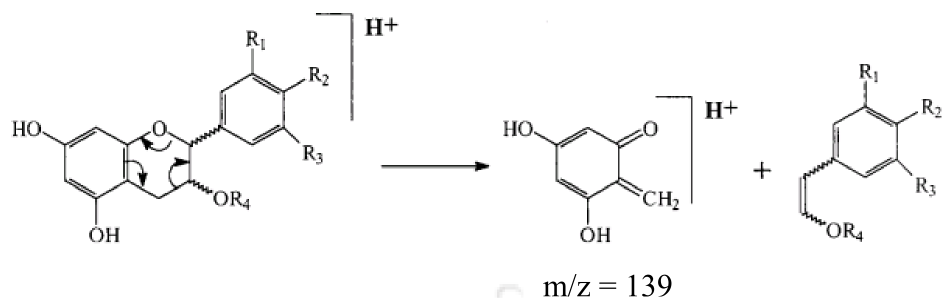

Scheme 4.1: Retro Diels Alder fragmentation (m/z=139) of a catechins (Zeeb et al., 2000).

4.4. BATCH KINETICS

Plant cell culture is a promising biotechnological approach, and has been adopted for production of various categories of secondary metabolites (Prakash et al., 2005). Although the lower yield of these compounds in cell culture has restricted the commercial utilization of the approach, several strategies have been proposed to enhance product yield and productivity in cell culture system (Choi et al., 2006). Specially, the cell suspension cultures offer a condensed biosynthetic cycle to study growth and production kinetics within a short cultivation time (about 2–4 weeks) with an added advantage of tunability which can help to implement optimal conditions for the production of a number of high value medicinal compounds in good quantities (Dörnenburg and Knorr, 1995; Singh and Chaturvedi, 2012). Undifferentiated plant cell suspension cultures, though are known to synthesize less number of secondary metabolites due to their lack of differentiation property of cell, have an indispensable advantage of fast growth rates along with an ease in scale-up (Omidi et al. 2010; Grover et al., 2012). The present investigation is focused on the establishment of tea cell suspension cultures from superior cell lines and development of optimal operating conditions in shake flask suspension cultures for biomass growth and metabolite production. Dynamic changes of parameters, such as medium, pH, wet and dry cell concentrations, consumption of major nutrients, carbon source concentration and agitation speeds, were investigated to understand the culture characteristics of suspended cells and their ultimate effect on catechins, caffeine and theophylline production.

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Singh and Chaturvedi (2012) reported that in *Spilanthes* the cell growth was faster in liquid medium than in semi-solid medium. It took only 18 days for the cell cultures to complete the growth cycle in liquid medium compare to the calli grown on semi-solid medium which needed at least 5 weeks to attain maximum growth. This may be because of facilitated nutrient uptake in liquid medium. Same pattern was observed in tea, in the present study, where cell growth was faster in liquid medium than the calli grown on semi-solid medium. In tea, it takes 30 days for cell cultures in suspension to complete the growth cycle compared to the calli which needed 60 days to achieve maximum growth in semi-solid medium. As a rule, 2,4-D promotes active proliferation of cells and the rate of cell growth depends on 2,4-D concentration in the medium and also characteristics of the cultivar (Butenko, 1999). Grover et al. (2012) received maximum cell biomass in suspension culture on MS + 2,4-D (26.8 µM) + BAP (4.44 µM). This confirms that high concentrations of auxin favor the cell growth in suspension cultures. Hazarika and Chaturvedi (2013) reported fast proliferation of gynogenic calli of TV18 cultivar of tea on MS + BAP (22.2 µM) + IBA (9.8 µM). In the present study, presence of 2,4-D in the medium supported cell growth in suspension culture but maximum cell biomass growth (11.2 g/l of DW) was achieved on medium MS + BAP (22 µM) + IBA (10 µM) at 27th day.

Sucrose has been extensively used as the principal carbon source for cultivation of plant tissue cultures. In general, it has been observed that the rate of cell growth of cultures is directly related to the sucrose concentration in the medium (Grover et al., 2012). In the current study, 3% sucrose was optimum for cell suspension culture. Grover et al. (2012) also observed maximum accumulation of biomass in tea suspension culture in presence of 3% sucrose. The effect of pH was observed in the present study and pH 5.8 was observed to be the best for maximum biomass accumulation in tea suspension culture. Similar type of observation was reported by Grover et al. (2012). Agitation speed in rpm directly affects the growth and viability of cells in culture due to aeration and agitation (Srivastava et al., 2011; Singh and Chaturvedi, 2012). Agitation speed is a very significant parameter for establishment of plant cell suspension cultures. It is primarily responsible for mixing the plant cells in the medium and, thus, to facilitate homogeneous nutrient uptake and also for providing a sufficient O_2 and CO_2 supply (Singh and

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Chaturvedi, 2012). In the present study, cells were grown at various agitation speed of 90, 120 and 150 rpm. Speed of agitation on growth and viability of cells in shake flask suspension cultures were assessed at 27th days. The maximum biomass accumulation with good viability of cells was observed at 120 rpm maximum at. At the other two rpm, the biomass and viability profile was highly unsatisfactory. At higher speed (150 rpm), the cells died due to rupturing and shear effect. At lower agitation (90 rpm), the cells died due to aggregation and clumping; only the cells at the outermost layer of the aggregate were alive and fluorescent green when stained with fluorescein diacetate (FDA). Similarly, Singh and Chaturvedi (2012) and Srivastava et al. (2011) observed maximum biomass accumulation and cell viability at 120 rpm. FDA is a non-fluorescing, non-polar dye which freely permeates through the plasma membrane. In the living cells, it is cleaved through esterase activity releasing the polar freely per portion i.e. fluorescein. It is unable to pass through the plasma membrane of living cells while in dead and broken cells it is lost. Hence, only the live, intact cells take up the stain and plasma green (Singh and Chaturvedi, 2012; Srivastava et al., 2011). Flavonoid production is growth associated in tea cell suspension culture (Shibasaki-Kitakawa et al., 2003). In present investigation also, it was observed that production of catechins, caffeine and theophylline is growth associated and maximum production were at 27th day i.e. in stationary phase. Similarly, production of triterpenoid in *Lantana camara* (Srivastava et al., 2011) and spilanthol production in *Spilanthes acmella* (Singh and Chaturvedi, 2012) were observed to be growth associated and maximum production being observed at stationary phase. Sucrose utilization occurred at a relatively faster rate and it was consumed completely from the medium by the cells at 27th day of culture. Therefore, it may be speculated that complete utilization of sucrose from the medium resulted in the onset of decline phase of cell growth in suspension culture and this was the major limiting nutrients for cell growth.

To date, this is the first scientific evidence that androgenic lines of Cambod type (TV19), Assam type (TV21) and China type (317/1) of tea possess high phenolic content with potential antioxidant activity. It can be a better source of effective natural antioxidants. The productivity of which can be further increased by media optimization and elicitor.

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Chapter 5

Conclusions and future prospects

The present study is an attempt to realize the potential of economically and medicinally important plant, *Camellia sinensis*, by biotechnological means using tissue culture techniques. Biochemical studies demonstrated the potential of tea anther cultures as source of important secondary metabolites. Antioxidant activity undertaken in all type of tea plants and in vitro cultures further confirmed the utility of these studies. Key points of the entire work are given in the bulleted list below.

- ✓ The present investigation establishes an efficient and reproducible protocol for in vitro production of haploids in anther cultures of tea by studying the effect of various stress treatments on embryo induction and plantlet development. Additionally, androgenic lines have also been evaluated for total phenolic contents, antioxidant activity and metabolite production.

- ✓ Cultivars from all the three taxa of tea, Assam, Cambod and China, were selected for the study. Anthers from 3-4 mm size flower buds, bearing microspores at early-to-late uninucleate stages, were used as explants. The entire androgenic process involved four steps: (1) callus induction, (2) callus multiplication, (3) regeneration via embryogenesis, (4) embryo maturation and germination.

- ✓ The androgenic haploid calli were induced in all the cultivars tested. However, callus multiplication in subsequent subcultures was obtained on cultivars TV19 (Cambod type), TV21 (Assam type) and 317/1 (China type). Calli were induced from inside the burst open locules of anthers on MS (with 6% Sucrose) + BAP (5 µM) + 2,4-D (1 µM) + NAA (1 µM) in TV19 and 317/1 cultivars while in TV21 cultivar, calli were induced on MS (with 6% Glucose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). Continuous dark incubation and

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pre-treatments were given to anthers. A Pre-treatment of 5ºC for 5 days before shifting to 25ºC was the best condition to induce calli from microspores.

- ✓ Calli were further multiplied, at every 8 weeks, on callus multiplication medium at reduced concentrations of carbon source. In TV19, the calli were multiplied and maintained on MS (3% Sucrose) + NAA (5 µM) + BAP (10 µM) medium and the rate of callus proliferation was (221.34 %). In the cultivar TV21, maximum callus proliferation in terms of fresh cell biomass increase (235.95±5.1%) was observed on MS (3% Sucrose) + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). While in case of 317/1, maximum cell biomass increase of 152.2% was obtained on MS (3% Sucrose) + 2,4-D (3 µM) + TDZ (18 µM) medium.

- ✓ Regeneration via embryogenesis was achieved only when the nodulated calli from callus multiplication media were transferred to the regeneration medium consisted of MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l). Asynchronous embryogenesis was observed in case of TV19 and TV21 cultivars on regeneration medium after two subcultures, each of 6 weeks duration. However, the calli from 317/1 cultivar did not show any sign of regeneration.

- ✓ Embryos later matured when transferred to 10 times rduced concentration of embryo induction medium, MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l) medium by developing bipolar structures with distinct radicular and plumular regions.

- ✓ Mature bipolar embryos germinated only when they were transferred to ½MS (major salts reduced to half strength) medium supplemented with BAP (10 µM) + IBA (1 µM) + GA_3 (0.5 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l).

- ✓ Cytological analysis of plantlets, regenerated from anther callus via embryogenesis, revealed that plantlets were haploids with chromosome number 2n=x=15. Flow cytometry analysis further confirms the haploid status of regenerants.

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- ✓ The young leaves from parent plant (control) and in vitro calli of TV19, TV21 and 317/1 cultivars, and embryos of TV19, TV21 cultivars, were subjected to extraction with hot water and various organic solvents, methanol, ethyl acetate and hexane. Of the three cultivars, TV19 (Cambod type), TV21 (Assam Type) and 317/1 (China type), the overall highest yield of extracts was obtained with the cultivar TV21. The yields of extracts from different solvents were obtained in the order: hot water > methanol > ethyl acetate > hexane. In general, the percent yield of extracts were maximum from young leaves of parent plants (control), followed by embryos and then calli.

- ✓ In general, TV21 extracts have more phenolic contents followed by TV19 and 317/1. The total phenolic contents were obtained in order of young leaves from parent plants > embryos > calli. Embryo extracts being the best among the androgenic lines contained 47.20 ± 2.5 mg GAE/g dry weight and 43.12 ±2.21 mg GAE/g dry weight of phenolics in hot water extracts of TV21 and TV19, respectively

- ✓ The antioxidant activity was performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) methods. DPPH inhibition pattern was greater in extracts prepared from leaves of parent plant (control) followed by embryos and then calli. IC_{50} values, generated from DPPH inhibition curves, revealed that TV21 showed higher radical scavenging activities than TV19 while 317/1 showed the least. Lower the IC_{50} values, higher is the antioxidant activities of the extracts. The IC_{50} values were in the order of young leaves from parent plant < embryos < calli. Hot water extracts has significantly very high antioxidant activity followed by methanol, ethyl acetate and hexane.

- ✓ Among the standard antioxidants used, epigallocatechin gallate showed excellent radical scavenging activity with IC_{50} value of 2.14±0.24 µg/ml which is 11.41-fold, 18.04-fold and 21.97-fold higher than the antioxidant activity of leaves (control), embryos and calli hot water extracts of TV21, respectively. While, vanillic acid

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showed very low radical scavenging activity with IC_{50} values as 4060 μg/ml which is 166.32-fold, 105.15-fold and 86.38-fold lower than the leaves (control), embryos and calli hot water extracts of TV21.

- ✓ FRAP assays also confirmed that all hot water extracts of TV21 showed higher antioxidant activity than TV19 while the least activity was observed in 317/1 cultivar. The obtained FRAP values of TV21 were 51.61 mg GAE/g for parent plant leaf (control), 35.79 mg GAE/g for embryos and 26.31 mg GAE/g for androgenic calli. The FRAP values in TV19 were 43.96 mg GAE/g for parent plant leaf (control), 26.19 mg GAE/g for embryos and 24.86 mg GAE/g for androgenic calli. The FRAP values in 317/1 were 27.89 mg GAE/g for parent plant leaf (control) and 19.21 mg GAE/g for androgenic calli.

- ✓ The leaves from parent plants (control), Calli and embryos from in vitro raised haploid culturs were analyzed for the production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophyline. HPLC and MS analysis revealed the accumulation of these compounds in all the in vitro raised cultures. Embryogenic cultures possessed highest content of all the compounds compared to that of dedifferentiated cultures (callus). In general, hot water extracts from leaves of field grown parent plant (control) contained highest amount of metabolites compare to that of in vitro androgenic cultures of 317/1, TV19 and TV21 cultivars.

- ✓ In batch kinetics studies, it was concluded that the drop in pH and complete utilization of sucrose from the culture medium, resulted in the onset of stationary phase and it was a major limiting nutrient for cell growth.

- ✓ The production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophyline was observed to be growth associated and showed an increase with the increase in cell biomass. It was revealed that the synthesis of these metabolites is initiated with the log phase of cultures and continues until the stationary phase upto 27th day and afterwards it declined. In general, in cell

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suspension cultures, the production of metabolites was higher than the callus cultures, but it is lesser than that produced in embryo cultures.

FUTURE PROSPECTS

- Diploidization of haploid plants to generate homozygous diploid lines.
- Further purification and characterization of components from extracts of tea and determination of their bioactive potential.
- Statistically optimization of media for enhanced production of metabolites.
- Elicitation and precursor feeding strategies can be carried out to increase the production of metabolites.
- The results of batch kinetics study can serve as a background for further scale-up related aspects in bioreactors.

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APPENDIX

***CAMELLIA SINENSIS* (L.) O. KUNTZE**

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Subclass Dilleniidae
Order Theales
Family Theaceae – Tea family
Genus *Camellia*
Species *sinensis*

***CAMELLIA ASSAMICA* SSP. *ASSAMICA* (MASTERS)**

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Subclass Dilleniidae
Order Theales
Family Theaceae – Tea family
Genus *Camellia*
Species *assamica*
Subspecies *assamica*

***CAMELLIA ASSAMICA* SSP. *LASIOCALYX* (PLANCH MS)**

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Subclass Dilleniidae
Order Theales
Family Theaceae – Tea family
Genus *Camellia*
Species *assamica*
Subspecies *lasiocalyx*

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List of Publications

In National/ International Journals

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Evaluation of chemical and physical parameters for callus induction from anther cultures of tea (*Camellia sinensis* (L.) O. Kuntze). Research J. of Biotechnology, 270-273, **2008.**

- Rakhi Chaturvedi, Rashmi Rekha Hazarika and **Vijay Kumar Mishra**. Assessment of regenerative potentiality of cotyledon explants of some indigenous varieties using varied concentration of cytokinins. Proceeding of 6th International Plant Tissue Culture & Biotechnology Conference, Dec. 3-5, 2010. Bangladesh Association for Plant Tissue Culture & Biotechnology (BAPTC&B), Dhaka, Bangladesh. pp. 27-40, **2010**.

- Rakhi Chaturvedi, **Vijay Kumar Mishra** and Rashmi Rekha Hazarika. Effect of age of donor seedlings and cytokine on organogenesis from *in vitro* cotyledon culture of *Citrullus lanatus* (Thunb.) Mastrum & nakai cv. Sugar Baby. Proceeding of 6th International Plant Tissue Culture & Biotechnology Conference, Dec. 3-5, 2010. Bangladesh Association for Plant Tissue Culture & Biotechnology (BAPTC&B), Dhaka, Bangladesh. pp. 41-51, **2010**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Assessment of He-Ne laser pretreatment of seeds on morphological, physiological and biochemical property of *B. juncea* seedlings. Assam Science Society, 52(1):1-4, **2011**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. In vitro haploid production – Fast forward technique for improved crop production, The Botanica 59-61: 35 – 42, **2012**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Effect of plant growth regulators, carbohydrate concentration and physical parameters on androgenesis of TV21 cultivar of *Camellia assamica* ssp. *assamica* (Masters) (*Under review*).

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. In vitro production of haploid plants via embryogenesis in anther cultures of tea *Camellia assamica* ssp. *lasiocalyx* (Planch MS) (*Under review*).

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Antioxidant potential of androgenic haploid lines of *Camellia assamica* ssp. *assamica* (Masters) and *Camellia assamica* ssp. *lasiocalyx* (Planch MS) determined using ferric-reducing antioxidant power (FRAP) and DPPH free-radical scavenging assays (*Under review*).

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, caffeine and theophylline in anther cultures of *Camellia sinensis* (L.). (*Communicated*).

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- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Production of (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, caffeine and theophylline compounds from cell suspension cultures of androgenic cell line of *Camellia sinensis* var. *assamica* (J. Masters) (*Under preparation*).

