



Faculty of Resource Science and Technology

***In vitro* Regeneration and Microscopic Studies of *Castanea sativa* Mill**

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In Vitro Regeneration and Microscopic Studies of *Castanea sativa* Mill.

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A thesis submitted

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Castanea sativa Mill. (European chestnut) belongs to the Fagaceae family and is known among multipurpose plants. It is a significant industrial crop that is appreciated and processed globally to produce goods in various industries including food, medicine, furniture, and timber industry. At present, it is considered a staple food throughout southern Europe, Turkey, southwestern Asia and eastern Asia, which infers the essential for rapid multiplication and higher yield production of the species. Yet, there is still a lack of research on the propagation of *C. sativa* Mill. especially in Malaysia. Thus, the present study focuses on investigating the optimum medium for the *in vitro* propagation of *C. sativa* to produce plantlets on a large scale besides establishing the sterilization protocol for seed germination. Initially, the selected seeds were surface sterilized and cultured on MS basal medium. After 2 months, the nodal segments of aseptic seedlings were sectioned into smaller pieces and cultured on various treatments for shoot induction. Then, the cultures were maintained in the culture room. Among the parameters recorded were the percentage of shoot production, mean number of shoots produced, mean shoot height, mean number of leaves, and mean leave length. Furthermore, the 6-month-old plantlets regenerated *in vitro* were subjected to morphological and anatomical studies. The present study has discovered the sterilization protocol with 80% seed germination. Besides, the stage of seedling growth and development have been examined starting with the seed germination on the first week of culture, followed by seedling emergence and the appearance of true leaves and hairy roots after one month of culture. Additionally, direct *in vitro* propagation of *C. sativa* is influenced by either internal or external factors. Among the main factors include plant growth regulators and plant additives. Current findings clarified the treatments tested mostly promoted 100% shoot

production whereby the explants could produce approximately 1-2 shoots per explant ($\approx$ 1 shoot per explant) with 1.0-2.0 cm shoot height ($\approx$ 2.0 cm) besides producing 1-4 leaves (2 leaves) and the leaves length within the range of 0.3-2.0 cm (1.0 cm) after 2 months of culture. Analysis of the results disclosed the most recommended medium for regeneration of the plant species *in vitro* was MS supplemented with CW + AC + 3.0mg/L BAP + 1.0mg/L NAA whereby 100% explants producing shoot within 2 months, with an average of 2 shoots per explant, 2.0 cm shoot height and 4 leaves with 1.0 cm length. Moreover, the morphological study has revealed the stomata dispersed widely on the abaxial leaf with the size of stomata opening, 12.45 μ m length and 2.7 μ m width. Meanwhile, the anatomical study disclosed unique internal structures of the leaf blade and midrib including the vascular bundles (phloem and xylem) and mesophyll cells (palisade and spongy). Hence, the present findings provide some basic information and understanding on the propagation of plant species *in vitro*. Nonetheless, further research should be performed to improve the protocol for seed sterilization and shoot induction of *C. sativa* Mill. *in vitro*. In fact, more parameters should be considered for data analysis as well as the other factors that could influence the growth and performance of *C. sativa* Mill.

Keywords: European chestnut, micropropagation, seed germination, shoot induction, plant growth regulators.

Pertumbuhan Semula In vitro dan Kajian Mikroskopik Castanea sativa Mill

ABSTRAK

Castanea sativa Mill. (buah berangan Eropah) tergolong dalam keluarga *Fagaceae* dan dikenali sebagai tumbuhan pelbagai guna. Ia merupakan tanaman perindustrian penting yang dihargai dan diproses secara global untuk menghasilkan barangan dalam pelbagai industri termasuk makanan, farmaseutikal, perabot, pengeluaran kayu dan lain-lain. Pada masa kini, ia menjadi makanan ruji di selatan Eropah, Turki, barat daya dan Asia timur yang menjadikan pembiakan dan pemuliharaan penting untuk kelestarian tumbuhan. Namun, masih terdapat kekurangan penyelidikan mengenai pembiakan in vitro *Castanea sativa Mill.* terutamanya di Malaysia. Oleh itu, kajian ini memfokuskan kepada pembiakan in vitro *Castanea sativa* untuk menyiasat atau mendapatkan medium optimum untuk pengeluaran anak pokok *Castanea sativa Mill.* (chestnut) dalam skala besar selain mewujudkan protokol pensterilan untuk percambahan biji benih. Pada mulanya, biji benih dipilih, disterilkan pada permukaan dan dikultur pada media asas MS. Selepas 2 bulan, segmen nod anak benih aseptik dibelah dan dikultur dalam pelbagai rawatan untuk induksi pucuk. Antara parameter yang direkodkan ialah peratusan penghasilan pucuk, purata bilangan pucuk yang dihasilkan, purata ketinggian pucuk, purata bilangan daun, dan purata panjang daun. Tambahan pula, anak pokok berusia 6 bulan yang dijana semula secara in vitro disediakan untuk kajian morfologi dan anatomi. Kajian ini telah menemui protokol pensterilan dengan 80% percambahan biji benih. Di samping itu, peringkat pertumbuhan dan perkembangan anak benih telah diperiksa bermula dengan percambahan benih pada minggu pertama kultur, diikuti dengan kemunculan anak benih dan penampilan daun sebenar dan akar rambut selepas satu bulan penanaman. Di samping itu, propagasi in vitro

Castanea sativa secara langsung dipengaruhi oleh sama ada faktor dalaman atau luaran. Antara faktor utama termasuk pengawal selia pertumbuhan tumbuhan dan bahan tambahan tumbuhan. Penemuan semasa menjelaskan rawatan yang diuji kebanyakannya menunjukkan pengeluaran pucuk 100% di mana eksplan boleh menghasilkan anggaran 1-2 pucuk setiap eksplan (≈ 1 pucuk setiap eksplan) dengan ketinggian pucuk 1.0-2.0 cm (≈ 2.0 cm) selain menghasilkan 1-4 daun (2 daun) dan panjang daun dalam julat 0.3-2.0 cm (1.0 cm) dalam 2 bulan kultur. Analisis ke atas keputusan mendedahkan medium yang paling disyorkan dalam penjanaan semula spesies tumbuhan secara in vitro ialah MS ditambah dengan CW + AC + 3.0mg/L BAP + 1.0mg/L NAA di mana 100% eksplan menghasilkan pucuk dalam tempoh 2 bulan, dengan purata 2 pucuk setiap eksplan selain ketinggian pucuk 2.0 cm dan 4 daun dengan panjang 1.0 cm. Selain itu, kajian morfologi telah mendedahkan stomata tersebar luas pada daun abaksial dengan saiz bukaan stomata, 12.45 μm panjang dan 2.7 μm lebar. Sementara itu, kajian anatomi mendedahkan struktur dalaman unik bilah daun dan pelepah termasuk berkas vaskular (floem dan xilem) dan sel mesofil (palisad dan span). Oleh itu, penemuan ini boleh memberikan beberapa maklumat asas dan pemahaman tentang pembiakan spesies tumbuhan secara in vitro. Walau bagaimanapun, kajian lanjut perlu dilakukan untuk menambah baik protokol pensterilan benih dan induksi pucuk *C. sativa* Mill. dalam vitro. Malah, lebih banyak parameter harus dipertimbangkan untuk analisis data serta faktor lain yang boleh mempengaruhi pertumbuhan dan prestasi *C. sativa* Mill.

Kata kunci: chestnut Eropah, pembiakan mikro, percambahan biji benih, induksi pucuk, pengawal selia pertumbuhan tumbuhan.

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St = stomata, GC = guard cell, Ad L – adaxial leaf, Ab L =
abaxial leaf, Ph = phloem and Xy = Xylem.

LIST OF ABBREVIATIONS

2.4-D	2,4-Dichlorophenoxyacetic acid
AC	Activated charcoal
ANOVA	Analysis of variance
BAP	Benzylaminopurine
CW	Coconut water
DKW	Driver & Kuniyuki
DMRT	Duncan Multiple Range Test
GD	Gresshoff & Doy
IAA	Indole-3-Acetic acid
IBA	Indole-3-Butyric acid
IBM SPSS	SPSS statistics software
ISTA	International Seed Testing Association
KIN	Kinetin
KNO ₃	Potassium nitrate
KOH	Potassium hydroxide
LP	Leaf primordia
LS	Linsmaier and Skoog
MMS	Modified MS

MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
NaOH	Sodium hydroxide
NH ₄ NO ₃	Ammonium nitrate
PGRs	Plant growth regulators
PVP	Polyvinyl Pyrrolidone
SAM	Shoot apical meristem
YLP	Young leaf primordia
SH	Schenk and Hildebrandt
TDZ	Thidiazuron
WPM	Woody Plant Medium
ZEA	Zeatin
ZiP	Isopentenyladenine

CHAPTER 1

INTRODUCTION

Castanea sativa Mill. commonly known as sweet chestnut is cultivated worldwide. It is generally found in the natural stands in small grooves (Bounous & Beccaro, 2002) as well as in the flora of the Northern Hemisphere (Sezgin & Dumanoglu, 2016). This sweet chestnut is synonymous with European chestnut, Marron, Portuguese chestnut, Spanish chestnut and Italian chestnut (Lim, 2012). In Malaysia and Indonesia, it is called Buah Berangan (Lim, 2012).

Besides European chestnut (*C. sativa*), there are three other chestnut varieties in the world including Chinese chestnut (*C. mollissima*), Japanese/Korean chestnut (*C. crenata*), and American chestnut (*C. dentata*) (Ertürk et al., 2006; De Vasconcelos et al., 2010; Otles & Selek, 2012) which are also among the ecologically and economically significant nut and timber-producing trees in the genus. They are considered as the “ideal trees” due to their importance and uses in numerous industries (Barakat et al., 2009).

Chestnut is classified as a deciduous tree with its character that shed its leaves every mid of the year and is well-known for various wildlife including bees for their pollination, others like birds and mammals for their sources of energy and nutrition. Historically, chestnut is initially introduced and cultivated by ancient Greek in the Mediterranean region (Conedera et al., 2004). It is usually consumed as an edible fruit and nowadays by people globally especially in the food industry. Previous evidence of carbonized nuts discovered in Japan discloses that chestnuts were used as food over 9,000 years ago (Chestnut in Asia, 2019.). In fact, it is one of the most important food resources in rural areas of Europe (Squillaci et al., 2018). Other than that, the raw astringent taste which derived from

processed fruits (raw and cooked) normally used in cooking and baking to improve the taste and aroma of dishes. Moreover, it has valuable compounds that promote biological activity which is essential in the pharmaceutical industry. It was found that the leaves, bark, nuts and shell could be used as a good source of tannins whereby all of the plant parts have potential therapeutic effects (antioxidant, anticancer, antibacterial, anti-inflammatory, hypoglycemic, treatment of obesity, and bleeding) against various diseases (Hu et al., 2021).

Chestnuts are commercially cultivated in about 27 countries and are popular mostly in Asia and Europe. Report by World Mapper in 2016 on the production of chestnut revealed that approximately 80% of the global production was from China. Then, followed by Bolivia, Turkey, South Korea and Italy (World Mapper, 2016). According to Food and Agriculture Organization of the United Nations, the global production of chestnuts was increasing gradually in 2018. The top three producers of chestnut are still the same viz. China(1965351 tonnes), Bolivia (84010 tonnes) and Turkey (63580 tonnes) whereby Asia ranked first with 86.7%, Europe in the second place with 9.4% and third place was America with only 3.8% for the global production of chestnut (FAO, 2019). Nevertheless, a slight change in the position of the top three producers of world chestnut production is noticed recently. China is still in the first place but the second and third place belongs to Spain and Bolivia, respectively (Atlas Big, 2022). Considering the significance of Chestnut in various industries besides their importance in global production specifically in Asia, thus a number of researchers have been conducted to produce chestnut plantlets on a large scale by different propagation techniques. Chestnut is a woody plant that is considered difficult to grow by vegetative propagation through cuttings and grafting (Tafazoli et al., 2013). Earlier study recommended the development of proper propagation methods for cultivar description,

identification and fruit trait characterization, especially in the selection of high-quality goods (excellent sensory and qualitative qualities, high bioactive chemical content) is required (Beccaro et al., 2020).