Chapters in Book

- Rashmi Rekha Hazarika, **Vijay Kumar Mishra**, Chaturvedi Rakhi. In Vitro Haploid Production - A Fast and Reliable Approach for Crop Improvement. In: Crop improvement under adverse condition, Eds. Tuteja, Narendra; Gill, Sarvajeet Singh. Springer publication, pp171-212, **2013**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Haploid production in tree species. Springer publication (*In Press*)

Abstract in international conference

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Evaluation of chemical and physical parameters for callus induction from anther cultures of tea (*Camellia sinensis* (L.) O. Kuntze). First International Society Biotechnology Conference (ISBT-2008) on Environmental Biotechnology, December 28-30, 2008. Sikkim Manipal Institute of Technology, Gangtok, Sikkim, India. Page No. 270, **2008**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Factors affecting callus induction and proliferation of anther cultures of *camellia Camellia sinensis* (L.) O. Kuntze. International conference on Biotechnological Solutions for Environmental Sustainability. October 21-23, 2009. At VIT University, vellore, Chnnai, Tamil Nadu, India. Page No. 251, **2009**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Effect of physical and chemical factors for induction of callus and proliferation through in vitro androgenesis in *Camellia sin*ensis (L.) O. Kuntze. International Conference on Frontiers in Biological Sciences (InCoFIBS- 2010). October 01-03, 2010. At National Institute of Technology, Rourkela, Orissa, India. Page No. 221, **2010**.

- Rakhi Chaturvedi, Rashmi Rekha Hazarika and **Vijay Kumar Mishra**. Assessment of regenerative potentiality of cotyledon explants of some indigenous varieties using varied concentration of cytokinins. Proceeding of 6th International Plant Tissue Culture & Biotechnology Conference, Dec. 3-5, 2010. Bangladesh Association for Plant Tissue Culture & Biotechnology (BAPTC&B), Dhaka, Bangladesh. Page No. 92, **2010**.

- Rakhi Chaturvedi, **Vijay Kumar Mishra** and Rashmi Rekha Hazarika.comparative study of TDZ and BAP on organogenesis from *in vitro* cotyledon culture of *Citrullus lanatus* (Thunb.) Mastrum & Nakai cv. Sugar Baby. Proceeding of 6th International Plant Tissue Culture & Biotechnology Conference, Dec. 3-5, 2010. Bangladesh

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- **Vijay Kumar Mishra** and Rakhi Chaturvedi. In vitro haploid production via anther culture of TV21 cultivar of *Camellia sinensis* (L.) O. Kuntze var. *assamica*. International Conference on New Horizons in Biotechnology (NHBT-2011) November 21-24, 2011; Trivandrum, Kerala, India. Page No. 221, **2011**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Identification and quantification of (+)-Catechin, (-)-Epicatechin and (-)-Epigallocatechin gallate in anther cultures of TV21 cultivar of *Camellia sinensis* (L.) O. Kuntze var. *assamica.* 18th international conference 2012 (post iscbc) on Perspective and Challenges in Chemical and Biological Sciences, 28th - 30th january, 2012, jointly organized by institute of advanced study in science and technology (IASST), guwahati, assam, india and indian society of chemists and biologists (iscb), lucknow, up, india. Page No. 319, **2012**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Determination of antioxidant activity and total phenolic contents in in vitro androgenic cultures of *camellia scinensis* (l.) o. kuntze. 12th congress of International Society for Ethnopharmacology [ISE] to be held at Science City, Kolkata,India during February 17-19, 2012. Page No. 56, **2012**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. In vitro androgenesis in anther cultures of TV21 cultivar of tea. 90th Anniversary Meeting 2012, by **The Society for Biotechnology, Japan (SBJ)**, at Kobe International Conference Center **Kobe, Japan,** during October 23 – 26, Page No 150, **2012**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. An Efficient Protocol for Androgenic Haploid Production in TV21 Cultivar of Tea (*Camellia assamica* ssp. *assamica* (Masters)). 2013 In Vitro Biology Meeting by Society For In Vitro Biology, at the Rhode Island Conference Center and The Omni Providence, **Providence, Rhode Island, USA** June15-19, **2013**, (*Accepted*).

National conference

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Callus proliferation from anther cultures of *Camellia sinensis* (L.) O. Kuntze. National Seminar on Exploration, Utilization and Strategy Action Plan for Sustainable Management of Plant Resources, February 27-28, 2009. Department of Botany, Gauhati University, Guwahati, Assam, India. Page No.33, **2009**.

- **Vijay Kumar Mishra** and Rakhi Chaturvedi. Assessment of He-Ne laser pretreatment of seeds on morphological, physiological and biochemical property of *Brassica juncea* seedlings, 55th Annual Technical Session of Assam Science Society, 15th February, 2010. Gauhati University, Guwahati, Assam, India. Page No.45, 2010.

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Regional seminar

- Attend regional seminar on “Intellectual Property and Innovation Management in Knowledge Era”, organized by National Research Development Corporation at Indian Institute of Technology Guwahati on 5th - 6th May **2010.**

Workshop

- Department of Biotechnology (DBT), Government of India sponsored training course on “Biotechnological tools & techniques for plant biodiversity and conservation study”, at North-East Institute of Science & Technology (NEIST) 19th Jan-31st Jan., **2009**.

TH-1176_07610612

FIGURE 1

PARENT PLANTS

Experimental plants growing at TRA, Tocklai, Jorhat

A- A 40-year-old TV21 cultivar of Tea bearing young flower buds

B- A 35-year-old TV19 cultivar of Tea bearing young flower buds.

C- A 35-year-old 317/1 cultivar of Tea bearing young flower buds.

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FIGURE 2

ANTHER CULTURE

Establishment of in vitro anther cultures of tea

A- Flowering twigs of tea bearing young flower buds (Bar = 5 cm)

B- Young flower buds of 4 mm size used for the study (Bar = 6 mm).

D- A cultured anther bearing early-late-uninucleate stage of microspores (Bar = 175 µm)

D, E, F- Uninucleate stage of microspores (Bar = 10 µm)

G, H- Tetrad stage of microspores (Bar = 10 µm)

I- Petri-dish having cultured 20 number of anther (Bar = 1.3 cm)

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FIGURE 3

ANTHER CULTURE

Callus induction

A- One-week-old culture showing enlargement of anther (Bar = 167 µm).

B- Same as figure **A**, after 2 weeks of culture initiation (Bar = 260 µm).

C- 6-week-old culture, showing longitudinal rupturing of anther walls along the entire length of the anther sacs (Bar = 382 µm).

D- 7-week-old culture of anthers where anther sacs burst open and small white calli can be seen inside (Bar = 382 µm).

E- Same as **D**, after 8 weeks, where anthers sacs open widely, releasing shiny, white, transparent callus from within the anther locules along with minute callusing along the edges of the wall (Bar = 0.7 mm).

F-I- 10-week-old culture showing profusely growing white, transparent callus from within the anther locules along with light brown granulated callus from the peripheral anther walls (shown by arrow) (Bar = 0.5 mm).

J- 12-week-old culture where entire anthers were covered with cream and brown growing calli (Bar = 0.8 mm).

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FIGURE 4

ANTHER CULTURE

Callus multiplication

A to C- 6-week-old callus culture, subcultured in the induction medium and maintained in dark. The calli grew well as white, hard callus after second subculture (Bar = 0.4 mm).

D- 6-week-old callus subculture in induction medium. The calli were shifted to diffused light. The green area developed in the white, hard and compact callus (Bar = 0.3 mm).

E- 4 week-old callus culture of TV21 on multiplication medium, MS + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l), where the calli multiply further and turned green, hard and compact. Some of the cells develop red color pigmentation (Bar = 0.23 mm).

F- Same as E, after 8 weeks. The calli multiply further and develop into light green callus with distinct bright green loci (arrow head) (Bar = 0.4 mm)

G, H- 8-week-old callus culture of TV19 on multiplication medium, NAA (5 µM) + BAP (10 µM). The calli turned hard, compact, bright green and nodulated (Bar = 0.4 mm). The nodules are very distinct in Figure **H**.

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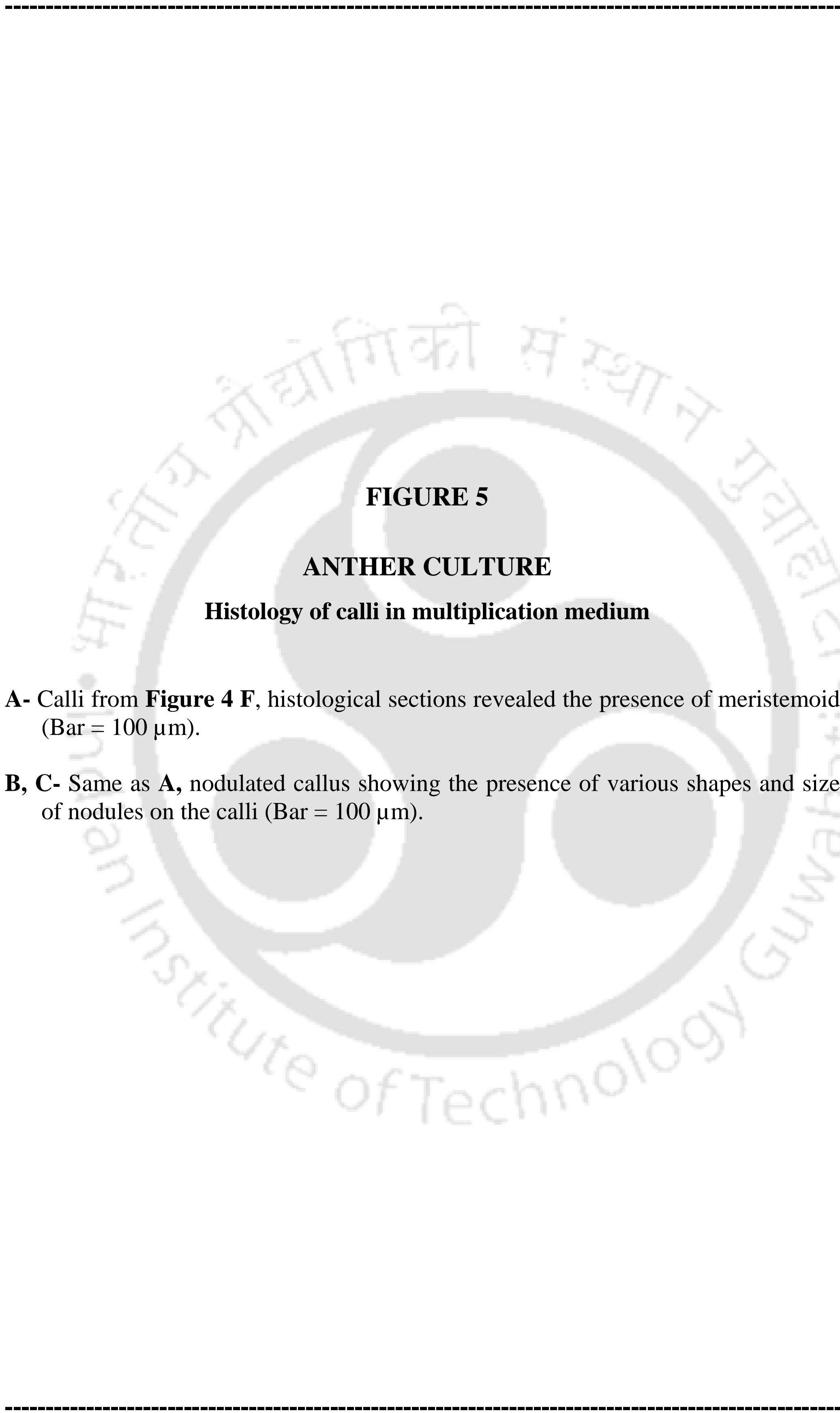

FIGURE 5

ANTHER CULTURE

Histology of calli in multiplication medium

A- Calli from **Figure 4 F**, histological sections revealed the presence of meristemoids (Bar = 100 µm).

B, C- Same as **A,** nodulated callus showing the presence of various shapes and sizes of nodules on the calli (Bar = 100 µm).

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FIGURE 6

ANTHER CULTURE

Embryogenesis in callus cultures

(G- Globular shape, H- Heart shape, T- Torpedo shape, C- Dicotyledonous stage)

A&B- Embryogenesis on BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) medium, in TV19 cultivar (Bar = 0.3 mm).

C&D- Embryogenesis on BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) medium, in TV21 cultivar (Bar = 0.2 mm).

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FIGURE 7

ANTHER CULTURE

Histology of in vitro embryos

A- The regenerating callus of TV19, showing the presence of shoot-tip (arrow marked) with two leaf-primordia (L) (Bar = 120 µm).

B- The globular embryo, developed from calli on MS (3% Sucrose) + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) (Bar = 100 µm).

C- Same as **B,** the heart-shape of embryo (**H**) and nodule (**N**) developed from peripheral tissue of calli (Bar = 100 µm).

D- Same a **B**, early torpedo shaped embryo showing the vascular strand (>) (Bar = 100 µm).

E- Same as **D**, an advanced stage of torpedo embryo with distinct notch (<) at plumlar end (Bar = 100 µm).

F- Same as figure **B**, an early dicot stage embryo. Clear notch is seen at plumular end with distinct closed vascular strand (Bar = 120 µm).

G- Same as figure **F**, late dicot stage, showing two cotyledons (C) and well developed closed vascular strand (< marked) (Bar = 120 µm).

H, I- Mature embryos showing distinct vascular strands, properly connected from radicular end to the plumular end (Bar = 100 µm).

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FIGURE 8

ANTHER CULTURE

Scanning Electron Micrograph (SEM) images of embryogenic cultures

A- A callus culture of TV19 cultivar on the multiplication medium, MS + NAA (5 µM) + BAP (10 µM), showing well nodules of various shapes and sizes (Bar = 100 µm).

B, C- Callus cultures of TV21 cultivar on multiplication medium, MS + 2,4-D (5 µM) + Kn (5 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l), showing nodules of various sizes (Figure B, Bar = 100 µm, Figure C, Bar = 2 µm).

D- Globular embryo of TV19, developed on regeneration medium, MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) (Bar = 20 µm).

E- Same as **D**, showing globular and heart-shaped (arrow head) embryos of TV21 cultivar (Bar = 20 µm).

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FIGURE 9

ANTHER CULTURE

Embryo development and maturation

A- 6-week-old culture, showing development of globular embryo (G) on MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) (Bar = 0.1 mm).

B- Same as **A,** showing heart shape of embryo (H) (Bar = 0.1 mm)

C- Same as **A,** showing early torpedo shaped embryo (T) (Bar = 0.15 mm)

D- Same as **A,** showing late torpedo shaped embryo (T) (Bar = 0.1 mm)

E- Same as **A,** showing dicotyledonous stage of embryo (Bar = 0.15 mm)

F- Same as **E**, 8-week-old matured dicot embryo on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), with distinct radicular to plumular regions (Bar = 0.8 mm).

G- Seed-like embryos (S) (Bar = 0.1 cm) on MS + BAP (10 µM) + GA_3 (3 µM) + L-Glutamine (800 mg/l) + L-Serine (200 mg/l) (Bar = 0.1 mm).

H- Same as **G**, on MS + BAP (1.µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing two enlarged cotyledons (C) and well developed root system (R) (Bar = 0.1 mm).

I- Same as **H**, after 8 weeks of culture on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing germinated embryo with distinct shoot at plumular region (Bar = 0.15 mm).

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FIGURE 10

ANTHER CULTURE

Monopolar embryo germination

A- 5-week-old culture of embryo on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing monoplar germination of embryo by giving raise to roots at radicular end (Bar = 0.3 mm).

B, C- 5-week-old culture of embryo on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing monopolar germination of embryo by giving rise to multiple shoots at plumular region (Bar = 0.7 mm)

D- Same as **B**, single shoot developed at the plumular region (Bar = 2.5 mm)

E- Same as **B**, multiple shoots developed at the plumular region during monopolar germination of embryo (Bar = 2.5 mm).

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FIGURE 11

ANTHER CULTURE

Abnormal embryos development

A- Embryos on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing sequential development with well defined radicular end but with abnormally thick plumular end (Bar = 0.3 cm).

B- An abnormal embryo with normal radicular end but with no cotyledon at the plumular region. Note the presence of cream callus at the plumular region (Bar = 0.1 cm).

C. An abnormal embryo with well developed radicular end and with completely closed, green, rounded, fused cotyledons (Bar = 0.2 cm).

D. An abnormal embryo with multiple, green colour, fused cotyledons forming cup-shaped structure (Bar = 0.1 cm).

E- A flower shape abnormal embryo with multiple fused green cotyledons (Bar = 3.5 cm).

F- Secondary embryogenesis at plumular region of germinating embryos. Note the presence of distinct radicular end (Bar = 0.7 mm).

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FIGURE 12

ANTHER CULTURE

Embryo germination and plantlet development in TV19 cultivar

A- 8-week-old fully matured embryo of TV19 cultivar on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing bipolar structure with long radicular end and small green plumular end (Bar = 0.5 mm).

B, C- 10-week-old germinated embryos on ½ MS (major salts reduced to half strength) + BAP (10 µM) + GA_3 (0.5 µM) + IBA (1 µM) L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing distinct roots and shoots (Bar = 0.5 mm).

D- Same as **B,** conversion of embryo into fully developed plantlet. Note the development of additional shoot at plumular region (Bar = 0.3 mm).

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FIGURE 13

ANTHER CULTURE

Embryo germination and plantlet development in TV21 cultivar

A to F- Fully matured embryos of TV21 cultivar on MS + BAP (1 µM) + GA_3 (0.3 µM) + L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing bipolar structures with long radicular end and small green plumular end (Bar = 6.0 mm).

G, H- 10-week-old germinated embryos on ½ MS (major salts reduced to half strength) + BAP (10 µM) + GA_3 (0.5 µM) + IBA (1 µM) L-Glutamine (80 mg/l) + L-Serine (20 mg/l), showing distinct roots and shoots) (Bar = 0.3 mm).

I, J- Same as G, conversion of embryos into fully developed plantlets. Note the development of additional shoot at plumular region (Bar = 0.8 mm).

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FIGURE 14

ANTHER CULTURE

Ploidy analysis of androgenic cultures of TV19 cultivar

A- Squash preparation of shoot-tip from field grown parent plant of TV19 cultivar showing diploid number of chromosomes as 2n=2x=30 (Bar = 5 µm).

B- Ploidy analysis by squash preparation from root-tip of in vitro regenerated plantlet of TV19 cultivar showing haploid number of chromosome as 2n=x=15 (Bar = 5 µm).

C- Flow cytometry histogram from leaves of field grown parent plants of TV19 cultivar (control). Note single channel peak at position 200.

D- Flow cytometry histogram from leaves of in vitro regenerated plants of TV19 cultivar.

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FIGURE 15

ANTHER CULTURE

Ploidy analysis of androgenic cultures of TV21 cultivar

A- Squash preparation of shoot-tip from field grown parent plant of TV21 cultivar showing diploid number of chromosomes as 2n=2x=30 (Bar = 5 µm).

B- Ploidy analysis by squash preparation from root-tip of in vitro regenerated plantlet of TV21 cultivar showing haploid number of chromosome as 2n=x=15 (Bar = 5 µm).

C- Ploidy analysis by squash preparation from root-tip of in vitro regenerated aneuploid plantlet of TV21 cultivar showing aneuploid number of chromosome as 2n=2x-4=11 (Bar = 5 µm).

D- Flow cytometry histogram from leaves of field grown parent plants of TV19 cultivar (control). Note single channel peak at position 200.

E- Flow cytometry histogram from leaves of in vitro regenerated plants of TV19 cultivar.

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FIGURE 16

ANALYSIS OF SECONDARY METABOLITES

High Performance Liquid Chromatography of standards

A- A chromatogram of (+)-catechin (C), eluted at retention time of 12.29±0.31 min.

B- A chromatogram of (-)-epicatechin (EC), eluted at retention time of 13.9 ± 0.32 min.

C- A chromatogram of (-)-Epigallocatechin gallate (EGCG), eluted at retention time of 20.9 ± 0.36 min.

D- A chromatogram of caffeine (CAF), eluted at retention time of 8.3±0.31 min.

E- A chromatogram of theophylline (T), eluted at retention time of 6.19±0.35 min.

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FIGURE 17

ANALYSIS OF SECONDARY METABOLITS

High Performance Liquid Chromatography of TV19 extracts

A chromatogram of hot water extracts

- **A-** from leaves of field grown parent plant showing elution of the four compounds CAF, C, EC and EGCG.
- **B-** from leaves of field grown parent plant showing elution of theophylline.
- **C-** from haploid calli showing presence of CAF, C, EC and EGCG
- **D-** from haploid calli showing presence of theophylline.

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FIGURE 17 (continue)

ANALYSIS OF SECONDARY METABOLITS

High Performance Liquid Chromatography of TV19 extracts

A chromatogram of hot water extracts

E- from haploid embryos showing presence of CAF, C, EC and EGCG.

F- from haploid embryos showing presence of theophylline.

G- spiked with the standards of CAF, C, EC and EGCG. The samples and standards were co-eluted at their respective retention times

H- spiked with the standard of theophylline. The samples and standards were co-eluted at the same retention time.

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FIGURE 18

ANALYSIS OF SECONDARY METABOLITS

High Performance Liquid Chromatography of TV21 extracts

A chromatogram of hot water extracts

A- from leaves of field grown parent plant showing elution of the four compounds CAF, C, EC and EGCG.

B- from leaves of field grown parent plant showing elution of theophylline.

C- from haploid calli showing presence of CAF, C, EC and EGCG

D- from haploid calli showing presence of theophylline.

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FIGURE 18 (continue)

ANALYSIS OF SECONDARY METABOLITS

High Performance Liquid Chromatography of TV21 extracts

A chromatogram of hot water extracts

E- from haploid embryos showing presence of CAF, C, EC and EGCG.

F- from haploid embryos showing presence of theophylline.

G- spiked with the standards of CAF, C, EC and EGCG. The samples and standards were co-eluted at their respective retention times.

H- spiked with the standard of theophylline. The samples and standards were co-eluted at the same retention time.

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FIGURE 19

ANALYSIS OF SECONDARY METABOLITS

High Performance Liquid Chromatography of 317/1 extracts

A chromatogram of hot water extracts

A- from leaves of field grown parent plant showing elution of the four compounds CAF, C, EC and EGCG.

B- from leaves of field grown parent plant showing elution of theophylline.

C- from haploid calli showing presence of CAF, C, EC and EGCG

D- from haploid calli showing presence of theophylline.

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FIGURE 19 (continue)

ANALYSIS OF SECONDARY METABOLITS

High Performance Liquid Chromatography of 317/1 extracts

A chromatogram of hot water extracts

E- spiked with the standards of CAF, C, EC and EGCG. The samples and standards were co-eluted at their respective retention times

F- spiked with the standard of theophylline. The samples and standards were co-eluted at the same retention time.

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FIGURE 20

ANALYSIS OF SECONDARY METABOLITS

Mass spectroscopy

Comparative positive mode electrospray ionization (+ESI) mass spectra of standard compounds and the purified samples

A- HPLC eluted caffeine standard.

B- HPLC eluted crude hot water extract.

C- HPLC eluted (+)-catechin standard.

D- HPLC eluted crude hot water extract.

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FIGURE 21

ANALYSIS OF SECONDARY METABOLITS

Mass spectroscopy

Comparative positive mode electrospray ionization (+ESI) mass spectra of standard compounds and the purified samples

A- HPLC eluted (+)-epicatechin standard.

B- HPLC eluted crude hot water extract.

C- HPLC eluted (+)-epigallocatechin gallate standard.

D- HPLC eluted crude hot water extract.

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FIGURE 22

ANALYSIS OF SECONDARY METABOLITS

Mass spectroscopy

Comparative positive mode electrospray ionization (+ESI) mass spectra of standard compounds and the purified samples

A- HPLC eluted Theophyline standard.

B- HPLC eluted crude hot water extract.

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FIGURE 23

SHAKE FLASK CULTURE

Effect of agitation speed on viability of cells

(Cells stained with 1 % fluorescein diacetate solution)

A, B- Cellular clump at 90 rpm, showing aggregate with loosely attached cells (bar = 20µm).

C-F- The cultures maintained at 120 rpm in the cell suspension, showing individual, live, healthy, fluorescent green stained cells (for C-E bar = 20µm; for F bar = 100µm).

G- The culture maintained at 150 rpm, showing dead (dark bodies) and sheared cells (bar = 20µm).

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Figure 1

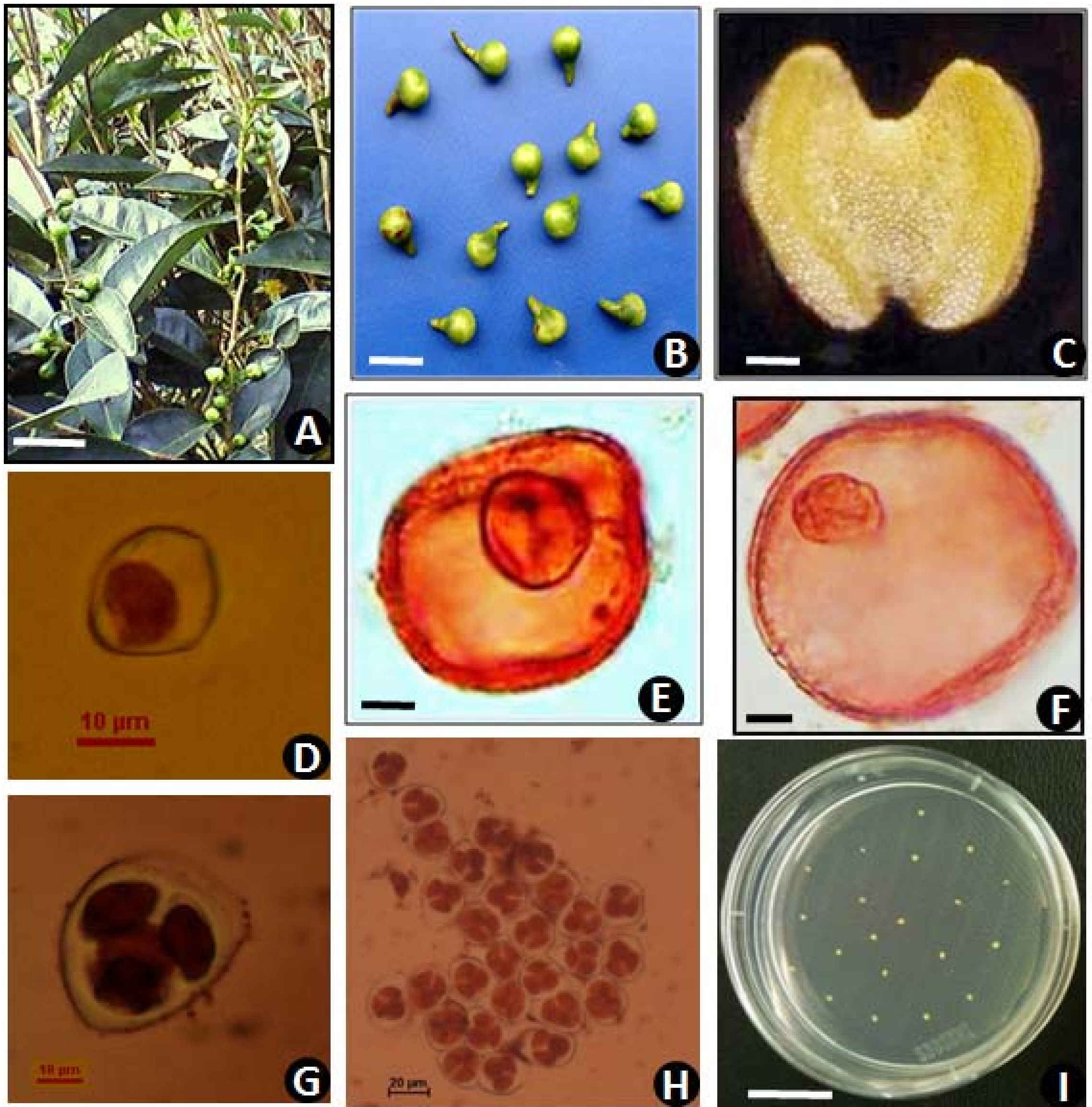

Figure 2

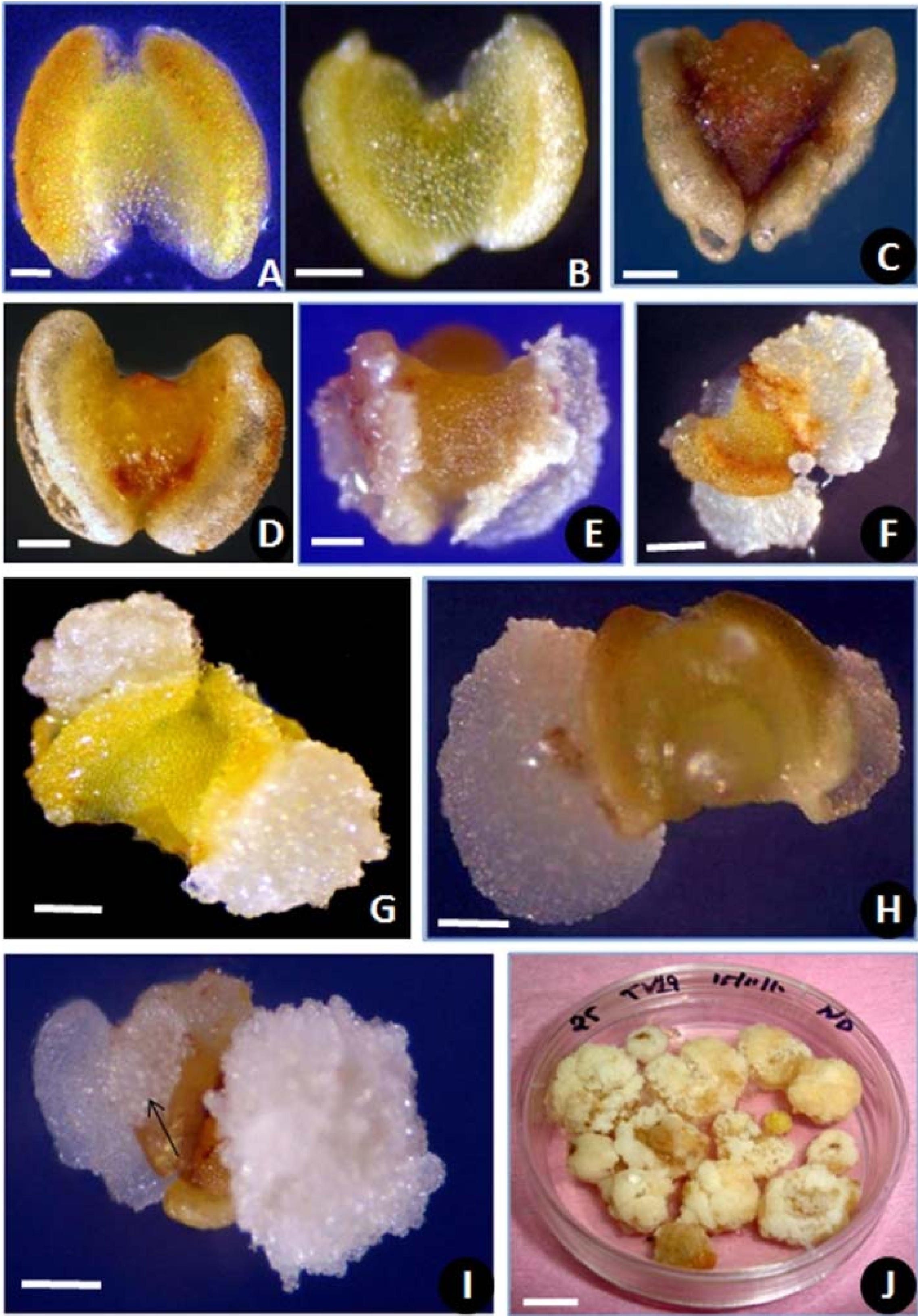

Figure 3

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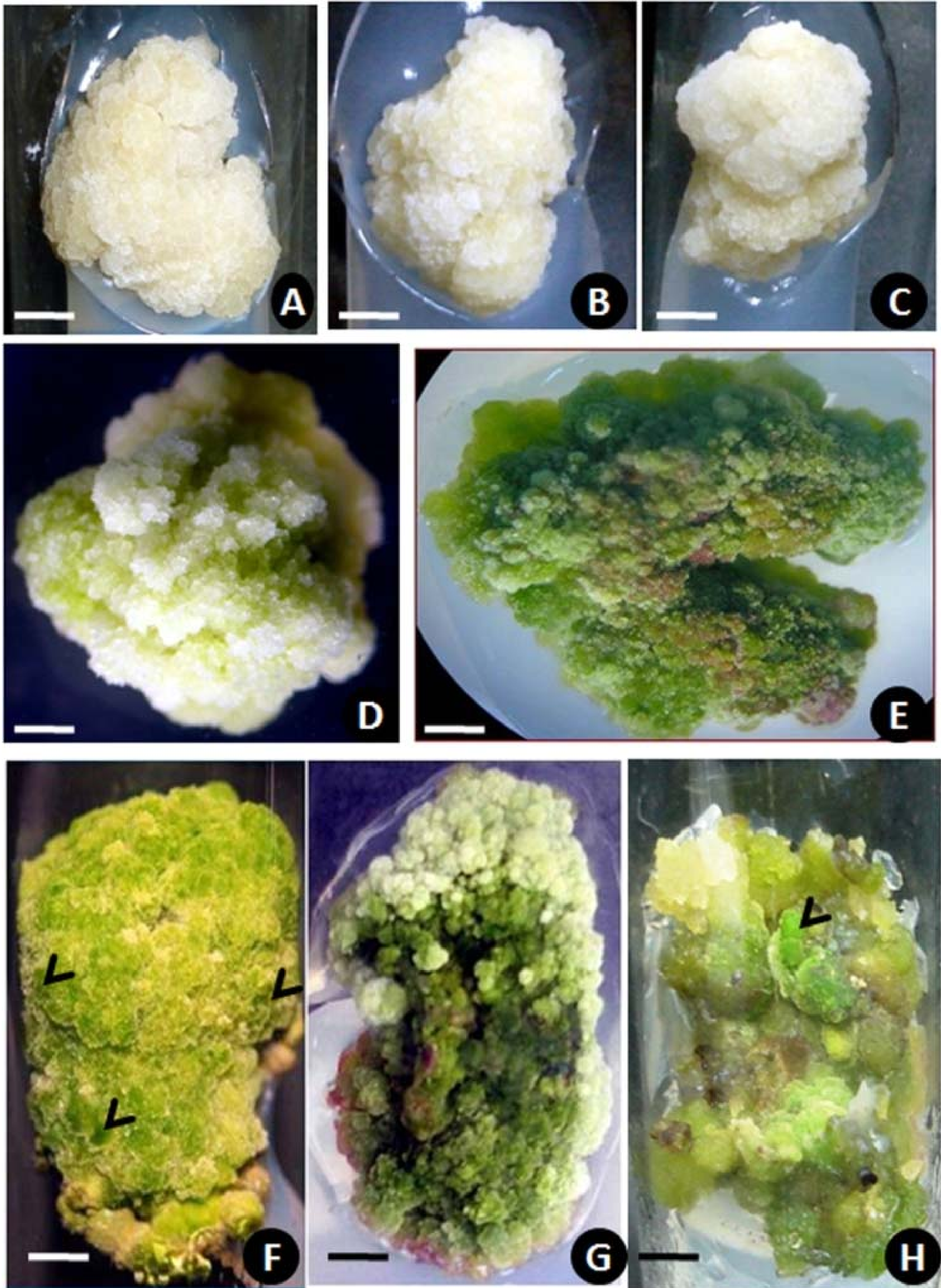