Chestnut is categorized as a monoecious plant that blooms in late June to early July and might be pollinated by the wind (more frequently when the weather is dry during flowering) or insects (predominant when the weather is moist) (Conedera et al., 2016). Traditionally, the chestnuts are propagated vegetatively or by using seedlings (Song et al., 2021). In Europe, rooted cuttings have been employed in the cultivation of chestnuts (Corredoira et al., 2017). On the other hand, seedlings of chestnut have been used to reintroduce the American chestnut to forests and are frequently used in landscapes (Vieitez et al., 2007; Corredoira et al., 2017). Nevertheless, several studies previously suggested the micropropagation is an effective vegetative propagation technique for industrial scale production of individual genotypes if compared to traditional propagation (Xing et al., 1997; Tetsumura & Yamashita, 2004; Vieitez et al., 2007; Chen et al., 2009; Nelson et al., 2014; Corredoira et al., 2017). Micropropagation produces the true-to-type plants of selected genotypes using *in vitro* culture technique (Debergh & Read, 1991; Laimer & Rucker, 2003). *In vitro* propagation comprises the technique which can undergo large scale multiplication in a short time prediction. The production of plants using *in vitro* propagation can be propagated through various plant parts such as seeds, pods (orchids), meristem leaves, flowers, stem cuttings, shoot buds, immature embryos and etc. Typically, micropropagation can be enhanced by a few processes which are axillary bud-breaking, direct or indirect production of adventitious buds and somatic embryogenesis (Smith, 2013).

There are several factors that could influence plant growth and development of *in vitro* propagation of chestnut trees. Recent investigation revealed one of the crucial factors influencing the growth and proliferation of shoots in American chestnut is the medium with basal salt combination (Liu et al., 2022). Woody Plant Medium (WPM) is the most often utilized basal medium in shoot proliferation and elongation of American chestnut (Carraway et al., 1997; Xing et al., 1997; Kong et al., 2014) and European chestnut (Capuana & Di Lonardo, 2013). Another well-known basal medium is Murashige and Skoog (MS) which is frequently applied in European chestnut (Tafazoli et al., 2013; Roussos et al., 2016), hybrid chestnut (Soylu & Ertürk, 1999; San-Jose et al., 2001), Japanese chestnut (Tetsumura & Yamashita, 2004) and Chinese chestnut (Hou et al., 2010) cultivars. Based on a finding by Liu et al (2022), Driver and Kuniyaki (DKW) basal medium is highly recommended for the overall growth of shoots in American chestnut by micropropagation system.

Besides the basal salt combination, the plant growth regulators (PGRs) applied in the media also play an important role in plant growth and development of *in vitro* propagation. They are known as the primary determinants of plant development and growth in tissue culture system (Liu et al., 2022). Three types of PGRs which regularly used to exhibit substantial cytokinin activity for *Castanea* spp. are Benzyladenine (BA), Thidiazuron (TDZ) and Zeatin. In the multiplication of shoot cultures, BA was discovered to be the most effective cytokinin for shoot induction and multiplication of *Castanea* spp. (Vieitez & Vieitez, 1983; Goncalves et al., 1998; Tetsumura & Yamashita, 2004; Fernandez-Lorenzo et al., 2005). Latest research by Song et al. (2021), recorded that the most effective media in promoting shoot proliferation and healthy shoot elongation is the combination of optimal

basic media with the addition of hormone; Woody Plant Medium (WPM) containing Zeatin Riboside (ZR).

Nowadays, *in vitro* propagation is preferred rather than traditional propagation because many plant species can adapt to this specific reproduction technique including woody plants, herbaceous plants, ferns, palms, and even a number of forest tree varieties that are very crucial in timber production. The advantage of *in vitro* propagation includes it is cost effective, whereby the cost is much cheaper compared to the cost for operation in traditional propagation. Yet, the large scale production can be done in a short duration for *in vitro* propagation. Furthermore, by *in vitro* propagation, the cash crop production could be increased throughout the year with faster and better quality plant production (plants have high resistance toward any type of diseases, free-disease plants, etc.) In current plant research, tissue culture technique is essential for various plant production worldwide. Besides, it provides imperative contributions to the improvement of preservation and conservation of biodiversity.

The study on *in vitro* propagation of *C. sativa* was proposed due to the quality of its hardwoods (Vazquez et al., 2015). Only *Eucalyptus* sp. and *C. sativa* are classified as hardwoods for timber design in Spain (Vega et al., 2012). *C. sativa* wood has historically been used in Portugal, Italy, and Northern Spain for carpentry, construction (without design) and furniture making (Vazquez et al., 2015). The current scope of chestnut use in Europe can be related to its application as a common food, charcoal, woodwork, medicine for the renowned confection marron glace, or as a flavouring for beer, particularly in France and Switzerland (Kosnovska, 2013). The enormous potential of chestnut trees for in fruit production as well as for use of the bark, leaves, and blossoms in pharmacopoeia due to the

astringent, antitoxic, stomatic and tonic properties of chestnut was first recognized by the Romans (De Vasconcelos et al., 2010).

According to numerous nutrient composition and health studies, chestnut trees have a lot of potential as functional foods and food ingredients. For example, chestnut polyphenolic has healthy compounds like gallic, ellagic acids and the ellagitannins that are good sources of antioxidant, antimicrobial, anti-inflammatory and anticancer (Kahkeshani et al., 2018; Squillaci et al., 2018).

Castanea sativa has been classified as Least Concern species on the official International Union for Conservation of Nature (IUCN) list (Barstow & Khela, 2018; IUCN, 2020). The huge massifs of Georgia's chestnut woods are at risk of extinction due to low self-renewal and harmful illnesses (Tavadze et al., 2012). Therefore, in accordance with IUCN Red List Categories and Criteria, sweet chestnut has been listed on the Red List of Georgia under the state Vulnerable (VU) (Gaidamashvili et al., 2021).

Seeds are mostly utilized to preserve plant genetic variation (Trusiak et al., 2023). Additionally, the seed is among the important stages in the plant life cycle because it serves as angiosperms' primary means of juvenile dispersal (Liu et al., 2022). Seeds of *Castanea sativa* are categorized as recalcitrant seeds, since they can shed at high water contents and are highly prone to desiccation and cold temperatures (Gaidamashvili et al., 2019). Recalcitrant seeds are important in the agriculture sector because they being used as food, animal feed and plant propagation resources (Du et al., 2020). Since the mid-1990s, Janeiro et al (1995), have documented the effective medium-term maintenance of chestnut hybrid clones. Moreover, other study clarified *Castanea* shoot cultures persisted during slow growth storage at 4 - 8°C for 5 to 18 months (Corredoira et al., 2017). For a very long time,

researchers have been working on techniques to overcome the chestnut's resistance to clonal multiplication and enable the mass production of chosen genotypes (Fernandes et al., 2020).

Castanea sativa (Sweet chestnut), is a very well-known fruit and is highly in demand not only in Malaysia but universally. The challenges on propagation of the plants include it takes a longer period to root, requires favourable environment to grow, and needs application of PGRs to grow and develop as it is in the woody plant category whereby propagation by seed or vegetative propagation through grafting and cuttings for mass production seems impractical. Consequently, tissue culture is a convincing system in the rapid production of plants. Besides, it is also an essential technique to notch up the achievement of sweet chestnut production in the industrial scale production. Despite earlier reports of the regeneration of sweet chestnut, *Castanea sativa* tissue culture success has not yet been regularly established. The success of *in vitro* process depends on the selection of a suitable culture medium for plant reproduction (Jain & Ishii, 2012). Better productivity of cultures in the optimization of culture conditions not only counts in terms of the number of plant production, but also involved the quality of plant itself during acclimatization in the natural environment.

Therefore, this research project mainly proposed mass production of sweet chestnut by considering a few objectives which might influence the growth and performance of the plant *in vitro* as outlined below:

- i. To establish the efficient protocol for sterilization of *Castanea sativa* seeds which is critical for the production of aseptic seedlings that would be utilized as the source of explants for further study in *in vitro* plant propagation.

- ii. To investigate the optimum medium for *in vitro* regeneration of *Castanea sativa* which could produce a good quality of plantlets that meet the industry demand for planting materials.
- iii. To study the microscopic studies that revealed stomata dispersion on abaxial leaf and histological studies that disclosed the unique internal structure of leaf structure of *Castanea sativa*.

CHAPTER 2

LITERATURE REVIEW

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3.1 *Castanea sativa* Mill.

3.1.1 Origin and Distribution of *Castanea* sp.

Chestnut is an important hardwood species that has economical value which is found in a natural stand, small groves and grown as nut orchards (Corredoira et al., 2005). It is considered a valuable forest species that have economic importance in the timber and nut production industry (Tafazoli et al., 2013). Chestnut is scientifically classified in the *Castanea* genus which consisted of 13 different species, belongs to the Fagaceae family and they are closely related to the beeches and oaks group.

Generally, chestnuts are mostly found in natural habitat in Northern Hemisphere, particularly in China, Korea, Japan and Southern Europe (Vossen, 2000; Corredoira et al., 2003; Sezgin & Dumanoglu, 2016). According to Sezgin and Dumanoglu (2016), *C. sativa* Mill. is originated in Anatolia, and it spreads throughout the Southern Europe, Northern Africa and Caucasia. *C. sativa* Mill. or sweet chestnut is most likely to be found in 25 countries of Europe (Manthos & Botu, 2013; Beccaro et al., 2019). The species also known as European, Spanish, Italian and Eurasian chestnut is also thought naturalized in Central, Western, Northern Europe and was introduced on the Pacific Coast of the USA, Australia and New Zealand (Saleh, 2003).

Manthos and Botu (2013) stated that Food and Agriculture Organization (FAO) recorded the highest production of chestnut fruit (2,023,019) in the year 2011 and with the largest area of chestnut fruit production (540,732 ha). The four main economic species of

chestnut are *C. dentata* (North American), *C. molissima* (Chinese), *C. sativa* (European) and *C. crenata* (Japanese) (Vossen, 2000; Kosnovska, 2013). The nuts in the genus *Castanea* should not be mixed up with those produced by horse chestnuts (*Aesculus* spp.) and tubers formed by water chestnuts (*Trapa* spp.) (Vossen, 2000) as they are obviously different in morphology.

2.1.2 Botany Description of *Castanea* sp.

Table 2.1 shows the taxonomical classification and nomenclature of *Castanea* sp.

Table 2.1: Taxonomic classification and nomenclature of *Castanea sativa*. Retrieved from <https://www.itis.gov/servlet/SingleRpt/SingleRpt#null>

Kingdom :	Plantae
Divisions :	Tracheophyta
Class :	Magnoliopsida
Order :	Fagales
Family :	Fagaceae
Genus :	<i>Castanea</i>

The trees of *C. sativa* Mill. is in the category of fast growing plants and it can reach heights up to 35 m. It has a notably strong trunk and a long lifespan (Gonzalez-Montelongo & Perez-Vargaz., 2021) with grey bark in colour and a retiform pattern that has deep furrows (Kosnovska, 2013). The sweet chestnut is a pioneer tree species that has a great degree of stability due to its deep taproots (Anna-Katharina & Manuel., 2018). Chestnut has

broad, long shaped and serrated leaves (Figure 1a) with five to seven inches (12.5 to 18 cm) length, oblong-lanceolate and pinnately veined (Vossen, 2000; Kosnovska, 2013; Corredoira et al., 2017).

Chestnut tree is a monoecious and self-incompatible species that generally flowers from June to July. It is reported to begin the flowering process after 8-10 years (Jacobs et al., 2016). A study made by Vossen (2000) stated chestnut flowers bloom from mid-June to mid-July and they bear annually as the flowers evade spring frosts. The flowers of chestnut trees appear in two types of catkins; slender male catkins in the lower part of shoots and female flowers clustered in the base of bisexual catkins that located around the terminal part of the shoot (Corredoira et al., 2017). Male staminate and bisexual catkins are both produced by chestnut trees. Generally, basal male catkins are the first to start developing pollen ten weeks after bud break. The basal staminate catkins produce their first pollen around the end of May to the middle of June. The females emerge to pollen-receptive a few days later and remain that way for one to two weeks. Bisexual catkins then begin to produce pollen. The majority of chestnut trees are self-sterile, so they require a pollinizer that is distinct from them (Metaxas, 2013).