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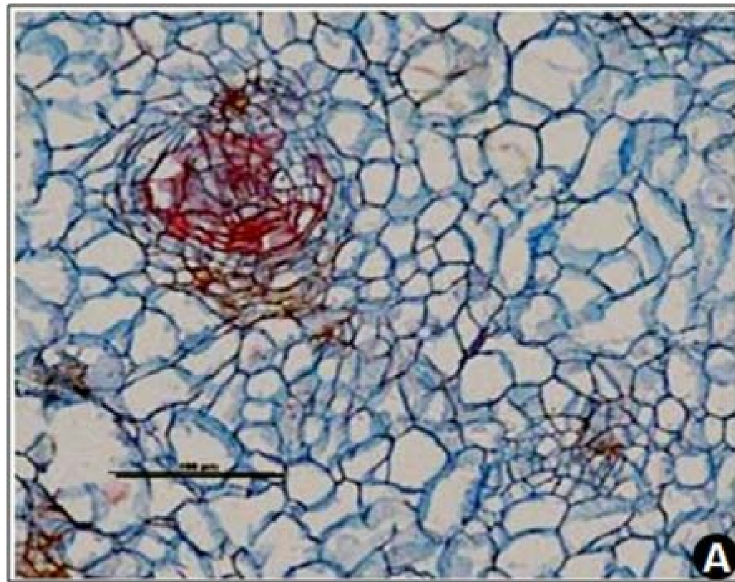

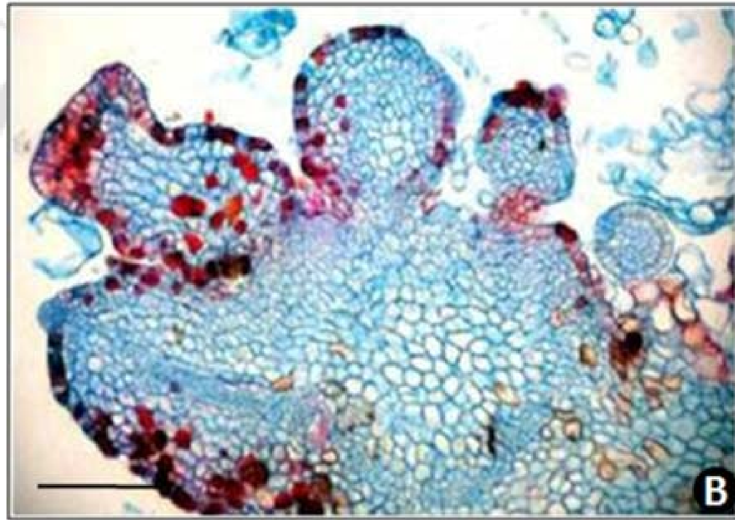

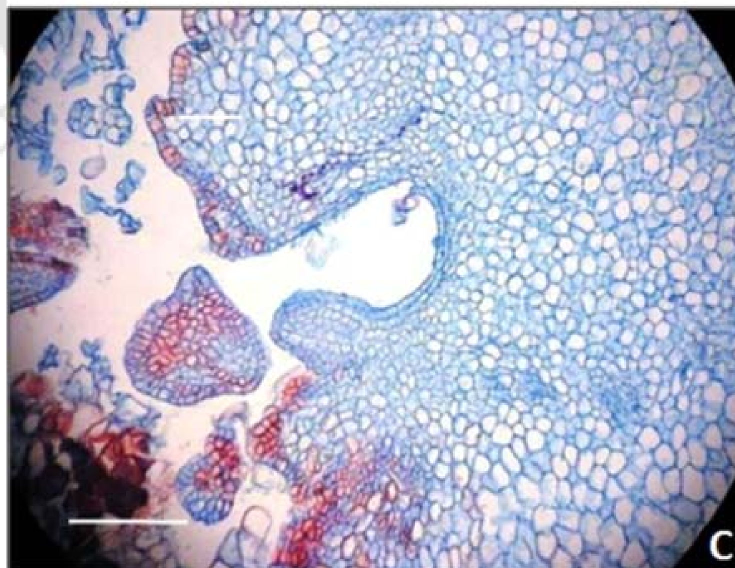

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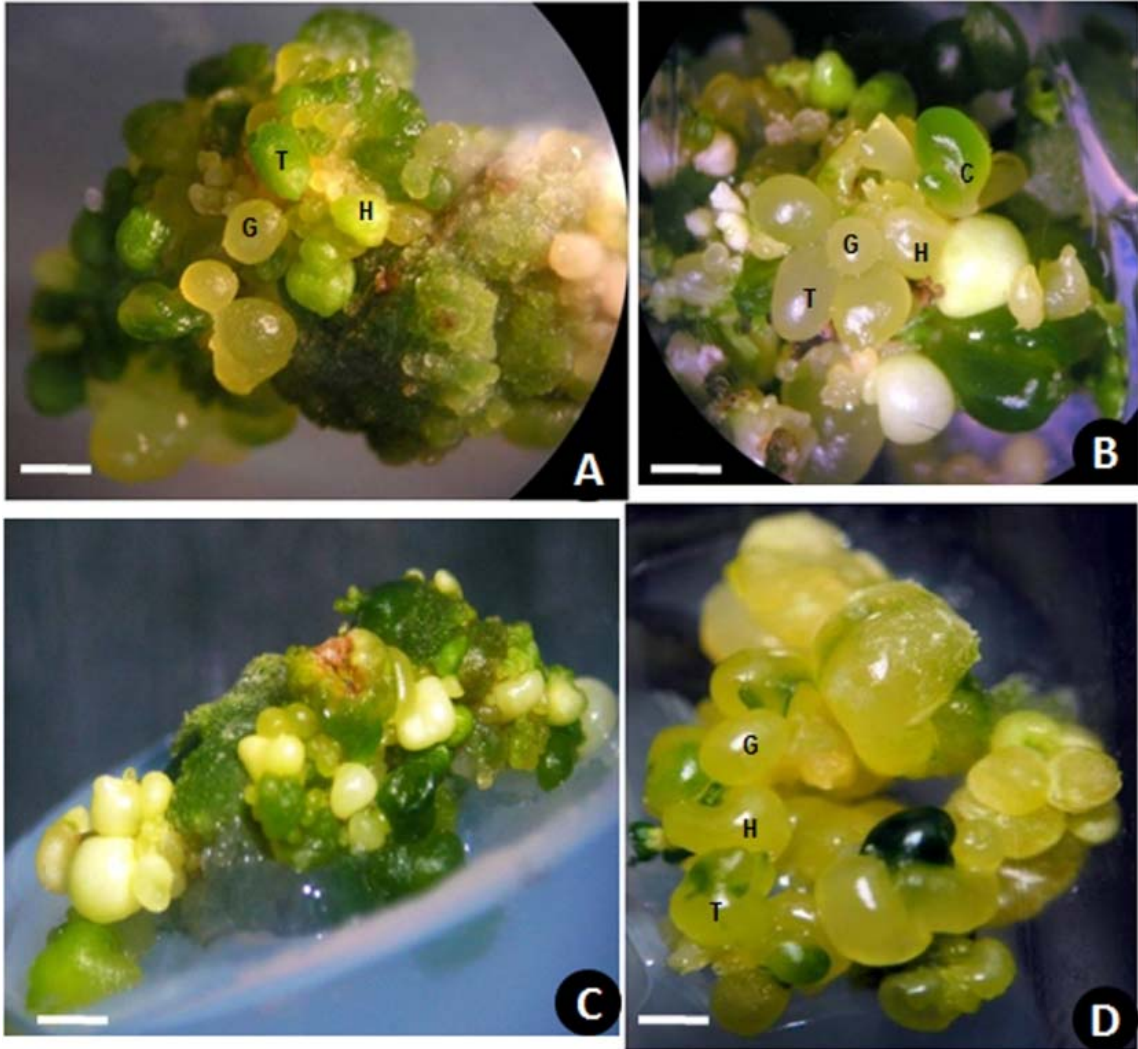

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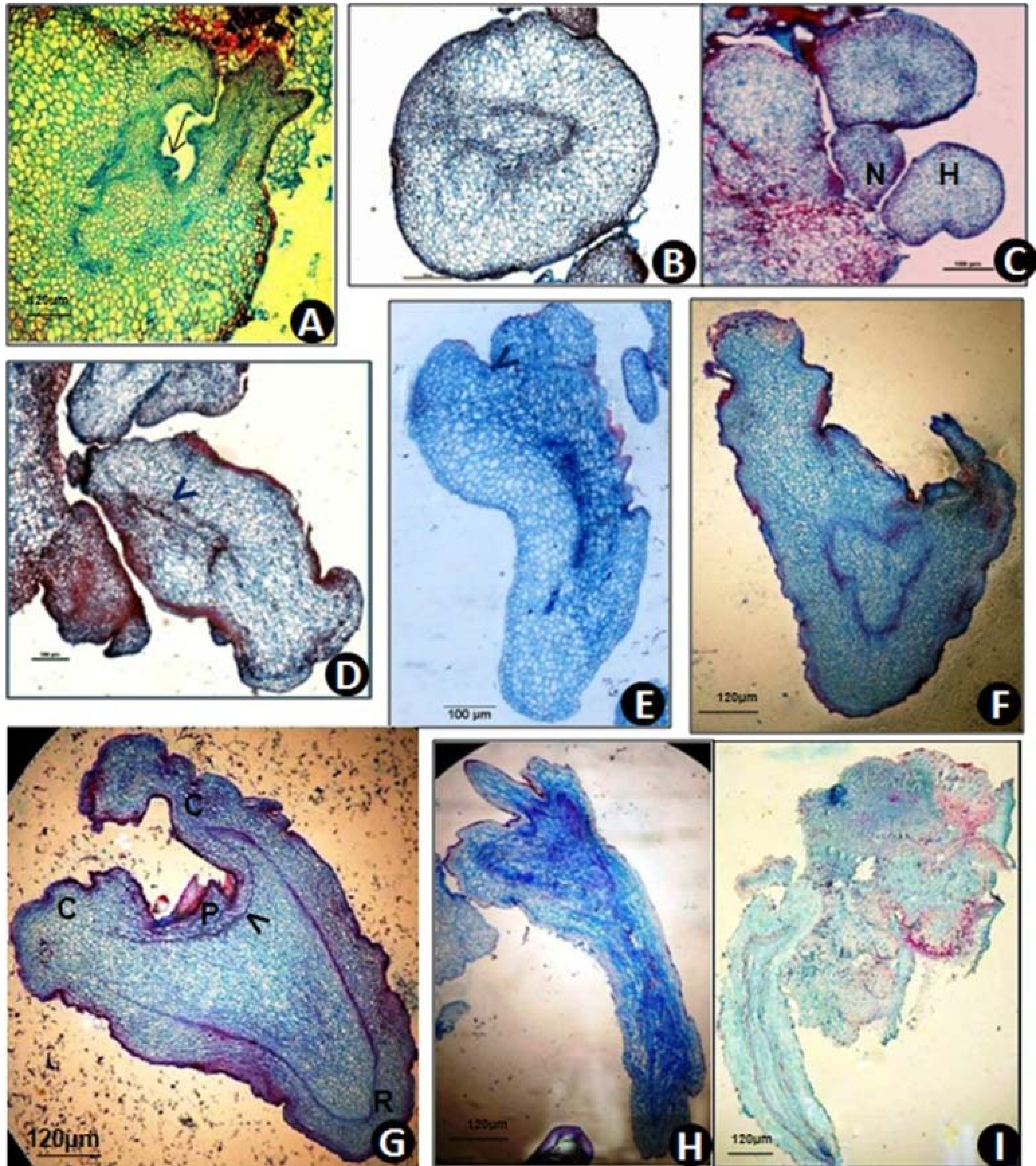

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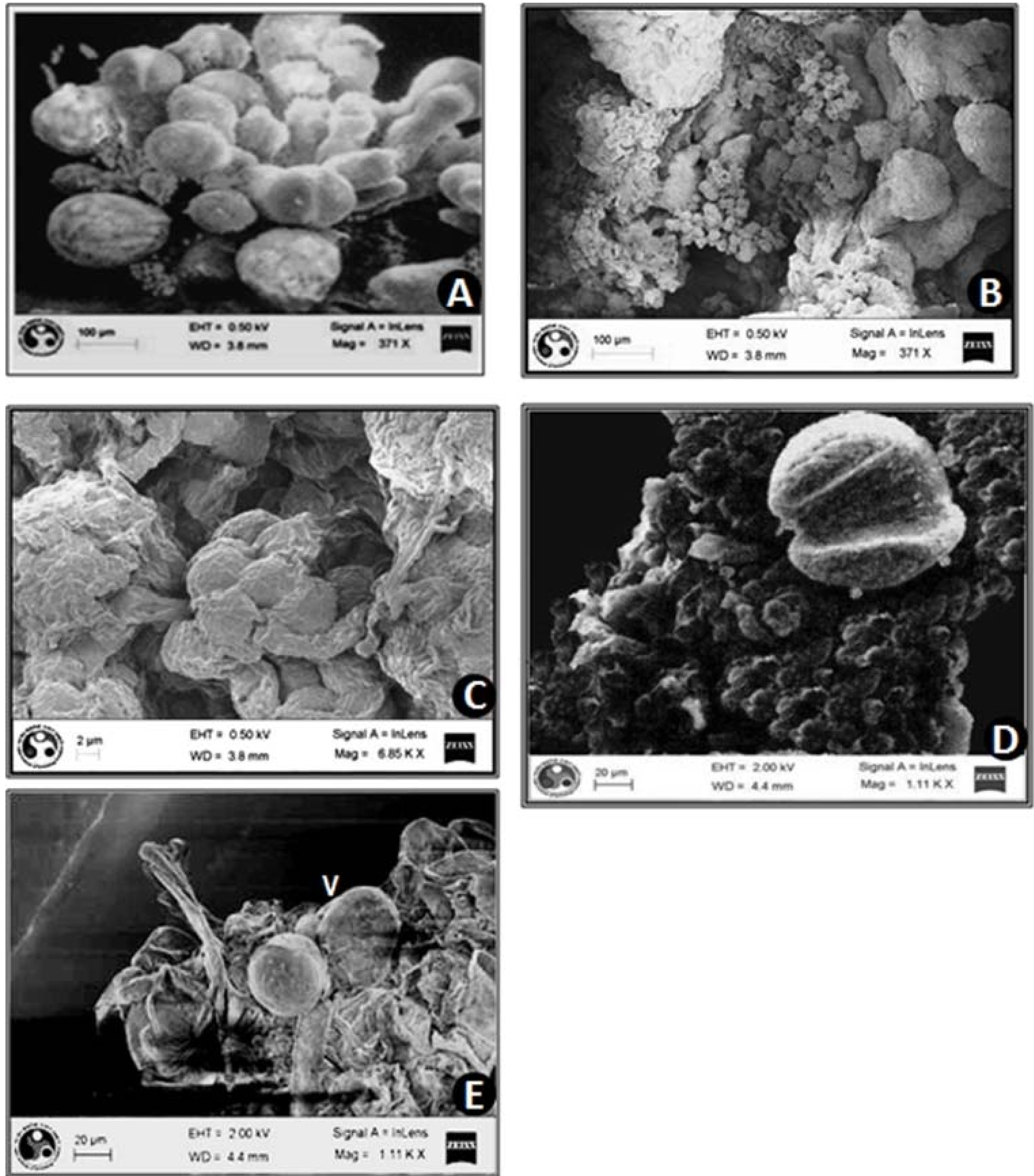

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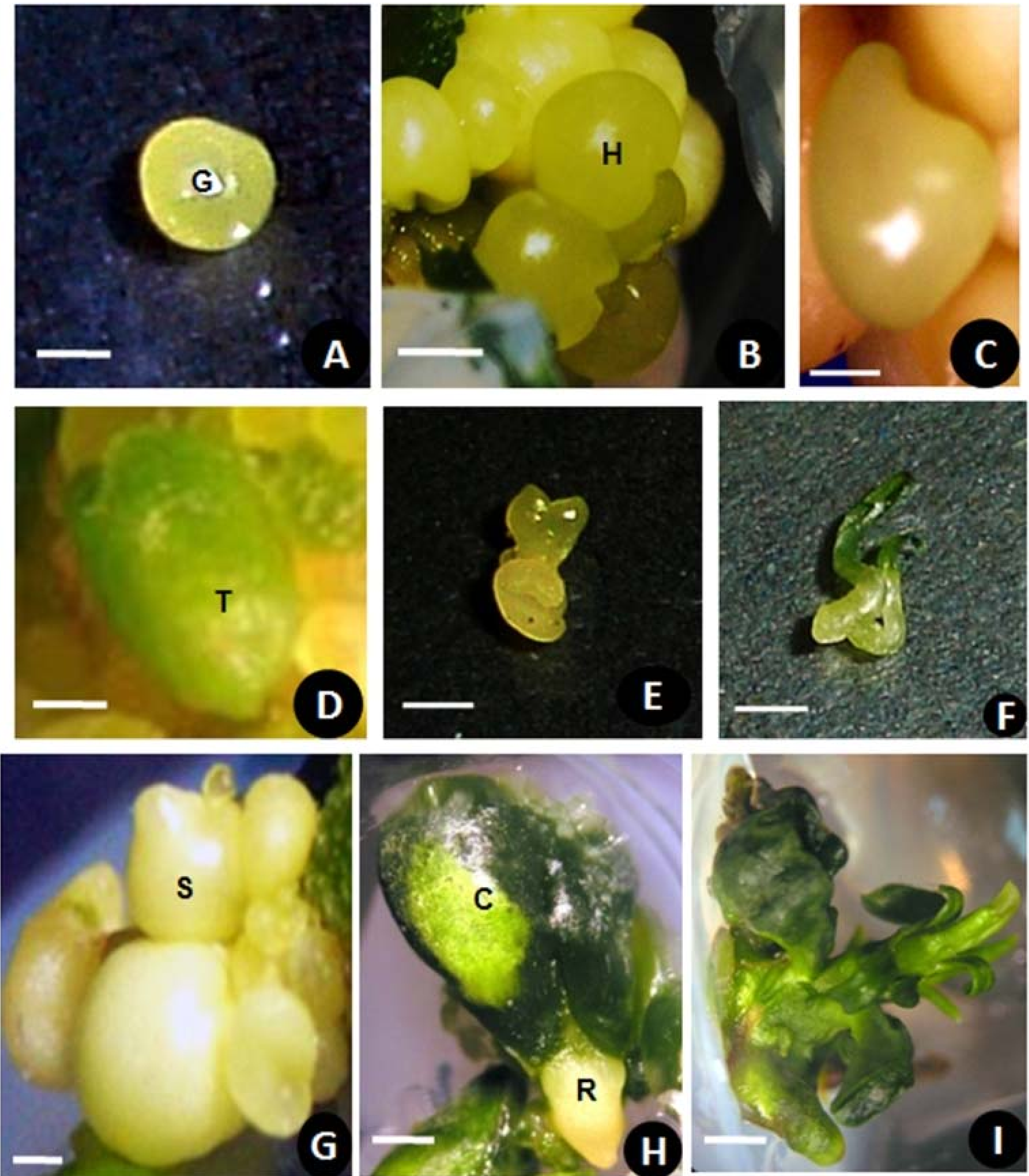

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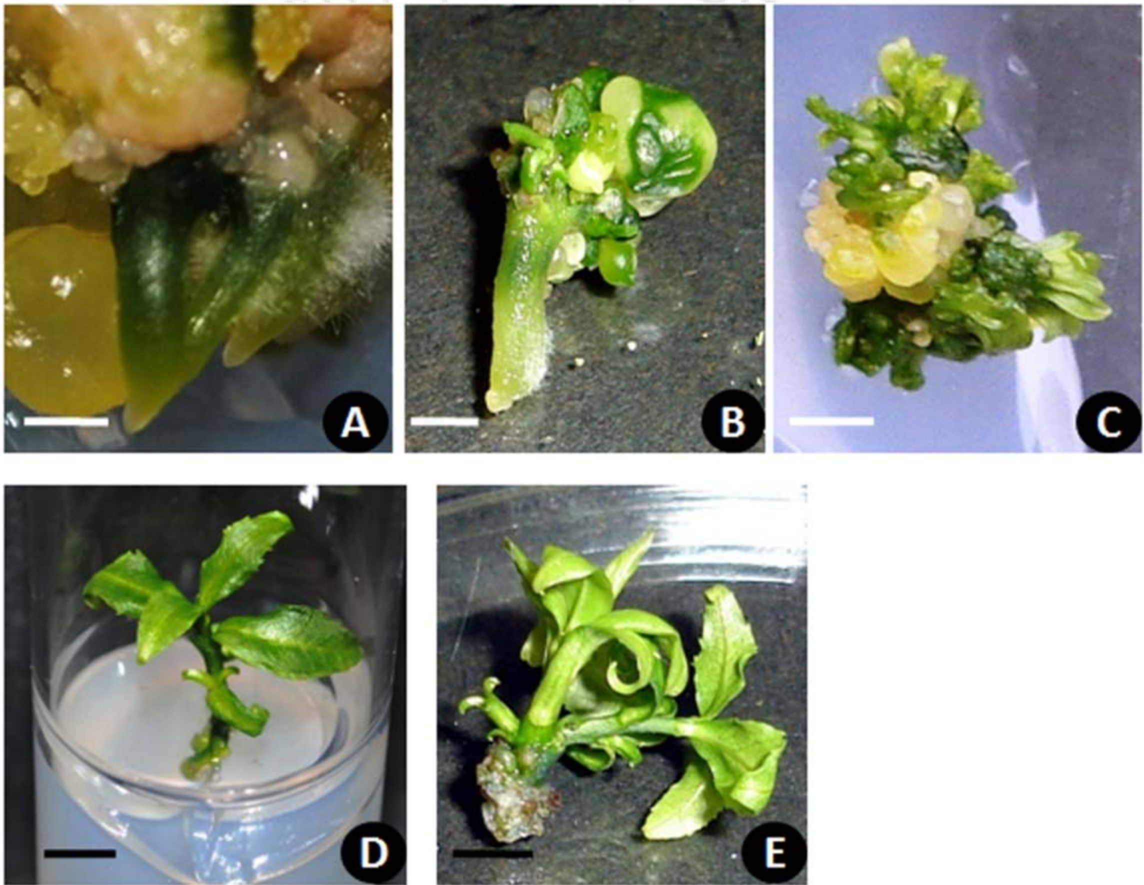

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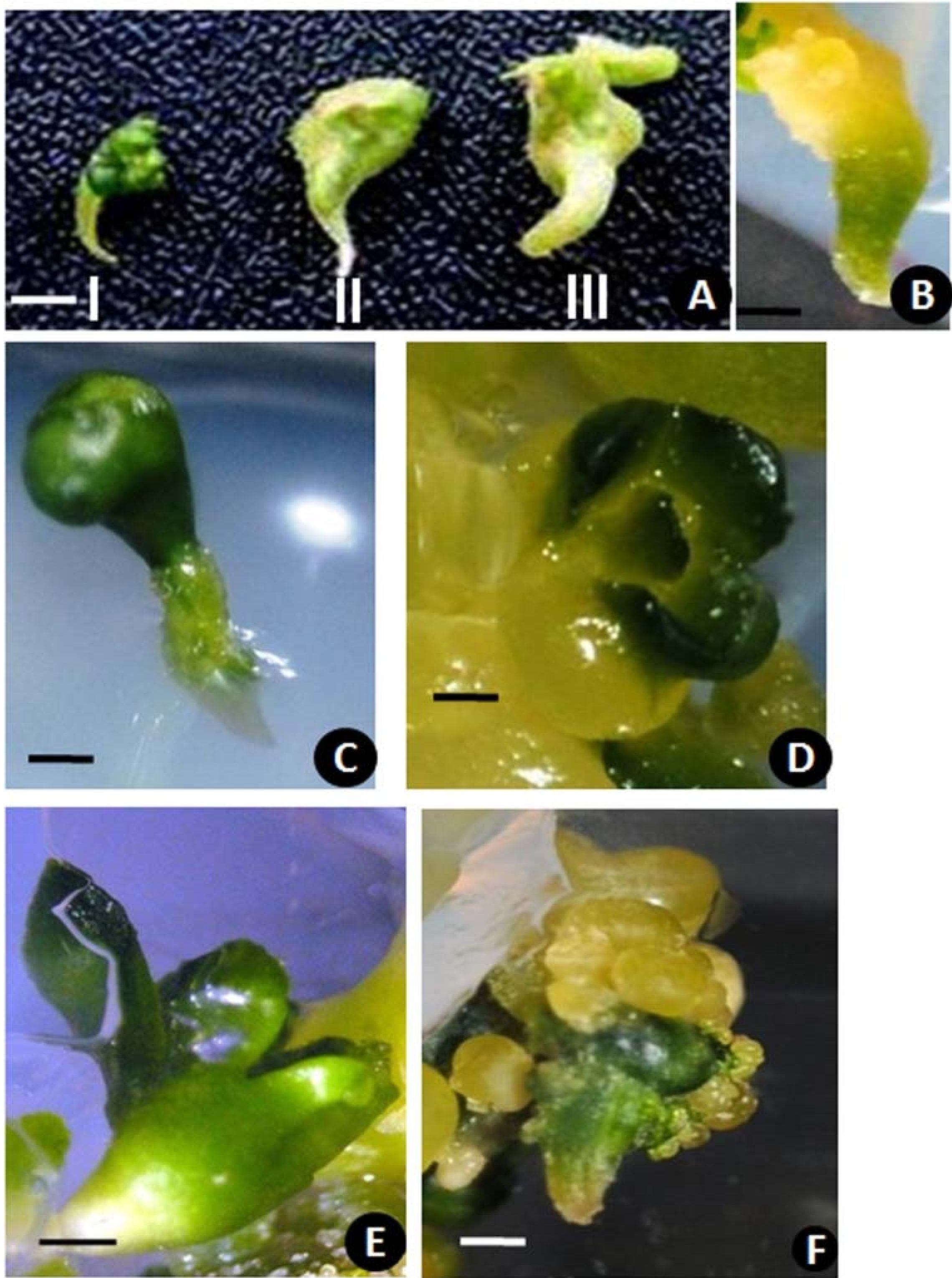

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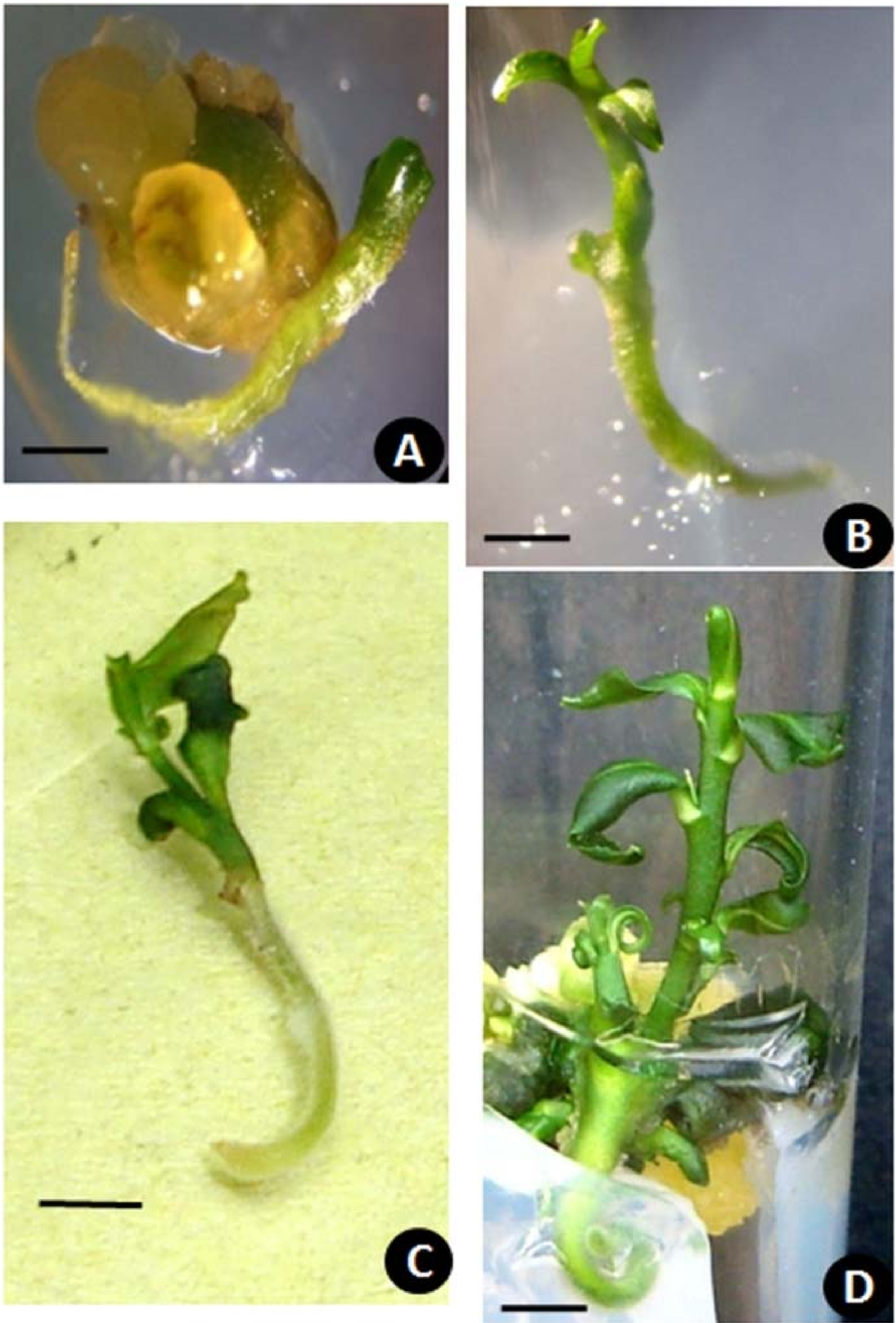

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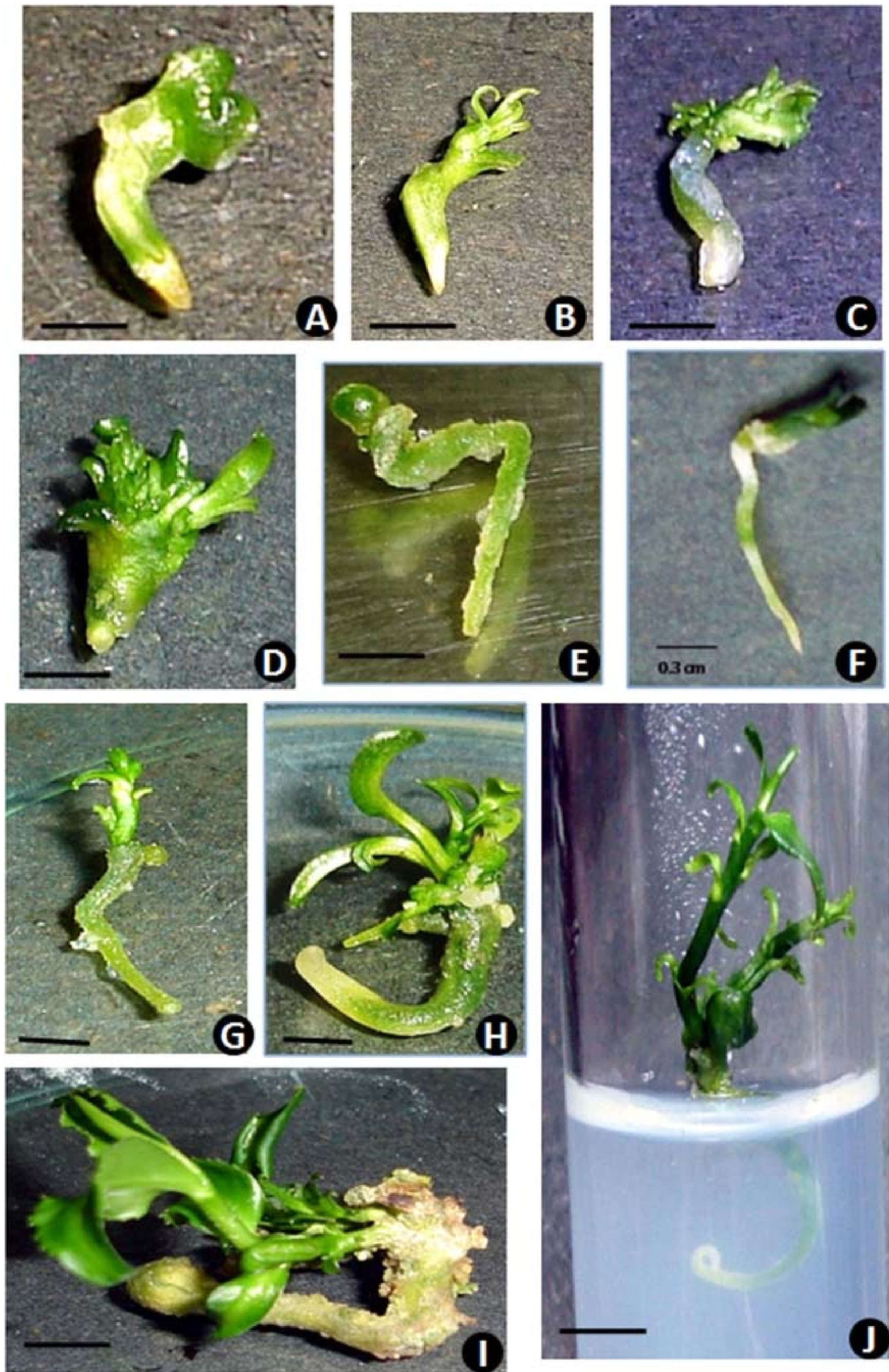