Chestnut bears fruit with a smooth surface and brown in colour. The fruit is made up of two to four segmented seeds, called burrs, that are coated in multiple needle-sharp, branching spines and hold the seeds, that are protected by a hard shell (Gray, 2013). Typically, a chestnut burr produces one to three fruits. One of which is an oval fruit with an inward-bent peak and the other two are intermediate products with a vertical flat peak (Brown & Kirkman, 1990; Mancarelli, 2001; Mujic et al., 2010). Based on Saleh (2003), chestnut fruits are develop in groups of one to three single-seeded nuts with 2-3.5 cm in

diameter. A large starchy nut with cream-coloured cotyledons covered with pellicle which in astringent membrane and its thickness ranges from papery to relatively thick woody tissue. Indeed, the chestnut fruit is essentially a grain growing on a tree and the nut contains about 40% carbohydrate which makes it similar to other starchy food such as potatoes, rice and cereal grains; thus it can be among the main food in food pyramid (Vossen, 2000). Figure 2.1 shows the morphology of *C. sativa* trees; shape of leaves, female flower and nut shape.



Figure 2.1: (a) Morphology of *C. sativa* trees leaves with serrated and toothed edge, (b) shape of female flower of *Castanea* sp. in cupules, (c) which is in spiny protective husks, (d) and it turns into nut shape. Retrieved from www.plantsoftheworldonline.org/taxon/urn:Isid:ipni.org:names:295349-1

2.1.3 Importance and Uses of *Castanea* sp

Chestnut has extensive economic importance due to the high nutritional content of its fruit, valuable pollen and precious hardwood that is being used for outdoor construction and furniture (Manthos & Botu, 2013). The timber of American chestnut can be used for many uses such as providing rot-resistant and tough lumber for log cabins, fence rails, telephone pole and coffins (Oakes et al., 2016). American chestnut was known as a premier tree which providing wood for railroad ties, house framing, barns, fences and highly pleasant for human and wildlife consumption (Vossen, 2000).

American chestnuts usually are used to make purees, stuffings, stew and French delicacy called *marrons glaces* (Vossen, 2000; Corredoira et al., 2017). The American chestnut was considered the grandest tree in United States in the production of timber and nut crops and it plays role in supporting the large and diverse wildlife population (Xing et al., 1997). Oakes et al. (2016) also mentioned that wildlife population; forest dwelling species for example wild turkeys, bear, deer and squirrel mostly stuffed by the richness of chestnut seeds, and leaf and coarse woody fragments of chestnut was suggested for food and territory stream-dwelling invertebrates which in turn providing more food for fish.

Chestnuts rich with tannins, a main source of phenolic bioactive compounds (Chiarini et al., 2013) that can be extracted from its bark can be used for tanning leathers (Oakes et al., 2016). Tannins can be divided into two major groups viz. hydrolysable and condensed tannins. Hydrolyzable tannins can be subdivided into two subclasses which are gallotannins and ellagitannins. These two components are present in some species of Fagaceae family (oak and chestnut). Research has been made and proven ellagitannins have anti-atherogenic, anti-thrombotic, anti-inflammatory and anti-oxidant activity (Chiarini et

al., 2013). Previous study done by Guimaraes et al. (2010), proved that chestnut leaves, and skins revealed better antioxidant properties than almond nuts. Other than that, the leaves of chestnut have been widely used in Mediterranean regions of Europe to treat various respiratory diseases such as asthma, cough, bronchitis, cold and bronchiole infections (Chiarini, 2013). In general, chestnut contains 40% - 44% of water content, 49% carbohydrate, 5% protein and 1% of fat, making it a complete food with high nutrition content (Corredoira et al., 2017). Vossen (2000) stated that chestnuts are considered as a health food unlike other nuts and it contains high-quality starch and protein.

2.1.4 Seed Germination of *Castanea* sp.

In normal circumstances, seeds are divided into three categories: orthodox, recalcitrant and intermediate seeds. Seeds are often described as orthodox and recalcitrant depending on their ability to tolerate desiccation (Xia et al., 2014). Orthodox seeds are known as the dry seeds that could be stored in a long time periods. In contrast, recalcitrant seeds are not preferred to be stored for a long time periods due to their viability decreased with time. Meanwhile, intermediate seeds are the seeds that have characteristics in between orthodox seeds and recalcitrant seeds. The intermediate seeds could be stored longer than recalcitrant seeds (generally less than 1 year) (Yulianti et al., 2019).

The recalcitrant seeds quickly lose viability when stored using conventional techniques while being metabolically active throughout their pre- and post-harvest development, resulting in being highly susceptible to desiccation and freezing (Kijak & Ratajczak, 2020). The lower number of plant species whose seeds do not survive (considerable) desiccation is referred to as "recalcitrant" (Robert, 1973). According to Fernandez et al. (2023), recalcitrant seeds frequently exhibit cold sensitivity and rapidly lose

viability. In addition, recalcitrant seed characteristics demonstrated the situation in which seed germination rate decreased as seed moisture content decreased (Choi et al., 2022). Numerous hardwood forest trees generate recalcitrant seeds, which are seeds that cannot be stored for extended amounts of time under normal circumstances and can be only stored by cryopreservation (Corredoira et al., 2017). Cryopreservation is seen as a supplementary storage option, especially for plant tissues with unique features (vegetatively propagated species) - strategies for the conservation of forest biodiversity (Corredoira et al., 2017).

Chestnut is among the recalcitrant seeds that are susceptible to moisture loss and degradation during storage, resulting in calcification and loss of economic value (Chen et al., 2022). This fact is supported by Du et al. (2020) as they stated, chestnut seeds are recalcitrant and showed dormancy, unlike the majority of other recalcitrant seeds. Due to its dormancy, the edible seeds have a longer shelf life and have a greater capacity to withstand adverse conditions. According to Leon-Lobos and Ellis (2018), *Castanea sativa* Mill. forest trees produce huge seeds that are shed at high moisture content with recalcitrant seed storage behaviour. The majority of the trees that are utilized sustain forest ecosystems and contribute significantly to the survival of wild animals and the ecological restoration of forests are included in the Fagaceae family (Liu et al., 2022). In an effort to overcome chestnut recalcitrance, a corpus of information outlining a wide variety of micropropagation methods has been developed (Fernandes et al., 2020).

Recent study on seed germination of *Castanea sativa* Mill. has been done *in vivo* (Tumpa et al., 2021). The study aimed to investigate the effect of seed size on germination and seedling growth of the species. In this study, the seeds were collected from 12 native

sweet chestnut population in Croatia. The seeds were then selected according to ISTA (International Seed Testing Association) rule (Tumpa et al., 2021).

2.1.5 Micropropagation of *Castanea* sp.

Earlier study reported that embryonic cultures from chestnut seed were established from leaf explants and juvenile zygotic embryos but only in low induction rates (Corredoira et al., 2005). In other study performed by Osterc et al. (2005) suggested the MS medium supplemented with BAP hormone promoted the strongest growth of newly formed chestnut shoots with 1.2 cm height on average and the finding concludes that the uses of BAP as cytokinin was the most productive hormone in the experiment. In contrast, the formation of chestnut embryoids also did give good results in treatment 10 mg/L 2,4-D with 0.5 mg/L Kinetin (Gonzalez et al., 1985). Several studies have shown that chestnut can be micropropagated from both young and adult plants using the axillary shoot development method and somatic embryogenesis (Vieitez et al., 2007). *C. sativa* (cv. “Montemarano”) shoot cultures also have undergone slow growth storage for 48 months at a temperature of 8°C. The results showed 82% of explants survived and were able to restart normal growth after being shifted to conventional culture conditions at 23°C (Capuana & Lonardo, 2013). According to Mullin (1987), nodal explants from a 2-year-old seedling and an adult selection (W1005) of *C. sativa* cultured on half MS medium fortified with 0.1mg/L BA gave 100% of axillary bud break within a month. Previous study reported by Algül and Dalkılıç (2014) clarified the shoot formation level in the MS medium added with $\frac{1}{2}$ NH_4NO_3 and KNO_3 was 41.48%, while in the GD + MS added with $\frac{1}{2}$ NH_4NO_3 and KNO_3 was 25.51%. Both findings showed the achievement of micropropagation of *C. sativa* Mill. in semi solid medium. Other most recent study on micropropagation of *Castanea* sp. clarified the growth of new shoots

is facilitated during the establishing phase by the application of a BAP concentration within the range of 0.5–2.0 mg/L (Pavese et al., 2022).

2.2 Principles of Plant Tissue Culture

According to Vasil and Vasil (1972), The Cell Theory of Schleiden and Schwann, originally in 1838–1839, and popularised by Virchow in 1858 with his famous phrase "omnis cellula e cellula," contains the idea of cellular totipotency (every cell from a cell). However, until 1878, when VSchting was successful in dividing plants up into small fragments and maintaining the fragments' viability and growth, no attempts were made to gather any experimental data to support the hypothesis of totipotency. Totipotency is a concept that describes the fact that cells retain their full ability for development and, given the right circumstances, can proliferate and produce all the different kinds of cells that make up a new creature (Grafi et al., 2011). Totipotency is known as the production of an entire plant from a few cells or a small section of a plant; the establishment of missing plant organs or parts (Mineo, 1990). Nowadays, tissue culture become a well-established technique for *in vitro* propagation that includes cell division, proliferation and embryo induction under precisely controlled conditions (Shahzad et al., 2017). Most of the methods of plant tissue culture are depends on technologies that were adopted over 20 years ago (Swamy et al., 2014). Plant tissue culture is an essential technique in plant biotechnology that has given a tremendous contribution to plant propagation and producing a high number of crop varieties in forestry, agriculture and horticulture (Handique & Das, 2002). Ordinarily, *in vitro* tissue culture or micropropagation is defined as the culture of diverse somatic cells, tissues or organs of plants under controlled *in vitro* conditions with the intent of producing many progeny plants which identical to the stock plants, in a short time compared to conventional propagation

(Ahmadian et al., 2013). Gaikwad et al. (2017), mentioned that the categories of tissue culture plants are basically disease free growth, higher survival rate and have healthier and fibrous root system.

The plant tissue culture is preferred rather than soil grown plants because the plant grown *in vitro* are easier to transport to any place (Gaikwad et al., 2017). *In vitro* propagation also become essential to meet the high demand requested from the world trade market and also for the conservation of valuable stock plants as it produces the planting materials in a large scale (Nilanthi & Yang, 2014). Around the mid twentieth century, this plant tissue culture technique is applied in the plant propagation industry worldwide (Akin-Idowu et al., 2009). Tissue culture technique is an alternative for the propagation of plants with no seed or the plants which require pollinators to produce seeds (Akin-Idowu et al., 2009). Although most of the plants can be propagated using conventional propagation such as grafting, stem cuttings and marcotting however the viability of plant production has been restricted due to epidemic of diseases such as root knot nematodes, mosaic virus and insects' pests (Swamy et al., 2014). By tissue culture technique, the production of plants in sterile conditions could reduce the chances of transmitting severe diseases (Akin-Idowu et al., 2009). Other advantages of the tissue culture technique are the production of good quality plants for commercialization, pharmaceutical purposes and production of secondary metabolites by considering nutritional and hormonal composition of nutrient medium, duration of culture and growth condition of plant species (Siwach & Gill, 2014). Thus, determine the optimum medium needed in order to get free disease plants and high quality of propagules.

2.3 Types of Media in Plant Tissue Culture

The main factors that commonly influence the mass production of plants are the types of medium, the type of explant used for *in vitro* regeneration and the physical environment (Swamy et al., 2014). In fact, there are many types of media used for *in vitro* propagation. Murashige and Skoog (MS), modified MS (MMS), Gamborg's B5 medium, B5 modifications, Woody Plant Medium (WPM), and Driver and Kuniyuki Woody Plant medium (DKW) are the most prominent basal media's composition to elicit and support various plant growth responses (Philips & Garda, 2019). MS medium is a basic medium that is often used in plant tissue culture. According to Saad and Elshahed (2011), other basic media that are frequently used include Woody Plant Medium (WPM), Gamborg B5 medium, Nitsch and Nitsch medium and Linsmaier and Skoog (LS) medium. Research by Kajani et al. (2012), reported numerous basal media such as Gamborg (B5), Murashige and Skoog (MS), Woody Plant Medium (WPM), Driver and Kuniyuki (DKW) and Schenk and Hildebrandt (SH) used to maintain, initiate callus culture and suspension cultures. Previous study by Nas et al. (2013) discovered that MS medium took 5 years to develop as a defined medium for the tissue culture of *Nicotiana tabacum*. Typically, the plant tissue culture media contain all the following elements: micronutrients, vitamins, amino acids, sources of carbon, undefined organic supplements, growth regulators and solidifying agents (Saad & Elshahed, 2011).