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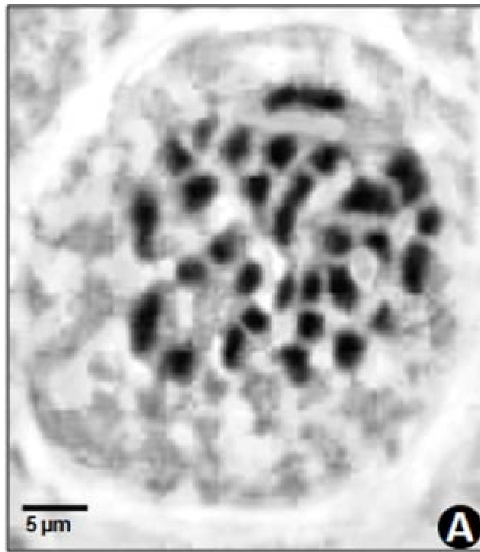

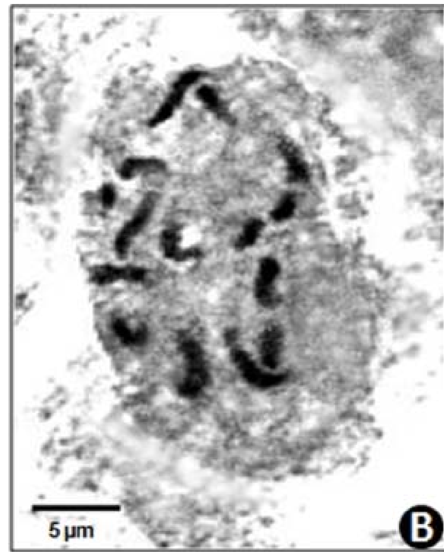

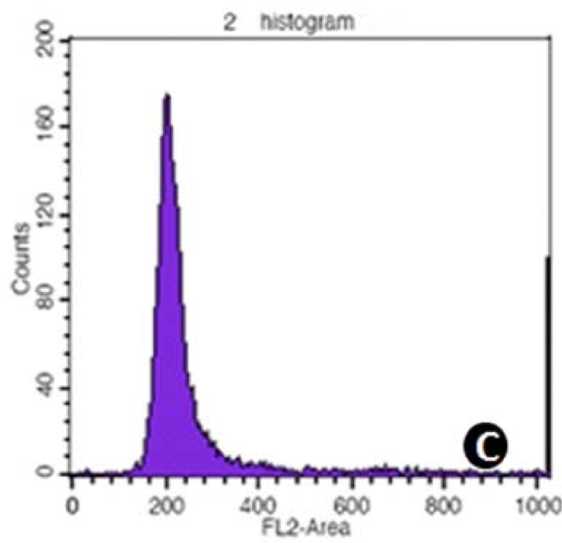

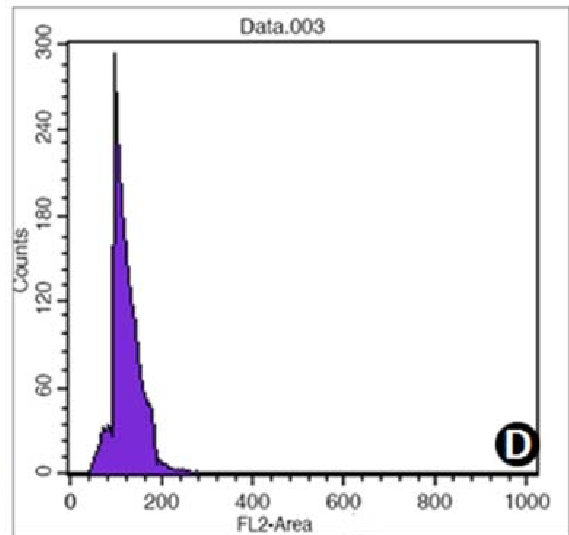

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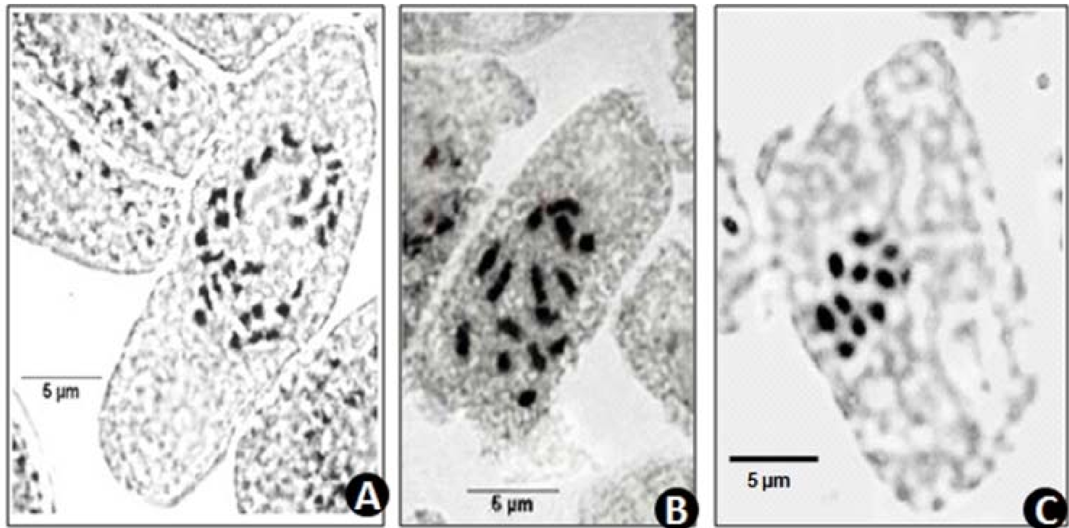

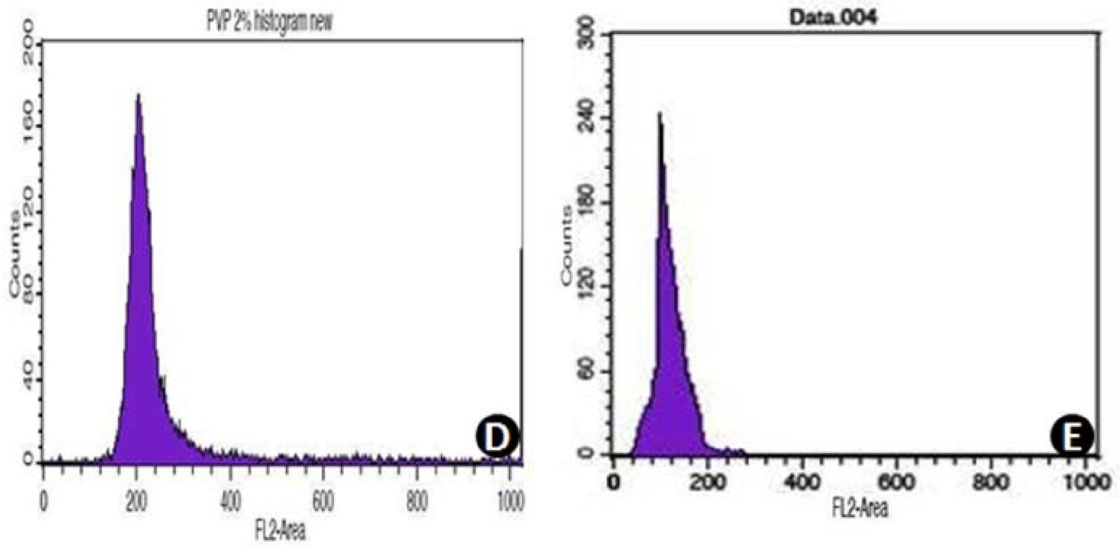

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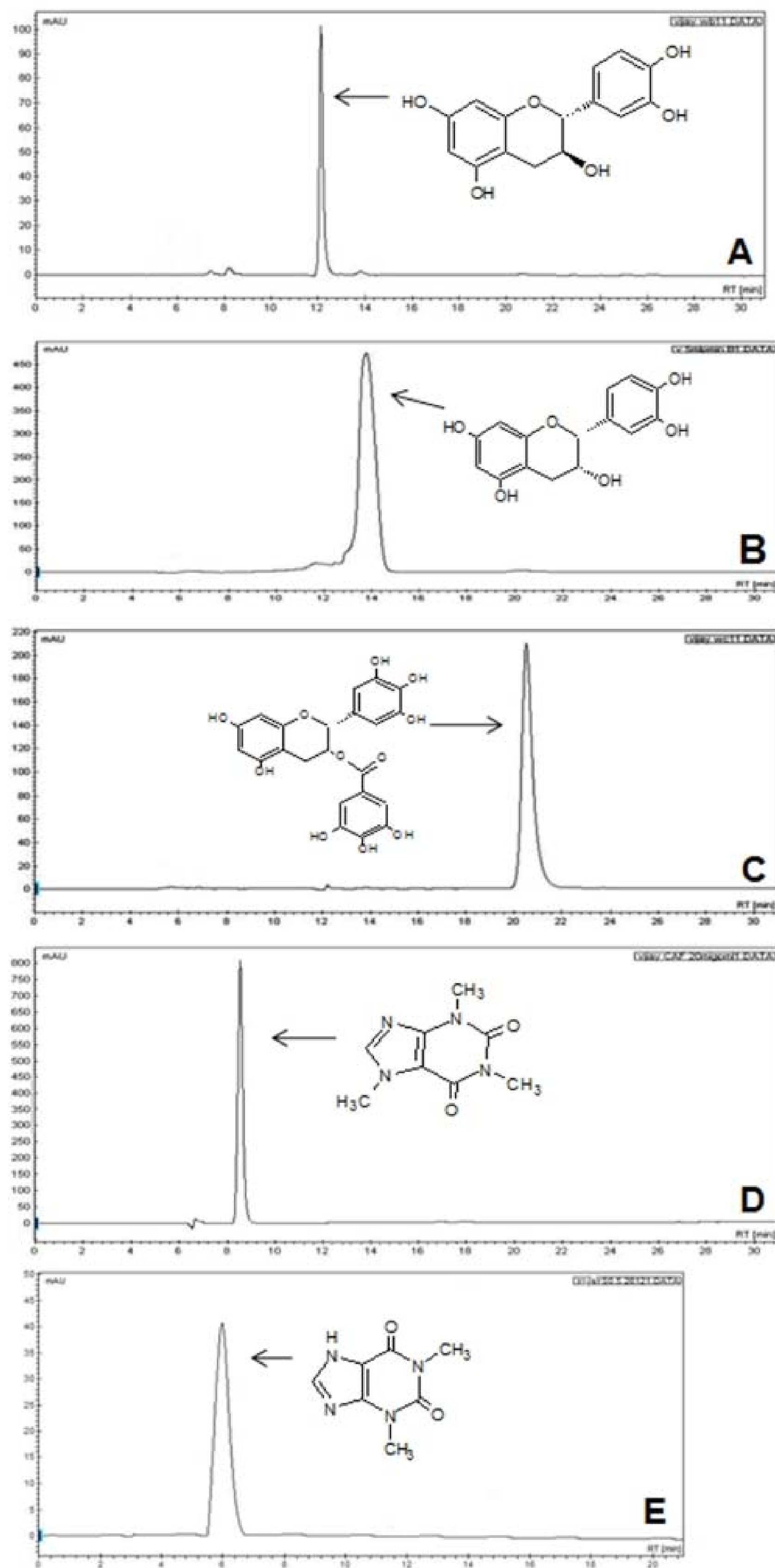

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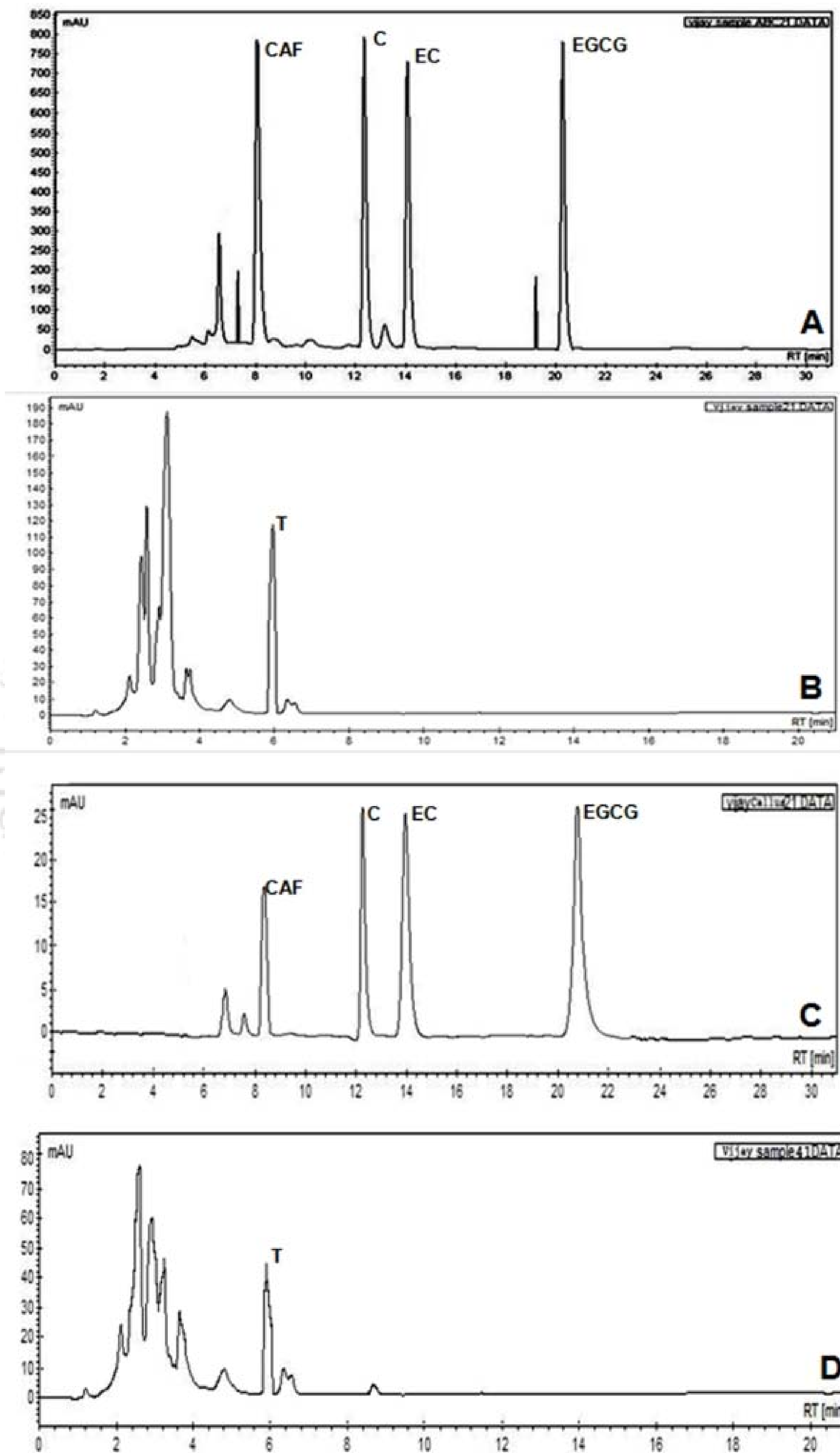