2.4 Roles of Sucrose

The role of carbon source is as a remedy of osmotic potential and capable in the induction of root. Source of carbohydrate, 3% sucrose is commonly used (Ahmadian et al., 2013). Sumaryono et al. (2012), indicated that carbohydrates usually added to tissue culture

medium because they can be applied as an energy source and an osmotic agent. As mentioned by Swamy et al. (2010), about 2-5% of sucrose is the most general carbon source that was used in tissue culture studies as it has efficient uptake across the plasma membrane. Mosaleeyanon et al. (2004), stated that the earliest growth and morphogenesis of buds and leaves are essential for subsequent photosynthetic activities if the sucrose is present in the culture medium. Sucrose is known as the best carbon source in previous research on *Mentha piperita*, *Stevia rebaudiana* and *Solanum nigrum* as it is the most common carbohydrate that found in the phloem sap of many plants (Mahadev et al., 2014).

2.5 Roles of Coconut Water

Organic supplements such as coconut water, hydrolyzed casein, fruit juices and yeast or malt extracts play a vital role in the plant tissue culture industry. Coconut water is one of the organic supplements that is widely used as a growth-promoting element in tissue culture medium (Yong et al., 2009). Coconut water become famous in the tissue culture industry due to its cytokinin-type activity that promotes rapid growth and helps in cell division. In 1941, coconut water was introduced by Van Overbeek et al., as new constituent of a nutrient medium for callus culture (Prando et al., 2014). Amongst all the organic supplements, coconut water is proven to be the best supplement for *in vitro* seed germination and seedling growth (Thejaswini & Narasimhan, 2017). Studies about coconut water validate that coconut water is rich in various type of cytokinins which are N⁶-isopentenyladenine, dihydrozeatin, *trans*-zeatin, kinetin, *ortho*-topolin, dihydrozeatin O-glucoside, *trans*-zeatin O-glucoside, *trans*-zeatin riboside, kinetin riboside and *trans*-zeatin riboside-5'-monophosphate (Yong et al., 2009). Earlier research by Gansau et al. (2016) suggested that coconut water is ample with beneficiary elements such as amino acids, organic acids, nucleic acids, plant regulators

with zeatin and minerals, carbohydrates and vitamins. Additional nutrients from coconut give benefits in the development and growth of plant species such as elongation of shoot and root, proliferation and induction of callus and somatic embryogenesis (Kamaruzzaman et al., 2018).

2.6 Roles of Activated Charcoal

In vitro plant propagation is always restrained by several issues such as somaclonal variation, exudation of phenolics and poor quality of plant species (Gnasekaran et al., 2012). Woody plants especially, contain high phenolic exudation which can activate the oxidative enzyme that would lead to the failure of plant tissue culture (Ahmad et al., 2013). The challenge usually related to injury caused during sectioning of explant is necrosis of plant tissue. Thus, activated charcoal is a suitable option to minimize tissue necrosis and eventually could reduce this problem (Furlan et al., 2018). Charcoal is among the carbon source with a strong adsorptive capacity for gases, vapours, and colloidal particles. It is formed through the destructive distillation of carbonaceous materials such as wood, peat, lignite, nut shells, bones, and vegetables (Pan & Staden, 1998). Because of its extraordinarily high surface area, very finely distributed pores, and high volume, activated charcoal has a phenomenal capacity for adsorption (Thomas, 2008). Usually, better growth of the plant species might be due to the addition of activated charcoal supplemented in the tissue culture medium (Ebert et al., 1993). Charcoal is used to promote morphogenesis including embryogenesis, formation of root induction and adsorption of secreted compounds from tissue culture (Bhatia & Ashwath, 2008). Besides, it is known for its carbon structures which are odourless, tasteless and nontoxic substances (Prizao et al., 2012). Study by Buckseth et al. (2018) suggested that activated charcoal can improve cell growth and development of

plant species as well as it can stimulate the uptake of nitrogen by shoot of plant species and promotes *in vitro* rooting in dark environment. By adsorbing inhibitors such as phenolic compounds and abscisic acid, activated charcoal inhibits the breakdown of the auxin supplied to the medium and stimulates plant growth (Simsek et al., 2017).

2.7 Types of Explants

Next, the type of explant also plays an important role in order to produce a large number of plant species. Shoot apices from *H. radiata* were the most effective part for tissue culture as it showed 33% of survival rate after 8 weeks of culture (Mitsukuri et al., 2009). Another research by Ibrahim and Daraj (2015) mentioned that among all sources of explant; cotyledon leaf, hypocotyl, shoot tip, nodal and root segments, only shoot tips and hypocotyl showed the highest response to the formation of shoots about 86.67% and 43.33% respectively. Embryonic structures such as seeds, cotyledons and root apices have high embryogenic potential because they are substantial in meristematic tissues and as the structure become matures (leaves, petiole and stems), this potential tends to decrease (Moura et al., 2017). This statement supported by Gitonga et al. (2010) that stated only nodal segments and cotyledonary explants signified possibility of shoot regeneration with 63% bud break within two weeks and fully formed shoots after five weeks. According to Sanchez et al. (1997), *C. sativa* and *C. sativa* x *C. crenata* shoot cultures were grown *in vitro* by utilizing shoot tips and nodes from stump sprouts, epicormic shoots and crown branches harvested from adult trees whereby, the result showed the basal nodal segment was the most effective explant type for subculturing, specifically when callus tissue was left on the subcultured basal segment. Previous research by Xiong et al. (2018) mentioned that one of the factors that influenced shoot proliferation rates and quality is the type of explant;

cotyledonary nodes of *C. henryi* resulted in high percentage of adventitious shoots (85.67%) with 0.1 mg/L 2,4-D.

2.8 Roles of Plant Growth Regulators (PGR)

Plant growth regulators are essential in plant tissue culture as they are significant in stem elongation, tropism and apical dominance of plants (Saad & Elshahed, 2011). There are several classes of plant growth regulators that are frequently used in plant tissue culture media, for instance auxins, gibberellins, abscisic acid (ABA), ethylene and cytokinins (Plant & Soil Sciences eLibrary, 2022). According to Hill and Schaller (2013), *Arabidopsis thaliana* easily endures de novo shoot organogenesis in high level of endogenous cytokinin. Nowadays, the most frequently used cytokinins in plant tissue culture are Kinetin (KIN) and Benzylaminopurine (BAP) (Gold Biotechnology, 2019).

Study by Jenik and Barton (2005) stated that auxin represents small molecules which include indole acetic acid (IAA) and indole butyric acid (IBA). Auxin plays a vital role that involves a wide array of development and growth processes, for instance to initiate root, flowers and leaves, including the formation of meristem (Hu et al., 2017). Auxins also generate the formation of roots, callus and did the extension development of stems (Efretuei, 2018).

Other than that, cytokinin activates the division of cells. According to Efretuei (2018), the division of cells can stimulate the establishment of shoot apical meristems following shoot buds initiation. Among all the cytokinin, Thidiazuron (TDZ) is the most active cytokinin-like substance for woody plant tissue culture (Huetteman & Preece, 1993). Cytokinin also acts as an essential tool for *in vitro* flowering as it can help in flower induction, initiation and floral development process (Mohamed et al., 2016). Several studies

agreed the most efficient PGRs for shoot proliferation of woody plants is a combination of cytokinin and auxin (Mathur et al., 2002; Siwach & Gill, 2011; Li et al., 2016). Additionally, (6-benzylaminopurine) 6-BA promoted an excellent outcome as a shoot-multiplication inducer for various woody plants, including American and European chestnuts (Rodriguez, 1982; Xing et al., 1997).

Gibberellins usually act as a signal to communicate stress tolerance between roots and shoots (Colebrook et al., 2014). Cell elongation has been shown to be a main component in stem elongation during early embryo development (Hays et al., 2002). The application of gibberellins also promotes stolon elongation and inhibits tuber formation (Xu et al., 1998). According to Gupta and Chakrabarty (2013), gibberellin initiates seed germination, activates the transitions from meristem to shoot growth, vegetative to flowering and determines the sex expressions. Gibberellins also performs as natural regulators in seed germination to generate the production of a hydrolytic enzyme (Gupta & Chakrabarty, 2013).

CHAPTER 3

MICROPROPAGATION OF *Castanea sativa* Mill.

3.1 Experimental Aims

Nowadays, numerous researchers have focused on the production of *C. sativa* via asexual propagation methods that offer promising prospects for commercialization. Commercial multiplication via asexual propagation is widely used and preferable to prevent genetic segregation and long juvenile phase during seed germinations (De Souza et al., 2013). Although grafting is the most frequent method used for conventional cultivation of *C. sativa*, micropropagation seems convincing of supplying sufficient elite genotypes, besides providing high quality yields (Vieitez et al., 2007) which need to be considered for rapid and large scale production of planting materials. Tissue culture techniques assist in the industrial production of improved crop varieties viz. disease-free plants, varieties tolerant to salinity, drought and heat stresses also enhance the production of secondary metabolites (Hussain et al., 2012). Several studies have been reported on successful micropropagation of *C. sativa* through direct regeneration however the results presented less shoot production (Osterc et al., 2005; Tafazoli et al., 2013). Meanwhile, previous studies on indirect regeneration via somatic embryogenesis of *C. sativa* are still not properly addressed (Corredoira et al., 2003; Corredoira et al., 2005). Therefore, this research attempts to establish the efficient protocol for seed sterilization that is essential for the production of aseptic seedlings as the source of explants in the present research. Besides, this research also investigates the optimum medium for *in vitro* regeneration of *C. sativa*, enhance the plantlet production and, study the microscopic studies to reveal the stomata dispersion meanwhile the histological studies to disclose a unique internal leaf structure of *C. sativa*.

Several *Castanea* species that have been successfully propagated include *Castanea dentata* Marsh through microcuttings grown in Woody Plant Medium (WPM) + 500 mg/l PVP + 0.89 μ M Benzyladenine (BA) (Xing et al., 1997) and somatic embryogenesis of *Castanea* spp. through different part of explants like immature and mature zygotic embryos and leaf sections (Corredoira et al., 2005). Besides, previous study by Gonzalez et al. (1985) also revealed the successful production of somatic embryos through cotyledon of hybrid, *C. sativa* x *C. crenata*. In another study, *C. sativa* was successfully propagated through nodal explants cultured in Murashige and Skoog medium (MS) + $\frac{1}{2}$ NO₃ + 1 mg/l 6-Benzylaminopurine (BAP) but with lower intensity of shoot formation (Osterc et al., 2005). The latest research on plant regeneration of *C. sativa* Mill. through indirect organogenesis discovered the highest shoot multiplication, 66.9% was observed in MS + 0.2 mg/l Thidiazuron (TDZ) (Tafazoli et al., 2013).

Typically, the growth of explants is affected by the type of plant growth regulators and explants used in the experiment. Therefore, in this chapter, the experiment aimed to study the effects of plant growth regulators (PGRs) such as 6-Benzylaminopurine (BAP) and Indole-3-Butyric Acid (IBA) applied singly with supplementation of coconut water (CW) and activated charcoal (AC) for *in vitro* regeneration of *C. sativa*. For shoot and root induction, nodal segments as the explants were cultured in various treatments with different concentrations of PGRs. In this experiment, parameters observed including the mean number of shoots per explant, mean percentage of explants produced shoots, mean shoot height of explants, mean number of leaves per explant and mean leaves length. The optimum medium for shoot induction was identified based on the data analysis.

Besides, other parameters have been observed on the epidermis of leaf surfaces, shoot tips structures, stomata structure, stomata density and sizes of stomata opening”. The location affects the internal structure of leaves whereby it was found that the mesophyll thickness reduced primarily attributed to a lower presence of palisade parenchyma. In contrast, the *C. sativa* leaves displayed significantly lower stomatal density, while the leaf area demonstrated the highest expression (Pinto et al., 2011).

3.2 MATERIALS AND METHODS

3.2 Source of Explants

Castanea sativa seeds bought at The Spring Shopping Mall in Kuching, Sarawak (Figure 3.1), were surface sterilized before being grown *in vitro* on MS basal medium. The seeds were germinated as early as the third days of culture whereby their roots appeared first in the culture medium. After two months, the aseptic seedlings grown on MS basal medium with a size within the range of 4-6 cm height were selected and sectioned into small pieces before cultured on fresh MS medium supplemented with different concentrations of 6-Benzylaminopurine (BAP), 1-Naphthaleneacetic acid (NAA) and Thiamine (applied singly or in combination) for shoot induction.