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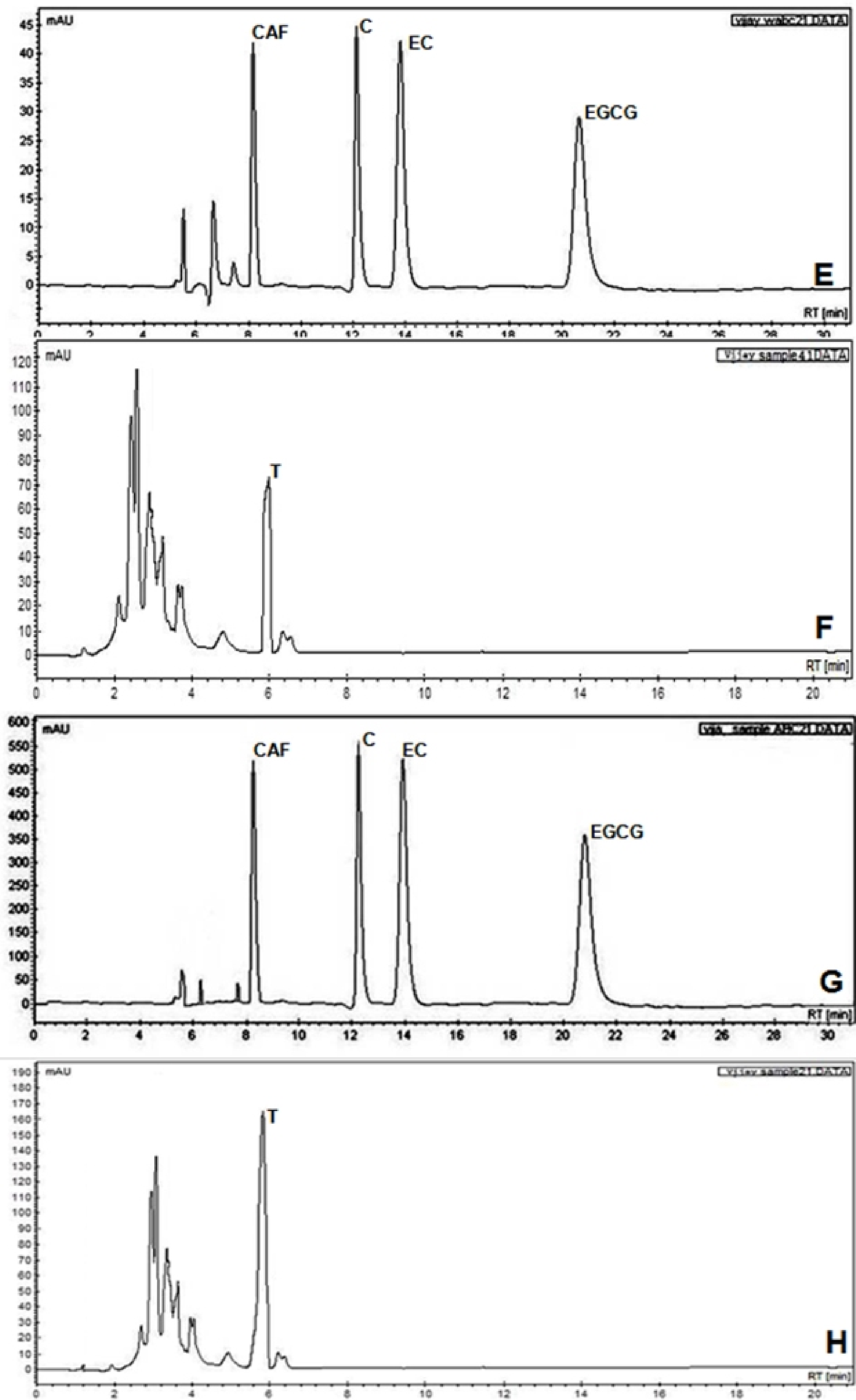

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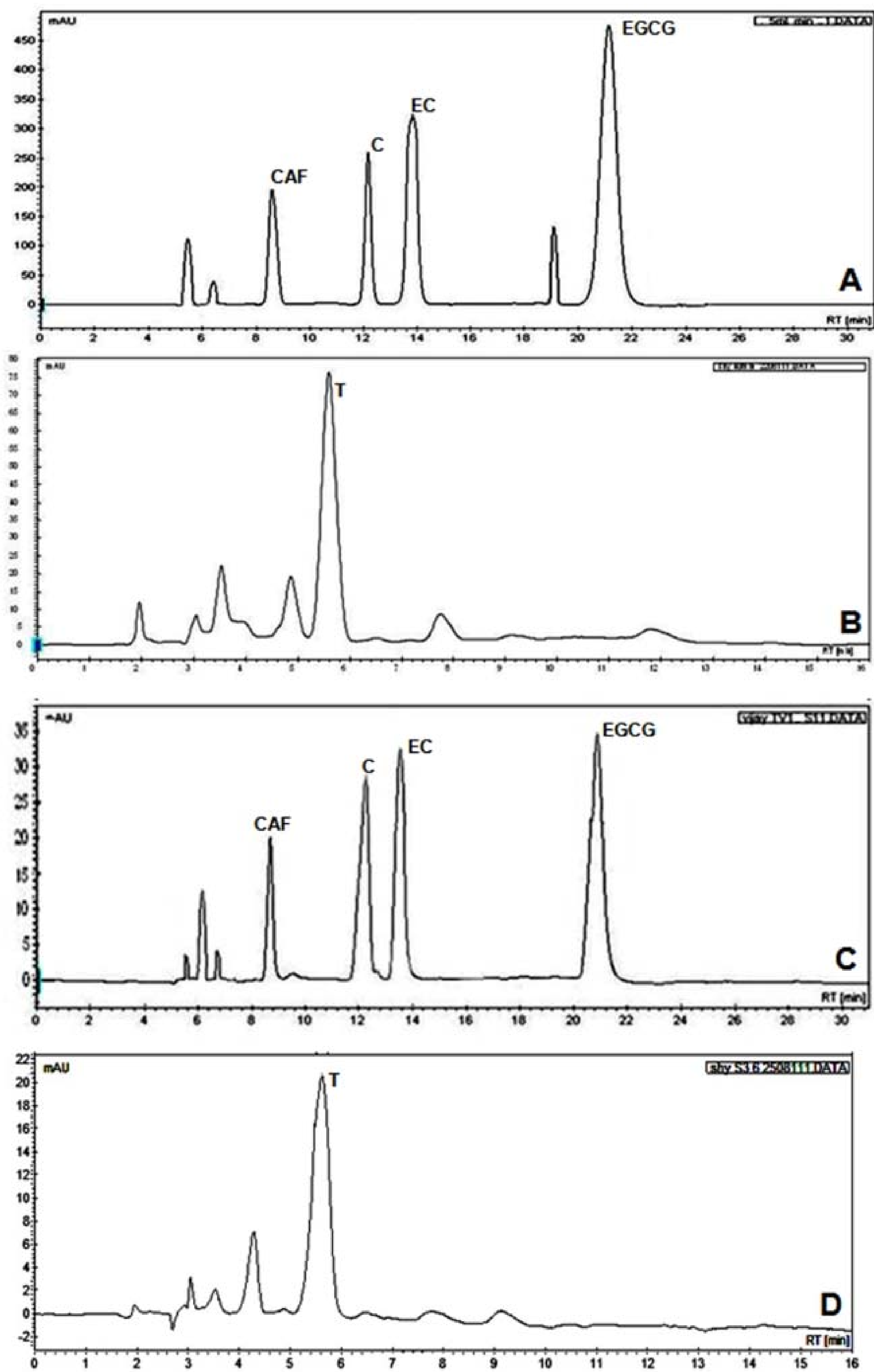

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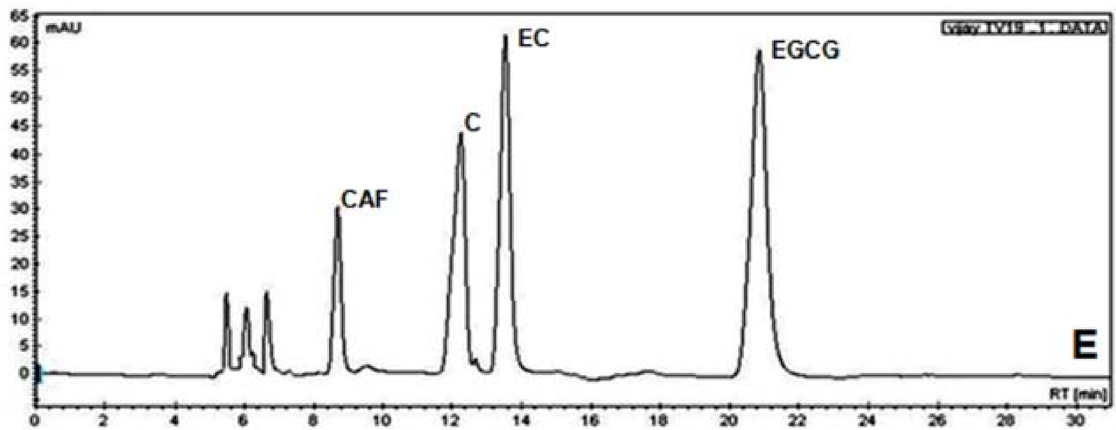

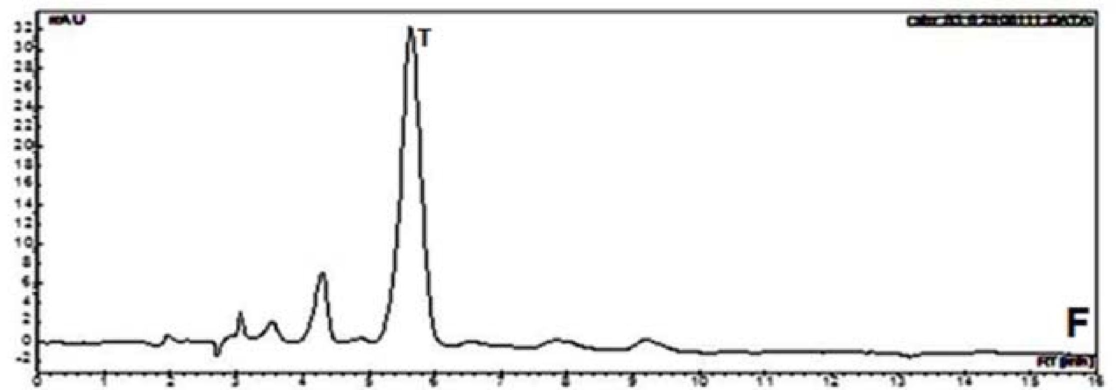

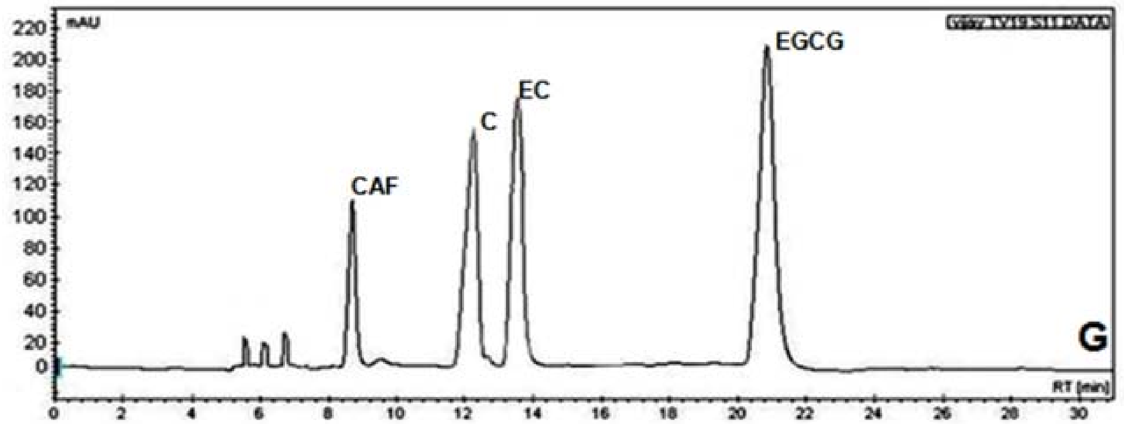

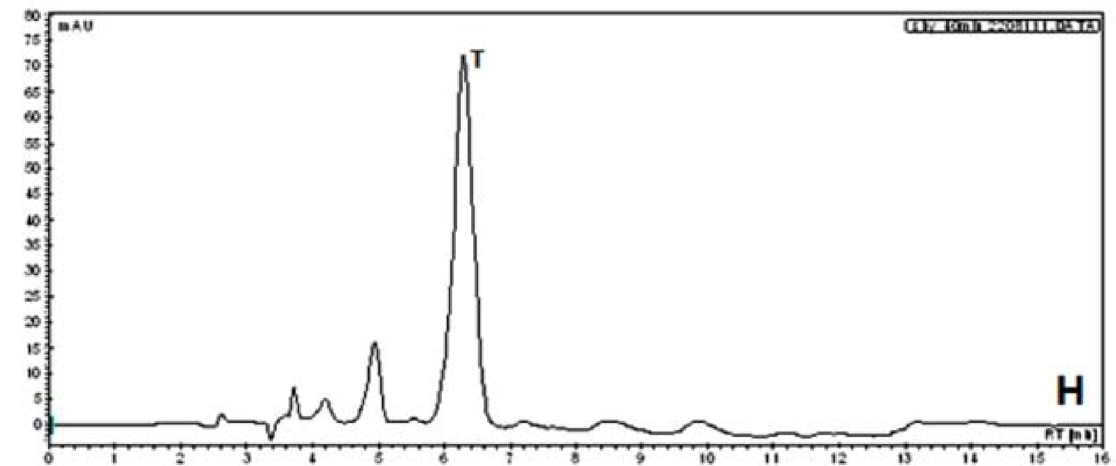

Figure 18

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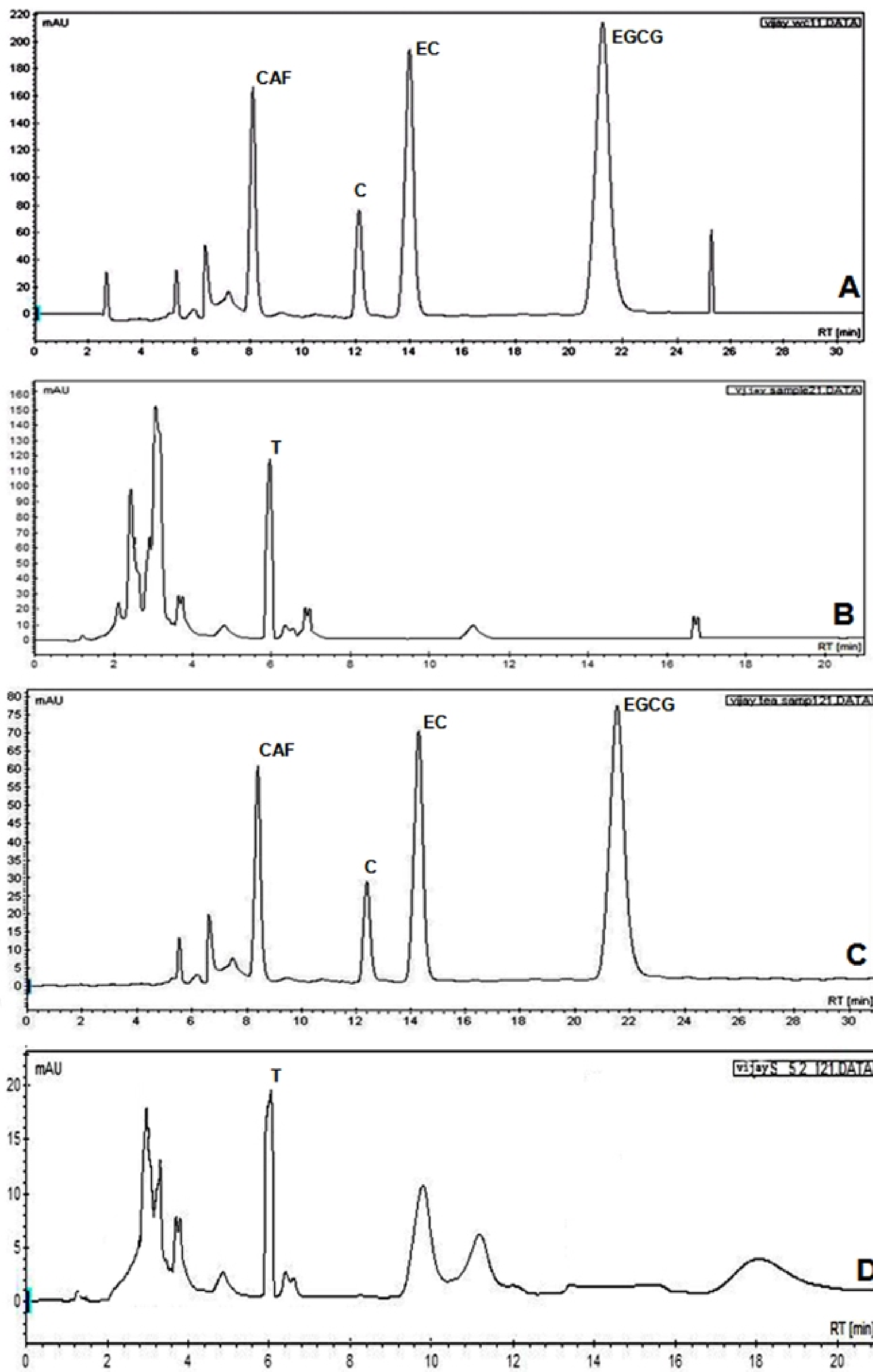
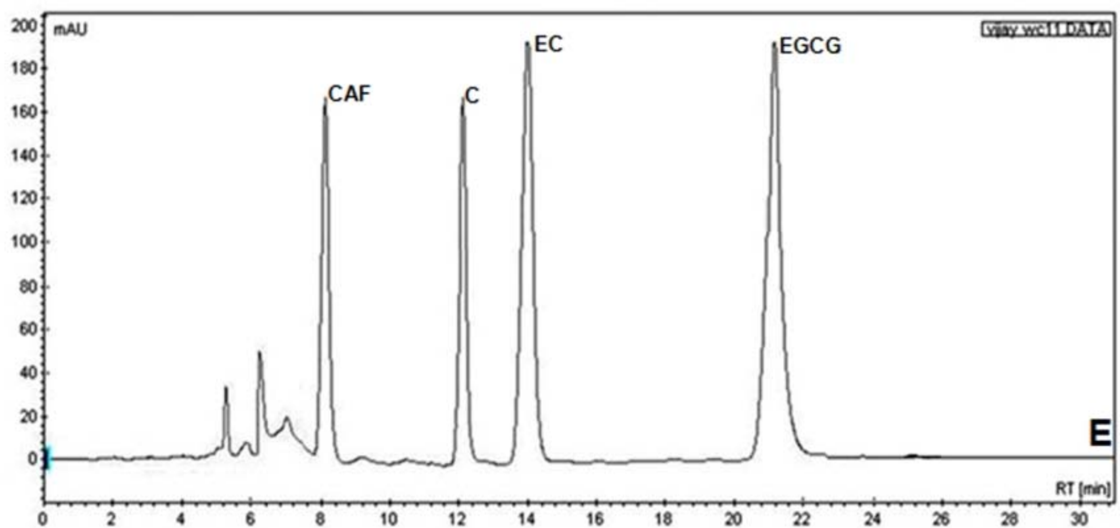