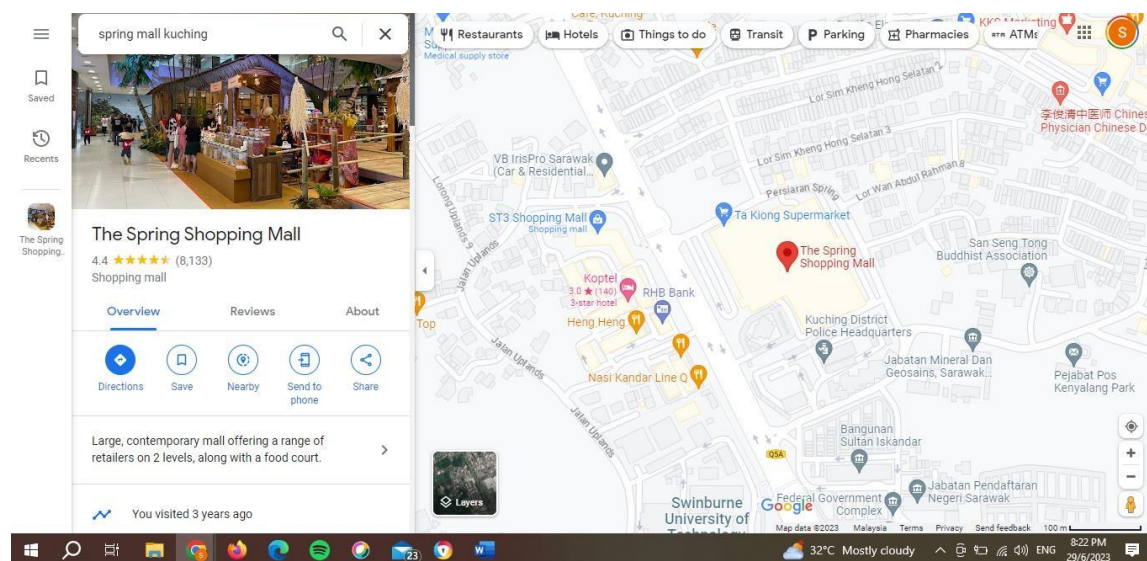


Figure 3.1: Map location of The Spring Shopping Mall in Kuching.

3.2.2. Preparation of MS Medium

Murashige and Skoog (MS) medium is the most recommended for *in vitro* plant propagation due to its feature possessing very high concentration of nitrate, potassium and ammonia (Misawa, 1994). Preparation of the medium was following the standard composition of Murashige and Skoog (MS) medium containing macronutrients, micronutrients, irons and vitamins (refer Appendix I). Besides, other practices using instant Murashige and Skoog (MS) medium in powder form which is commercially prepared and purchased from suppliers also applied for *in vitro* plant regeneration.

To prepare 1 litre medium, 4.4 g of instant Murashige and Skoog (MS) powder was weighed and dissolved in 800 ml of distilled water in 1 litre Schott bottle. After that, 30 g of sucrose was added to the solution before the medium was topped up to 1 litre by distilled water. The MS basal medium was placed on the hot plate and stirred using a magnetic stirrer until the sucrose and MS powder completely dissolved. Next, the pH of MS basal medium

was adjusted between 5.79-5.82 with some addition of 1.0 N potassium hydroxide (KOH) or 1.0 N sodium hydroxide (NaOH) to increase the pH readings while 1.0 N hydrochloric acid (HCl) is applied to reduce the pH readings. Subsequently, 3g of gelrite was added to Murashige and Skoog (MS) medium solution before the medium was autoclaved at 121°C with a pressure of 104 kPa (15 psi).

3.2.3 Preparation of Stock Hormone

Stock hormones (PGRs) were prepared earlier before being added to the Murashige and Skoog (MS) medium solution (treatments for shoot and root induction). For the preparation of 1 mg/ml of stock hormone, 100 mg of hormone (PGR) in powder form was dissolved with a few drops of NaOH/KOH and HCl. Next, 100 ml of distilled water was added when a clear solution of stock hormone was obtained. After that, the stock hormone was wrapped with aluminium foil and kept in a freezer or used immediately in media preparation. In this research, only 6-Benzylaminopurine (BAP) and Indole-3-Butyric Acid (IBA) were applied to investigate the most favourable medium for direct regeneration of this species.

3.2.4. Sterilization of Seeds

Seeds of *C. sativa* were rinsed with Dettol and scrubbed with a clean toothbrush until the seeds were completely clean. After that, the seeds were soaked in tap water while stirring using a magnetic stirrer on a hot plate to remove the dirt on the shell of the chestnut. The seeds were then washed under running tap water for 2 hours. Next, the seeds were soaked in 70% Clorox added with a few drops of Tween 20 for 15 minutes. The seeds were subsequently rinsed with sterile distilled water three times. The sterilization process was continued by soaking the seeds in different concentrations of Clorox (50% and 30%). A

similar procedure was used for each step. Then, the seeds were soaked in 70% ethanol for about 1 minute and rinsed with sterile distilled water. This step was carried out in the laminar air flow whereby the seeds were then dried on sterilized paper before the shell of chestnuts were peeled off afterwards. Once the outer shell was completely removed, the seeds were soaked again in 70% ethanol for 1 minute and rinsed with sterile distilled water to ensure the inner skin and contaminants were fully eliminated.

After drying, the sterilized seeds were eventually cultured on Murashige and Skoog (MS) basal medium (20 seeds/replicate for each treatment). Overall, 140 aseptic seedlings were successfully grown on Murashige and Skoog (MS) basal medium. After 2 months of culture, the explants (nodal segments) were selected for further study on *in vitro* shoot regeneration. In this experiment, the explants were cultured on Murashige and Skoog (MS) medium added with coconut water (CW), activated charcoal (AC) and fortified with different concentrations of 6-Benzylaminopurine (BAP), 1-Naphtaleneacetic acid (NAA) and Thiamine (applied singly or in combination). All apparatus necessary for culturing such as forceps, scalpel and etc. were wrapped with aluminium foils and autoclaved before use. Laminar air flow surfaces need to be sterilized with 70% ethanol before culturing is conducted. After culturing, all the cultures were kept in the culture room at $25 \pm 2^{\circ}\text{C}$ under 16 hours light provided by fluorescent light (36W), PHILIPS.

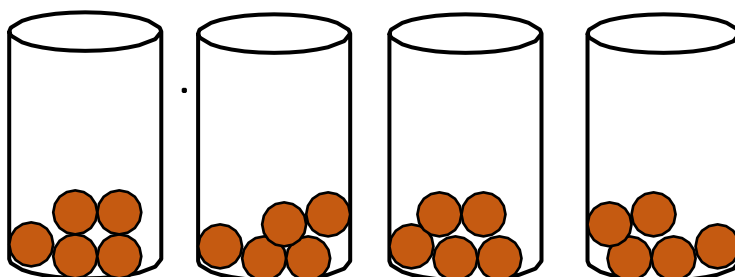


Figure 3.2: The diagram showed 5 seeds for each beaker, a total of 20 seeds per replicate for each treatment.

3.2.5. Seed Germination and Seedling Development

In seed germination and seedling development, healthy seeds were selected. The selection of seeds was following the International Seed Testing Association, International Rules for Seed Testing, International Seed Testing Association, Basserdorf, Switzerland (ISTA, 2008). The seed's weight (g) was subsequently measured using analytical balance and classified accordingly such as small (< 5g), medium (5 – 8 g) and large (> 8g). Then, the seeds were subjected to sterilization procedures before being cultured in the media provided; Murashige and Skoog (MS basal). Observations were done every week on the stages of seed germination. Meanwhile, seed germination rate, percentage of rooted seedlings, percentage of shoot and leave production were measured after 1 month of culture.

3.2.6. *In Vitro* Plant Regeneration

3.2.6.1. Effects of CW, AC, BAP, NAA, Thiamine Applied Singly or in Combinations on *In Vitro* Plant Regeneration

In plant regeneration *in vitro*, 2 months of aseptic seedlings derived from the previous study were used as the explants (nodal segments). The selected explants were sectioned into an average of 1.0 cm length before being cultured in all treatments tested. There were 20

explants cultured in each treatment whereby, one explant was placed per jar. The explants were cultured in the culture medium containing coconut water of approximately 1% (10 ml of coconut water added in 1 litre medium) was collected from Matag variety purchased at Samarahan market and was incorporated in the media along 1% of activated charcoal (0.01g of charcoal added in 1 litre medium), the other substances including hormones (applied singly or in combination) and vitamin in this experiment. All treatments tested throughout this study were listed as follows;

1. MS (Control/medium without any supplementation of hormone)
2. MS + 1% CW
3. MS + 1% CW + 1% AC
4. MS + 1% CW + 1% AC + 1.0 mg/l BAP
5. MS + 1% CW + 1% AC + 2.0 mg/l BAP
6. MS + 1% CW + 1% AC + 3.0 mg/l BAP
7. MS + 1% CW + 1% AC + 4.0 mg/l BAP
8. MS + 1% CW + 1% AC + 1.0 mg/l BAP + 1.0 mg/l NAA
9. MS + 1% CW + 1% AC + 2.0 mg/l BAP + 1.0 mg/l NAA
10. MS + 1% CW + 1% AC + 3.0 mg/l BAP + 1.0 mg/l NAA
11. MS + 1% CW + 1% AC + 4.0 mg/l BAP + 1.0 mg/l NAA
12. MS + 1% CW + 1% AC + 1.0 mg/l BAP + 1.0 Thiamine HCL
13. MS + 1% CW + 1% AC + 2.0 mg/l BAP + 1.0 Thiamine HCL
14. MS + 1% CW + 1% AC + 3.0 mg/l BAP + 1.0 Thiamine HCL
15. MS + 1% CW + 1% AC + 4.0 mg/l BAP + 1.0 Thiamine HCL
16. MS + 1% CW + 1% AC + 1.0 mg/l BAP + 1.0 mg/l NAA + 1.0 Thiamine HCL

17. MS + 1% CW + 1% AC + 2.0 mg/l BAP + 1.0 mg/l NAA + 1.0 Thiamine HCL

18. MS + 1% CW + 1% AC + 3.0 mg/l BAP + 1.0 mg/l NAA + 1.0 Thiamine HCL

19. MS + 1% CW + 1% AC + 4.0 mg/l BAP + 1.0 mg/l NAA + 1.0 Thiamine HCL

3.2.6.2. Microscopic Studies

Further studies on the morphology of *in vitro* propagated plantlets were done. In this study, the 6 months old plantlets (control and MS + 1% CW + 1% AC + 4.0 mg/l BAP) were selected and subjected to microscopy analysis using a field emission scanning electron microscope (FESEM). Observations were made on the epidermis of leaf surfaces, shoot tips structures, stomata structure, stomata density and sizes of stomata opening. The methods of preparing samples for field emission scanning electron microscope (FESEM) was included in Appendix 2.

3.2.7 Data Analysis

Data obtained in *in vitro* propagation were statistically analyzed using Duncan's Multiple Range Test (DMRT). Means with the same letters in the same column were not significantly different at $p \geq 0.05$. Data for the percentage of seed germination, mean number of shoots, mean number of leaves and mean length of leaves were analyzed using One-Way ANOVA. Meanwhile, for microscopic studies, the data including the mean size of stomata opening and mean number of stomata were analyzed using independent samples T-test ($p = 0.05$) using the IBM SPSS statistical package version 35.

3.2.8 Experimental Outline

Figure 3.1 shows the overall experimental outline of this research.

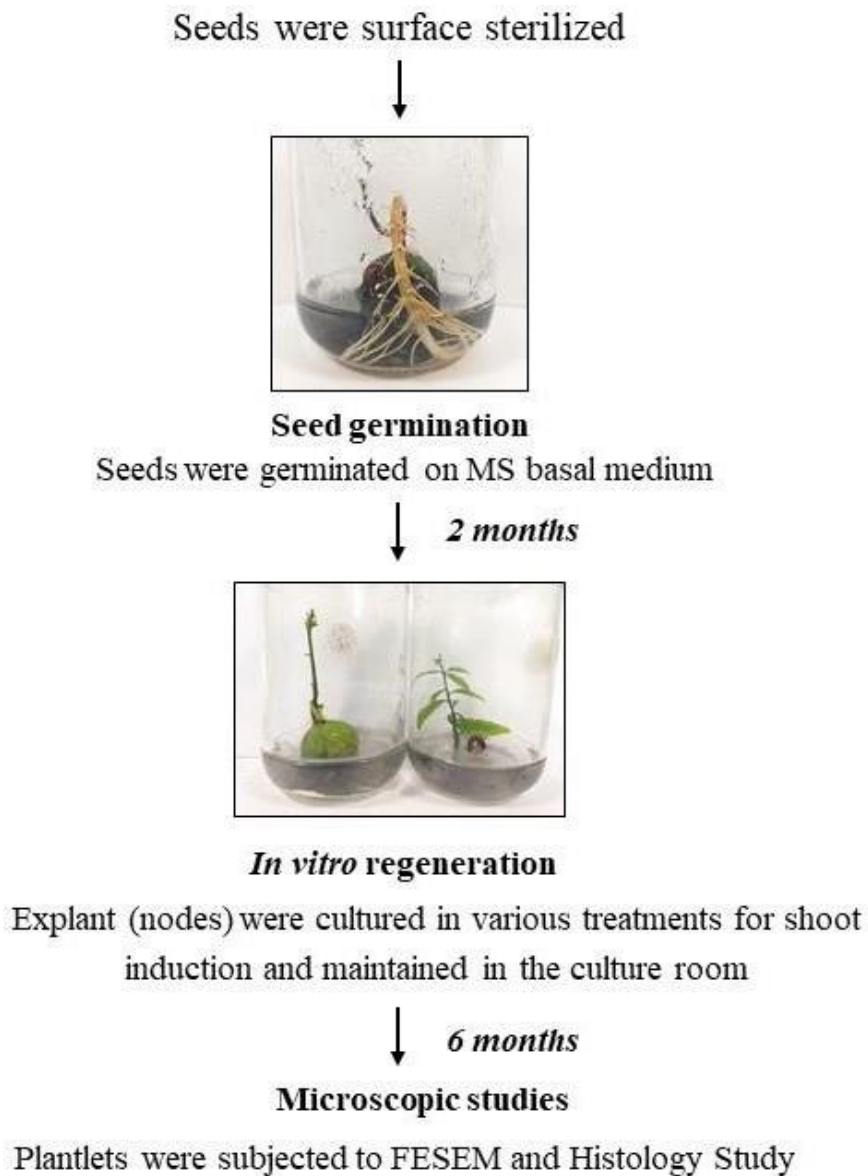


Figure 3.3: The overall experimental outline of this research

CHAPTER 4

RESULTS

Seed sterilization is crucial for the production of aseptic seedlings that would be used as explants for further studies on *in vitro* plant regeneration. Initially, the seeds were subjected to different sterilization procedures to identify the efficient procedure for the production of aseptic seedlings. In this experiment, about 20 seeds of *C. sativa* were used in each trial. After sterilization, the seeds were cultured in glass jars containing MS basal medium, one seed per jar. Observations were done every week to ensure the aseptic seedlings that grew in the basal medium were free from any contamination and get enough nutrients for plant growth and development.

4.1 Effects of Different Sterilization Procedures

In the first batch of trials, a basic sterilization procedure was used whereby the seeds were directly washed under running tap water for 2 hours. Next, the seeds were soaked in different concentrations of Clorox (70%, 50% and 30%) for 15 minutes, respectively. Then, the seeds were rinsed three times with sterile distilled water for 15 minutes before being cultured on MS basal medium. The result recorded was unsatisfactory because the contamination that occurred was quite severe. Observations revealed that the explant contaminated with fungus could be seen within 48 hours after culture. After one week, most of the explants were contaminated with a very low rate of germination, 20% in data analysis. Some of the explant contamination could be clearly observed as the explant was overgrown by the fungus (Figure 4.1). Thus, the sterilization technique applied in the first batch was insufficient for *C. sativa* seeds. Considering the high rate of contamination that occurred in the first batch, several modifications in the previous procedures were done for the second

batch of trials. Figure 4.1 shows severe contamination was observed in the first batch of trials after one month of culture.

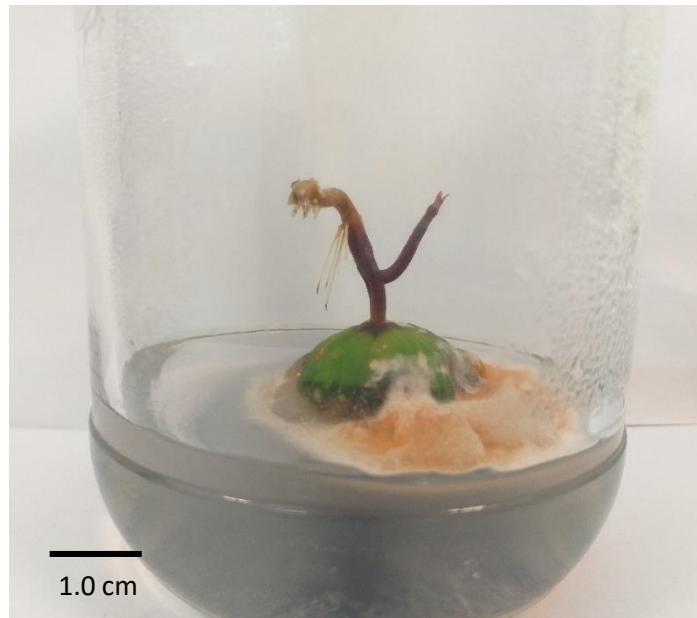


Figure 4.1: Severe contamination was observed in the first batch of trials after one month of culture. Fluffy orange and creamy colour of fungus grown on the explants cultured on MS medium.

In the second batch of trials, another 20 seeds of *C. sativa* were sterilized using concentrations of 1% Dettol and 1% Betadine before the seeds were soaked in 70%, 50% and 30% Clorox for 15 minutes, respectively. After that, the seeds were rinsed with sterile distilled water thoroughly until it was completely clean. Then, the seeds were soaked in 90% Ethanol for 1 minute and subsequently rinsed three times with sterile distilled water. The seeds were left in the laminar air flow for 30 minutes until they were completely dried. The dried seeds were peeled and cultured in the MS basal medium as in the previous trial. A better result was obtained as the contamination was under controlled with a higher rate of seed germination, 60%. However, the growth of the aseptic seedling was quite slow (Figure

4.2). Thus, further modification was done in the third batch of trials by reducing the times taken for the seed sterilization. Figure 4.2 shows seed germination of *C. sativa* grown on MS basal medium after 1 month of culture.



Figure 4.2: Seed germination of *C. sativa* grown on MS basal medium after 1 month of culture.

In the third batch of trials, the same sterilization procedures as the second batch was applied with a few modifications. The seeds were washed under running tap water for 1 hour and the times taken to soak the seeds in various concentrations of Clorox were reduced to 10 minutes each. The result obtained was better compared to the first and second batch of trials. Observation recorded almost 80% of seed germination.

4.2 Seed Germination and Seedling Development

Stages for growth and development of *C. sativa* were recorded (Figure 4.3). Observation clarified that *C. sativa* promoted hypogeal germination whereby, the cotyledons

remain in the growing substrate during the germination process. In general, the seeds cultured on MS basal medium changed their colour from pale to bright yellow after a few days of culture (Figure 4.3a). The yellow colour of seeds slowly turned to greenish yellow which was followed by the emergence of the radicle. The radicle started to grow downward after 1 week of culture. Most of the seeds turned to green colour after 2 weeks of culture (Figure 4.3b). A plumule subsequently emerged and this process was continued by the elongation of epicotyl. The plumule has grown and eventually developed into a shoot tip. The shoot initiation (Figure 4.3c) from apical meristem could be detected as early as three weeks of culture. After 1 month, the shoots within the range of 1-2 cm height continue producing true leaves and roots (Figure 4.3d). Data recorded after 1 month revealed the aseptic seedlings promoted 100% of root production. Meanwhile, the shoot and leaf production were only about 33.33% and 22.22%, respectively. The healthy aseptic seedlings aged 2-month-old (Figure 4.4) were selected for further studies on *in vitro* regeneration of *C. sativa*. Figure 4.3 shows stages for seedling development of *C. sativa* and Figure 4.4 shows healthy aseptic seedling (2-month-old) of *C. sativa* grown vigorously was selected for further studies on *in vitro* plant regeneration.

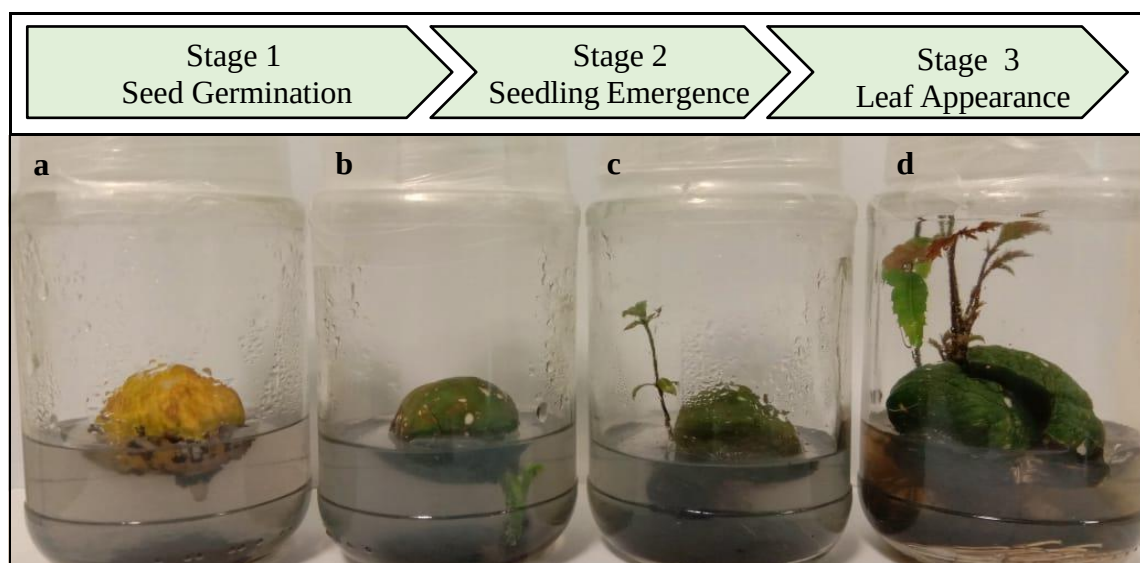


Figure 4.3: Stages for seedling development of *C. sativa*. (a) Seed cultured on the medium changed colour from pale to bright yellow and slowly turned to greenish yellow with the radicle grows downward after 1 week of culture. (b) Seed turned to green after 2 weeks of culture. (c) Aseptic seedling continues growing and (d) produced true leaves and hair roots after 1 month of culture.



Figure 4.4: Healthy aseptic seedling (2-month-old) grown vigorously was selected for further studies on *in vitro* plant regeneration.

4.3 *In Vitro* Plant Regeneration

4.3.1 Effects of CW, AC, BAP, NAA, Thiamine Applied Singly or In Combination on *In Vitro* Plant Regeneration.

The results on *in vitro* plant regeneration of *C. sativa* were presented in Table 4.1 and Table 4.2. Table 4.1 presented the observations on the mean number of shoots produced, percentage of explants producing shoots and mean shoot height. In general, the mean number of shoots produced per explant within 2 months were within the range of 1-2 shoots per explant and the average of shoot produced for all treatments was 1.16 ($\approx$ 1 shoot per explant). Overall, there was no significant difference in the mean number of shoots produced between all treatments and control (MS basal medium). The study revealed the highest mean number of shoots was 1.45 ($\approx$ 2 shoots per explant) which was observed in two treatments viz. BAP alone (MS + CW + AC + 3 mg /L BAP) (Figure 4.5b) and a combination of BAP and NAA (MS + CW + AC + 3.0 mg/L BAP + 1.0 mg/L NAA) (Figure 4.5a). Then followed by treatment with BAP alone supplemented with Thiamine HCL (MS + CW + AC + 2.0 mg/l BAP + 1.0 mg/l Thiamine HCL) and treatment with combination of BAP and NAA supplemented with Thiamine HCL (MS + CW + AC + 4.0 mg/l BAP + 1.0 mg/l NAA + 1.0mg/l Thiamine HCl) that produced 1.40 and 1.30 mean number of shoots ($\approx$ 1 shoot per explant), respectively. The result was comparable with the control and most of the treatments tested. The lowest mean number of shoots produced was 0.85, observed in treatment with MS + CW + AC.