Figure 19



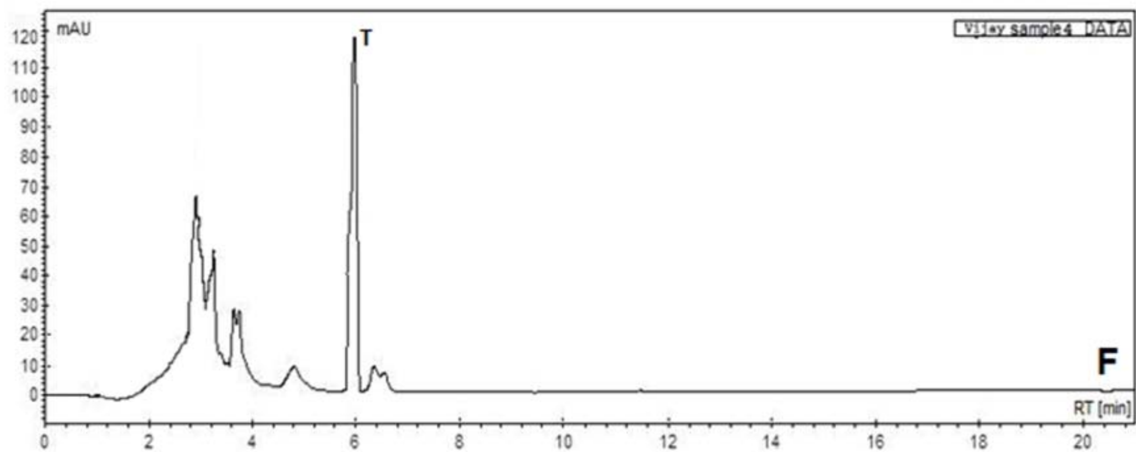


Figure 19

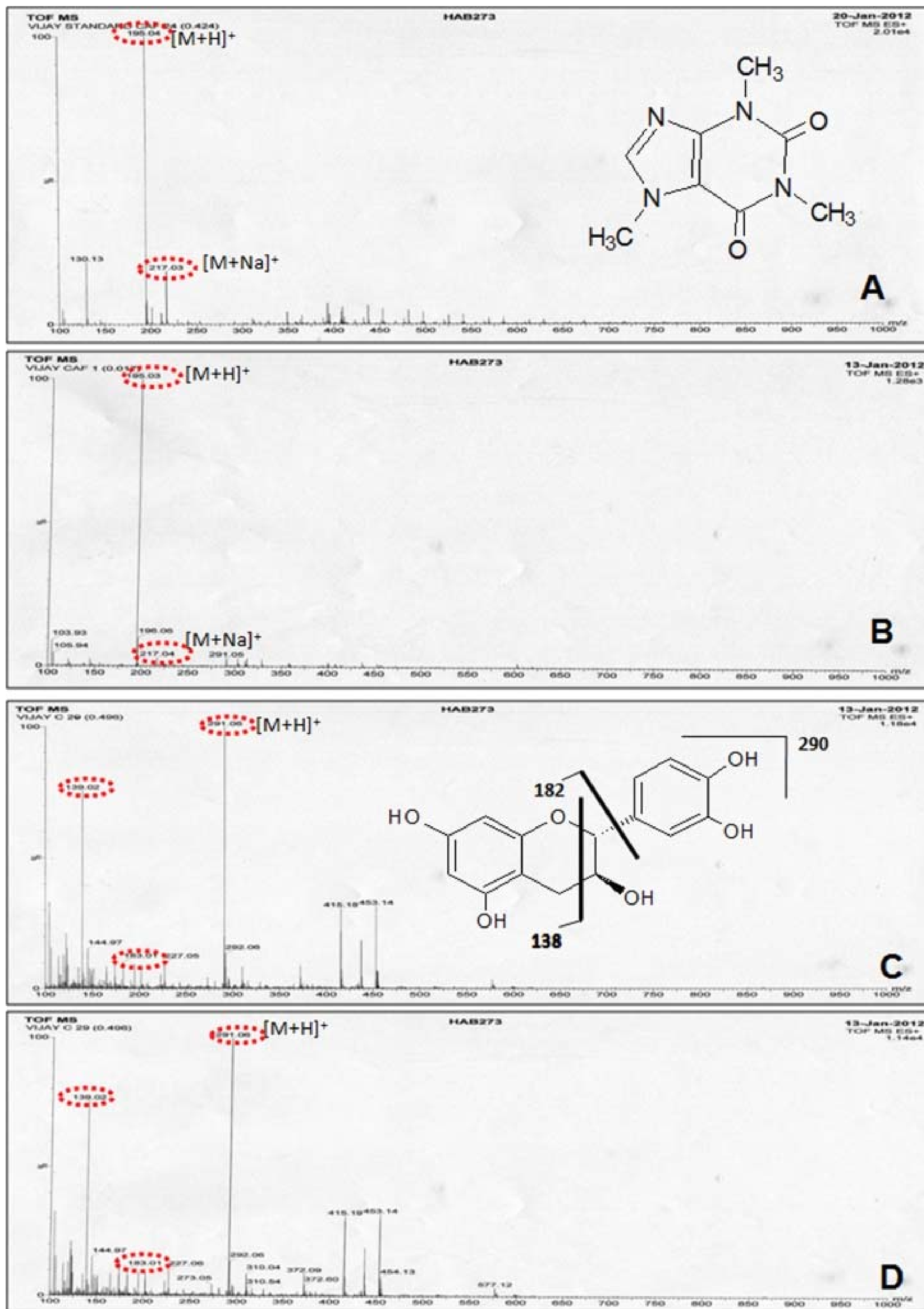


Figure 20

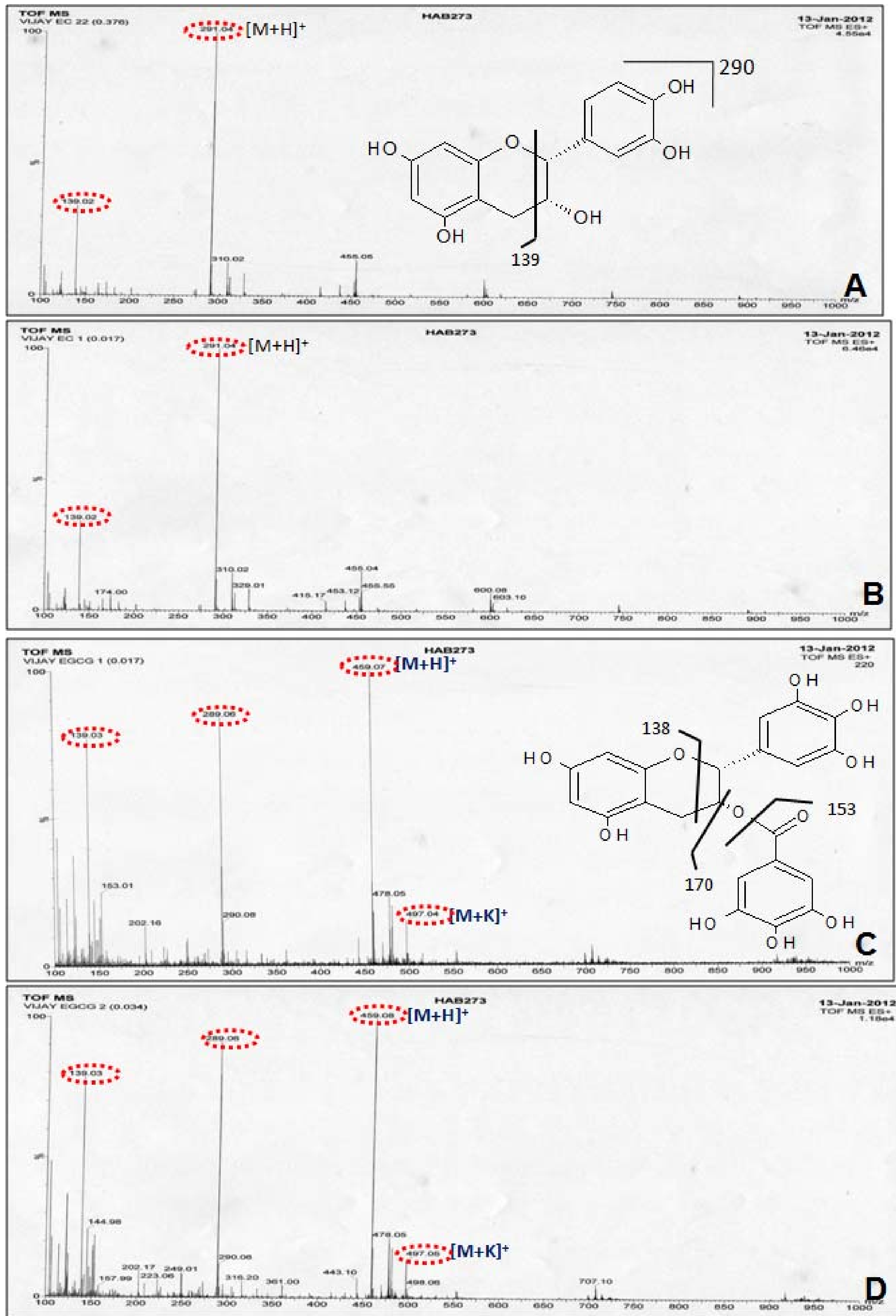


Figure 21

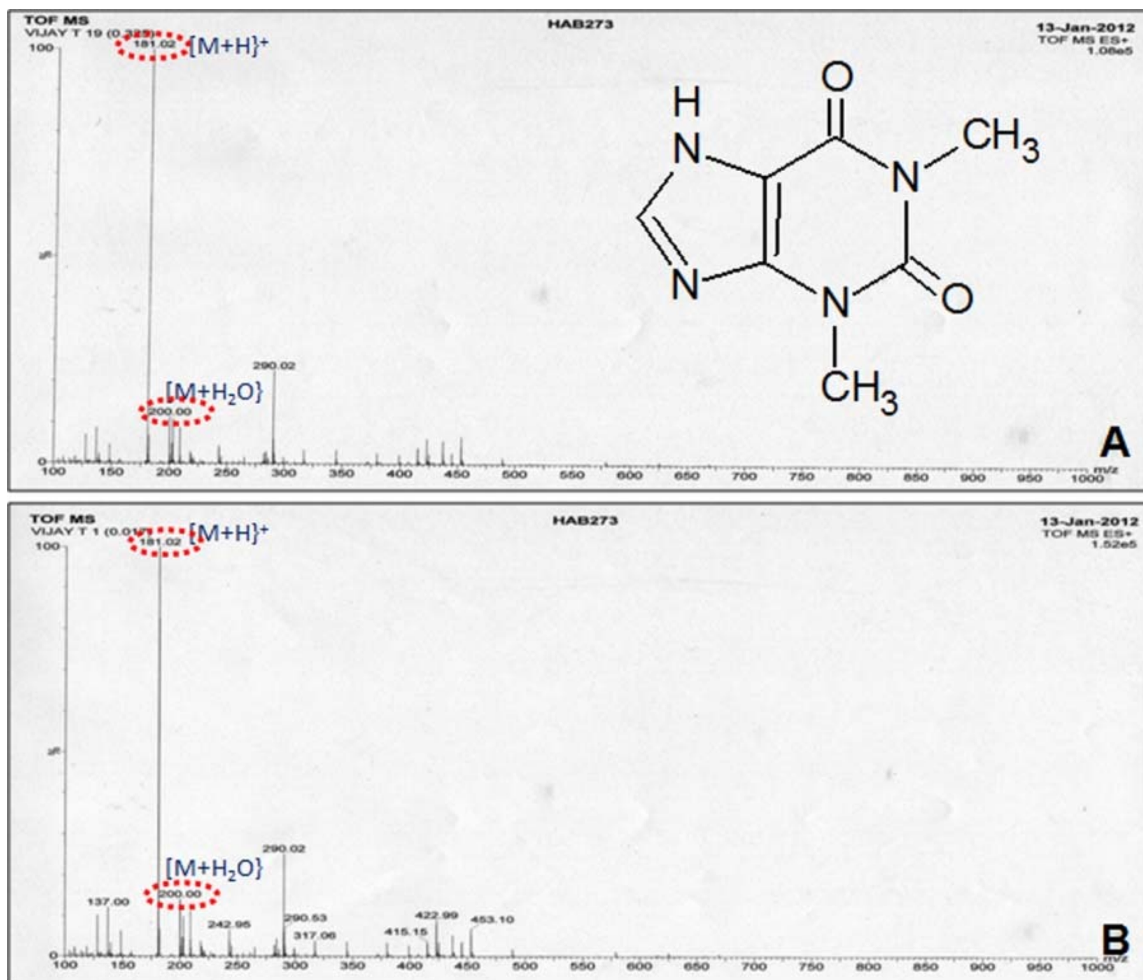


Figure 22

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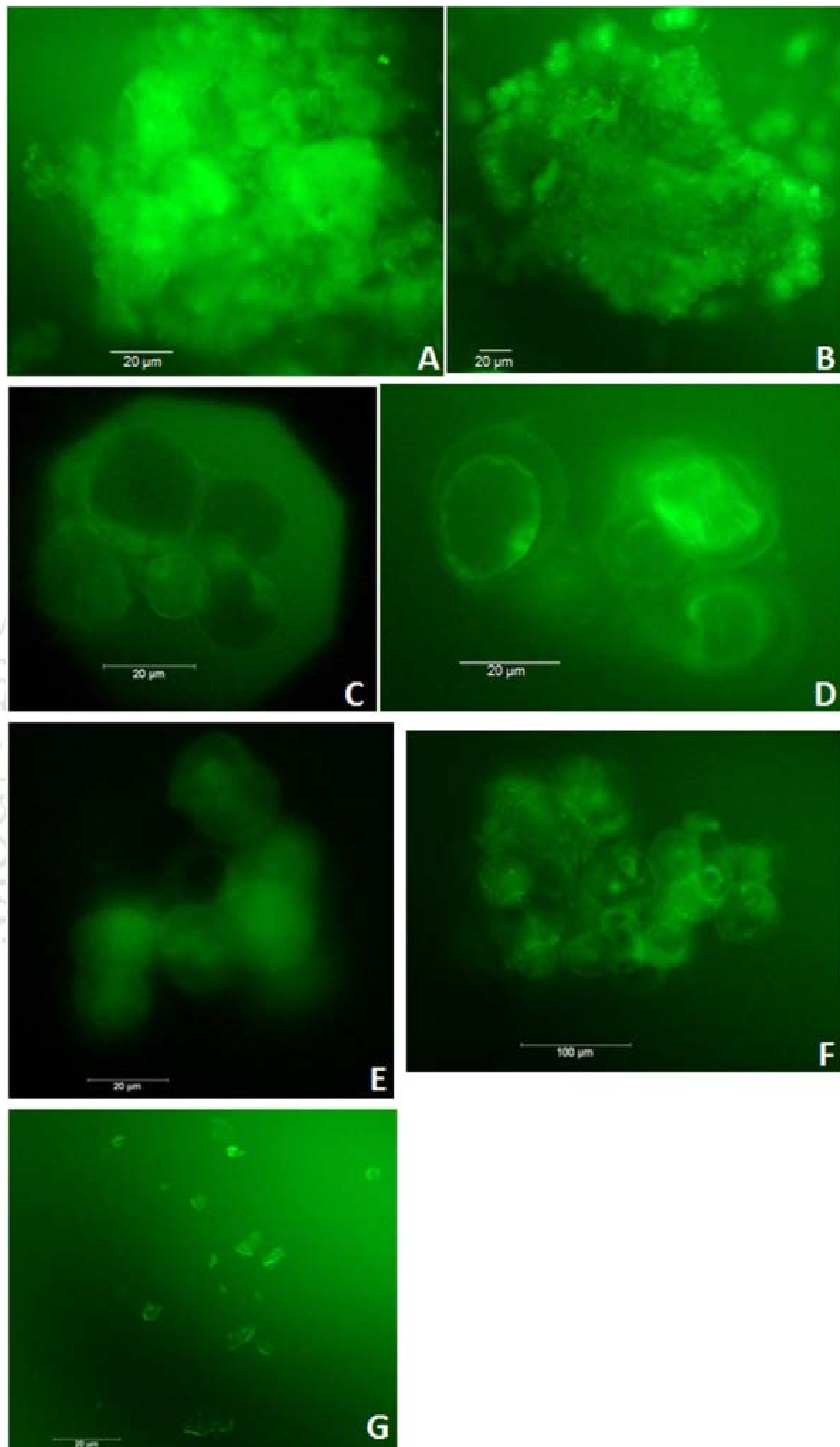

Figure 23

TH-1176_07610612