Almost all the treatments including control recorded 100% of explant producing shoots (Table 4.1). 100% of explant producing shoots were obtained in all treatments supplemented with Thiamine HCl. Meanwhile, 100% of explants producing shoots were

observed in half of the treatments in BAP alone (MS + CW + AC + 1.0 mg/l BAP, MS + CW + AC + 2.0 mg/l BAP) and half of the treatments in the combination of BAP and NAA (MS + CW + AC + 3.0 mg/l BAP + 1.0 mg/l NAA, MS + CW + AC + 4.0 mg/l BAP + 1.0 mg/l NAA). Hence, the addition of Thiamine HCl at lower concentrations of BAP alone (1 and 2 mg/l BAP) and higher concentrations of BAP and NAA in combination (3 and 4 mg/l BAP + 1 mg/l NAA) induced shoot production. Higher concentrations of BAP alone (3 and 4 mg/l BAP) resulted 90 - 95% of explant producing shoots. In fact, only several treatments (MS + CW + AC + 1 mg/l BAP + 1 mg/l NAA, MS + CW + AC, MS + CW + AC + 2 mg/l BAP + 1 mg/l NAA) promoted 70 – 80% (lower percentage) of explant producing shoots, which were significantly different with control.

Additionally, the results recorded in Table 4.5 showed the mean shoot height was within the range of 1-2 cm. In general, the average shoot height was 1.53 cm after 2 months of culture. All of the treatments supplemented with Thiamine HCl gave 2 cm shoot height. Similar observations were recorded in higher concentrations of BAP alone and also in combination with BAP and NAA (3 and 4 mg/l BAP), respectively. The highest mean shoot height, 2.15 cm was obtained in the treatment with BAP alone (3 mg/l BAP) which was significantly different from the control (1.17 cm). Then, followed by the treatments with Thiamine HCl either in BAP alone (4 mg/l BAP) and, BAP and NAA in combination (2 mg/l BAP + 1 mg/l NAA), which gave 1.95 cm and 1.90 cm, respectively. In contrary, the lowest mean shoot height, 0.71 cm was recorded in the treatment with MS + CW + AC which was not significantly different from control. Figure 4.5 shows the growth of plantlet in medium with hormones.

Table 4.1: Effects of different concentrations of hormone applied singly and in combinations on the mean number of shoots, percentage of shoot production and mean shoot height after 2 months of culture

Treatments	Mean no of shoots per explant $\pm$ SE	Percentage of explant producing shoots (%) $\pm$ SE	Mean shoot height (cm) $\pm$ SE
MS	1.30 $\pm$ 0.22abc	100.00 $\pm$ 0.00a	1.17 $\pm$ 0.06def
MS + 1% CW	1.05 $\pm$ 0.11abc	90.00 $\pm$ 0.07ab	1.06 $\pm$ 0.13def
MS + 1% CW + 1%AC	0.85 $\pm$ 0.11c	80.00 $\pm$ 0.09bc	0.71 $\pm$ 0.12f
MS + 1% CW + 1% AC + 1.0 mg/L BAP	1.05 $\pm$ 0.05abc	100.00 $\pm$ 0.00a	1.00 $\pm$ 0.13ef
MS + 1% CW + 1% AC + 2.0 mg/L BAP	1.45 $\pm$ 0.21a	100.00 $\pm$ 0.00a	1.42 $\pm$ 0.16bcde
MS + 1% CW + 1% AC + 3.0 mg/L BAP	1.10 $\pm$ 0.12abc	90.00 $\pm$ 0.07ab	2.15 $\pm$ 0.42a
MS + 1% CW + 1% AC + 4.0 mg/L BAP	1.30 $\pm$ 0.26abc	95.00 $\pm$ 0.05a	1.86 $\pm$ 0.40abc
MS + 1% CW + 1% AC + 1.0 mg/L BAP + 1.0 mg/L NAA	0.95 $\pm$ 0.18bc	70.00 $\pm$ 0.11c	0.76 $\pm$ 0.13f
MS + 1% CW + 1% AC + 2.0 mg/L BAP + 1.0 mg/L NAA	1.20 $\pm$ 0.22abc	80.00 $\pm$ 0.09bc	1.20 $\pm$ 0.19cdef
MS + 1% CW + 1% AC + 3.0 mg/L BAP + 1.0 mg/L NAA	1.45 $\pm$ 0.15a	100.00 $\pm$ 0.00a	1.86 $\pm$ 0.17abc
MS + 1% CW + 1% AC + 4.0 mg/L BAP + 1.0 mg/L NAA	1.00 $\pm$ 0.00abc	100.00 $\pm$ 0.00a	1.85 $\pm$ 0.16abc
MS + 1% CW + 1% AC + 1.0 mg/L BAP + 1.0 Thiamine HCL	1.15 $\pm$ 0.08abc	100.00 $\pm$ 0.00a	1.69 $\pm$ 0.13abcd
MS + 1% CW + 1% AC + 2.0 mg/L BAP + 1.0 Thiamine HCL	1.40 $\pm$ 0.13ab	100.00 $\pm$ 0.00a	1.60 $\pm$ 0.22abcde
MS + 1% CW + 1% AC + 3.0 mg/L BAP + 1.0 Thiamine HCL	1.15 $\pm$ 0.08abc	100.00 $\pm$ 0.00a	1.63 $\pm$ 0.16abcde
MS + 1% CW + 1% AC + 4.0 mg/L BAP + 1.0 Thiamine HCL	1.15 $\pm$ 0.11abc	100.00 $\pm$ 0.00a	1.95 $\pm$ 0.27ab
MS + 1% CW + 1% AC + 1.0 mg/L BAP + 1.0 mg/L NAA + 1.0 Thiamine	1.05 $\pm$ 0.05abc	100.00 $\pm$ 0.00a	1.84 $\pm$ 0.17abc

HCL			
MS + 1% CW + 1% AC + 2.0 mg/L BAP + 1.0 mg/L NAA + 1.0 Thiamine HCL	1.15 ± 0.08abc	100.00 ± 0.00a	1.90 ± 0.17ab
MS + 1% CW + 1% AC + 3.0 mg/L BAP + 1.0 mg/L NAA + 1.0 Thiamine HCL	1.00 ± 0.00abc	100.00 ± 0.00a	1.55 ± 0.18abcde
MS + 1% CW + 1% AC + 4.0 mg/L BAP + 1.0 mg/L NAA + 1.0 Thiamine HCL	1.30 ± 0.13abc	100.00 ± 0.00a	1.85 ± 0.19abc

Data represents mean value ± standard error (SE) with 20 explants in each treatment. Means with different letters in the same column are significantly different at p=0.01 according to Duncan's multiple range test (DMRT).

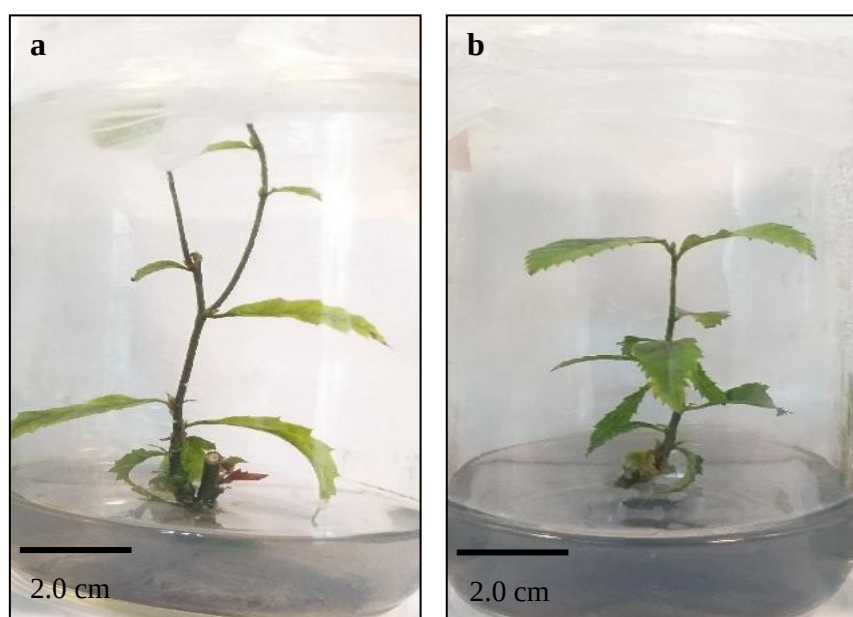


Figure 4.5: (a) Plantlet with 2 shoots observed in medium with hormones in combination, MS + 1% CW + 1% AC + 3.0 mg/l BAP + 1.0 mg/l NAA and, (b) Plantlet grown vigorously which promoted the highest shoot height in medium with hormone applied singly, MS + 1% CW + 1% AC + 3.0 mg/l BAP after 2 months of culture (scalebar = 2 cm)

Table 4.2 outlines the observations on the mean number of leaves and mean leaf length. Overall, the number of leaves produced per explant was within the range of 1-4, with an average of 2 leaves (1.93) after 2 months of culture. The minimum mean calculated, 0.55 (≈ 1 leaf) was obtained in the combination treatment supplemented with Thiamine HCl (3 mg/l BAP + 1 mg/l NAA + 1 mg/l Thiamine HCl). Comparable result with the production of 1 leaf per explant also recorded in several treatments including MS + CW, MS + CW + AC, MS + CW + AC + 1.0 mg/l BAP, MS + CW + AC + 4.0 mg/l BAP + 1.0 mg/l NAA, MS + CW + AC + 1.0 mg/l BAP + 1.0 Thiamine HCl, MS + CW + AC + 3.0 mg/l BAP + 1.0 Thiamine HCl. Besides, the appearance of 2 leaves per explant were observed in some treatments such as MS basal medium, MS + CW + AC + 2.0 mg/l BAP, MS + CW + AC + 1.0 mg/l BAP + 1.0 mg/l NAA, MS + CW + AC + 2.0 mg/l BAP + 1.0 mg/l Thiamine HCl, MS + CW + AC + 1.0 mg/l BAP + 1.0 mg/l NAA + 1.0 mg/l Thiamine HCl, MS + CW +

AC + 2.0 mg/l BAP + 1.0 mg/l NAA + 1.0 Thiamine HCl, MS + CW + AC + 4.0 mg/l BAP + 1.0 mg/l NAA + 1.0 Thiamine HCl. Only one treatment (MS + CW + AC + 2.0 mg/l BAP + 1.0 mg/l NAA) promoted 3 leaves per explant. Meanwhile, a few treatments resulted in 4 leaves per explant (MS + CW + AC + 3.0 mg/l BAP + 1.0 mg/l NAA, MS + CW + AC + 4.0 mg/l BAP) which were significantly different from the control. The maximum mean calculated was 4.15 which was obtained in the treatment with BAP alone (3 mg/l BAP).

The result recorded in Table 4.2 shows the leaf length was within the range of 0.3 – 1.6 cm with the average calculated was 0.82 ($\approx$ 1 cm). Most of the treatments were not significantly different from control except for treatment in BAP alone (MS + CW + AC + 4.0 mg/l BAP) and treatments in BAP alone supplemented with Thiamine HCl (MS + CW + AC + 2.0 mg/l BAP + 1.0 mg/l Thiamine HCl, MS + CW + AC + 3 mg/l BAP + 1.0 mg/l Thiamine HCl). The lowest leaf length, 0.34 cm was recorded in treatment with MS + CW. Furthermore, there was only one treatment in BAP alone (MS + CW + AC + 1.0 mg/l BAP) and two treatments in the combination of BAP and NAA (MS + CW + AC + 2.0 mg/l BAP + 1.0 mg/l NAA, MS + CW + AC + 3 mg/l BAP + 1.0 mg/l NAA) produced leaf length less than 0.5 cm. All treatments supplemented with Thiamine HCl, both for BAP alone and in combination produced $\approx$ 1 cm leaf length except for treatment with 3.0 mg/l BAP + 1.0 mg/l Thiamine HCl which promoted $\approx$ 2 cm leaf length. A similar result was recorded in the treatment with BAP alone (4 mg/l BAP) which resulted in the highest leaf length of 1.62 cm

Table 4.2: Effects of different concentrations of hormones applied singly and in combinations on the mean number of leaves and mean leaf length after 2 months of culture

Treatments	Mean no of leaves $\pm$ SE	Mean leaf length (cm) $\pm$ SE
MS (control medium)	1.50 $\pm$ 0.55de	0.54 $\pm$ 0.23cd
MS + 1% CW	1.20 $\pm$ 0.41de	0.34 $\pm$ 0.31d
MS + 1% CW + 1% AC	1.05 $\pm$ 0.35de	0.60 $\pm$ 0.21cd
MS + 1% CW + 1% AC + 1.0mg/l BAP	1.20 $\pm$ 0.50de	0.37 $\pm$ 0.13d
MS + 1% CW + 1% AC + 2.0mg/L BAP	2.04 $\pm$ 0.55bcde	0.93 $\pm$ 0.21abcd
MS + 1% CW + 1% AC + 3.0mg/L BAP	4.15 $\pm$ 1.04a	1.11 $\pm$ 0.24abc
MS + 1% CW + 1% AC + 4.0mg/L BAP	3.45 $\pm$ 0.91abc	1.62 $\pm$ 0.31a
MS + 1% CW + 1% AC + 1.0mg/L BAP + 1.0mg/L NAA	1.80 $\pm$ 0.41cde	0.57 $\pm$ 0.24cd
MS + 1% CW + 1% AC + 2.0mg/L BAP + 1.0mg/L NAA	2.55 $\pm$ 0.41abcd	0.35 $\pm$ 0.13d
MS + 1% CW + 1% AC + 3.0mg/L BAP + 1.0mg/L NAA	3.65 $\pm$ 0.61ab	0.55 $\pm$ 0.20 cd
MS + 1% CW + 1% AC + 4.0mg/L BAP + 1.0mg/L NAA	0.95 $\pm$ 0.29de	0.37 $\pm$ 0.13d
MS + 1% CW + 1% AC + 1.0mg/L BAP + 1.0 Thia HCL	1.25 $\pm$ 0.35de	0.93 $\pm$ 0.21abcd
MS + 1% CW + 1% AC + 2.0mg/L BAP + 1.0 Thia HCL	2.20 $\pm$ 0.72bcde	1.31 $\pm$ 0.27ab
MS + 1% CW + 1% AC + 3.0mg/L BAP + 1.0 Thia HCL	1.25 $\pm$ 0.40de	1.51 $\pm$ 0.32a
MS + 1% CW + 1% AC + 4.0mg/L BAP + 1.0 Thia HCL	2.20 $\pm$ 0.57bcde	0.81 $\pm$ 0.19bcd
MS + 1% CW + 1% AC + 1.0mg/L BAP + 1.0mg/L NAA + 1.0 Thia HCL	1.65 $\pm$ 0.42de	1.21 $\pm$ 0.22abc
MS + 1% CW + 1% AC + 2.0mg/L BAP + 1.0mg/L NAA + 1.0 Thia HCL	1.60 $\pm$ 0.43de	1.16 $\pm$ 0.15 abc
MS + 1% CW + 1% AC + 3.0mg/L BAP + 1.0mg/L NAA + 1.0 Thia HCL	0.55 $\pm$ 0.21e	0.62 $\pm$ 0.22bcd
MS + CW + AC + 4.0mg/L BAP + 1.0mg/L NAA + 1.0 Thia HCL	2.40 $\pm$ 0.63bcde	0.68 $\pm$ 0.18bcd

Data represents mean value $\pm$ standard error (SE) with 20 explants in each treatment. Means with different letters in the same column are significantly different at $p=0.01$ according to Duncan's multiple range test (DMRT).

4.4 Microscopic Studies

A few observations were made on the anatomy and morphological characteristics of *C. sativa* regenerated *in vitro* via FESEM and light microscope. A preliminary study on leaf anatomy clarified *Castanea sativa* possess a hypostomatic leaves where the presence of spherical and elliptic anomocytic stomata structure, also known as Ranunculaceous could be only seen on the abaxial of leaves (Figure 4.6). The stomata comprise guard cells which are surrounded by cells similar in shape, size and arrangement as the other epidermis cells. Each stoma is enclosed with two guard cells and surrounded by five or more subsidiary cells that are not differentiated from the normal epidermal cells (Metcalf & Chalk, 1950). Meanwhile, the epidermal cells of *Castanea sativa* were observed in polygonal shaped with two types of anticlinal wall patterns; sinuous on the abaxial (Figure 4.6a) and slightly straight on the adaxial surface of leaves (Figure 4.6b).

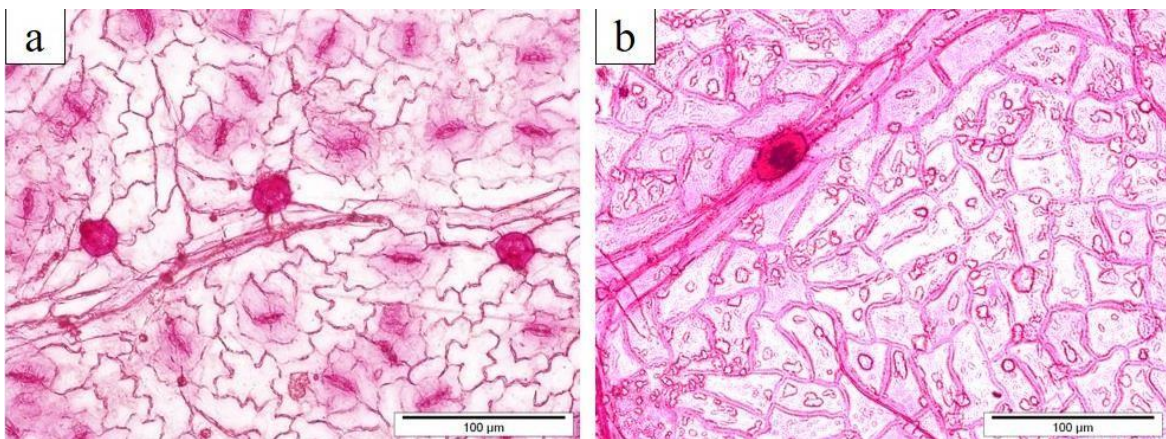


Figure 4.6: Epidermal layers of *Castanea sativa* leaves observed on (a) Abaxial surface, (b) Adaxial surface. Scale bar = 100 µm (40X magnification).

Further study in ultrastructural includes clarification on the stomata density and sizes of stomata opening that could only be done for (1) terminal shoot tip and (2) abaxial leaf part (Figure 4.7) due to the absence of stomata on (3) adaxial leaf part (Figure 4.8). Analysis of the results revealed the difference in stomata density for both samples, terminal shoot tip and abaxial leaf. Table 4.3 presented the mean number of stomata recorded both for the abaxial leaf part and terminal shoot tip. The mean number of stomata per area (1 mm^2) observed on the abaxial leaf part was 48.60 ± 1.57 which is significantly different from the terminal shoot tip, 13.6 ± 0.24 . Hence, the result suggested stomata disperse widely on the abaxial leaf part (Figure 4.9) compared to the terminal shoot tip (Figure 4.10). Nevertheless, a similar size of stomata pore aperture was recorded for both samples. Table 4.4 indicated the mean stomata opening both for the abaxial leaf part and terminal shoot tip showed no difference in statistical analysis. The mean size of stomata opening for terminal shoot tip samples with $17.72 \text{ }\mu\text{m}$ of length, $7.41 \text{ }\mu\text{m}$ of width was slightly higher than the size of stomata opening for abaxial leaf part with $12.45 \text{ }\mu\text{m}$ of length, $2.70 \text{ }\mu\text{m}$ of width. Figure 4.7 shows the *in vitro* sample of *Castanea sativa* used in the ultrastructural study meanwhile Figure 4.8 shows the FESEM micrograph that illustrated the absence of stomata on the adaxial surface of the *Castanea sativa* leaf sample.

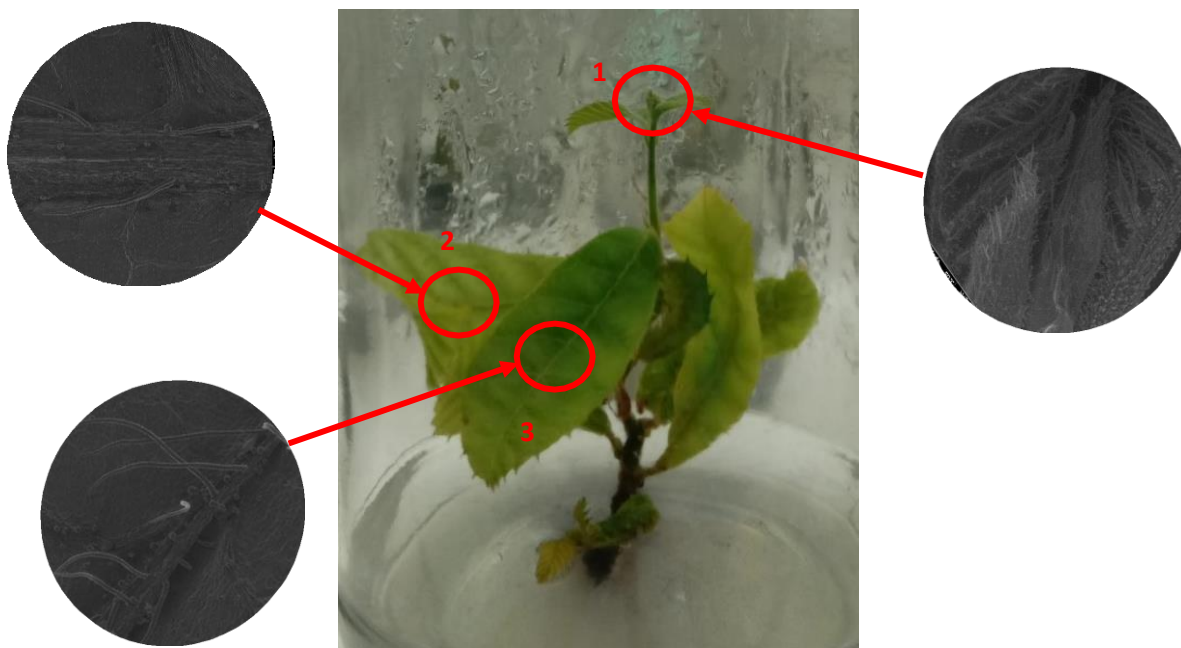


Figure 4.7: *In vitro* sample of *Castanea sativa* (6-month-old) used in ultrastructural study. (1) Terminal shoot tip, (2) Abaxial leaf and, (3) Adaxial leaf part.

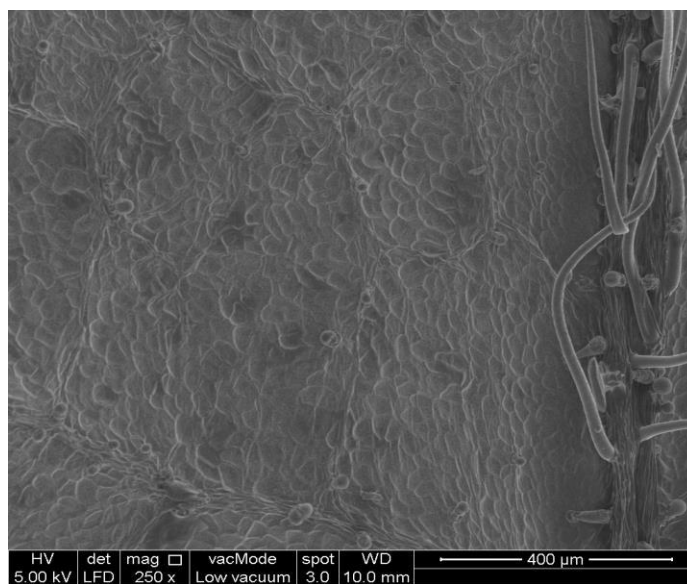


Figure 4.8: FESEM micrograph (Magnification: X 250) illustrated the absence of stomata on the adaxial surface of the *Castanea sativa* leaf sample.

Table 4.3: Comparison of the mean number of stomata per area (mm²) for abaxial and terminal shoot samples (MS + 1% CW + 1% AC + 4mg/l BAP) viewed under FESEM.

Replicates	Mean number of stomata per area (mm ²)	
	Abaxial leaf	Terminal shoot
1	50	15
2	41	12
3	41	15
4	49	13
5	62	13
Mean ± SE	48.60 ± 1.57*	13.6 ± 0.24*

Data represents mean value ± standard error (SE) with 5 replicates corresponding to 5 different areas captured on the epidermis surfaces. * Means are significantly different at p=0.05 (independent samples T-test).

Table 4.4: Comparison of the length and width of stomata pores aperture (µm) for the abaxial and terminal shoot samples viewed under FESEM.

Replicates	Opening stomata (µm)			
	Abaxial leaf		Terminal leaf	
	Length	Width	Length	Width
1	13.28	3.56	17.91	7.41
2	13.06	2.41	16.45	7.37
3	11.63	1.97	17.63	6.42
4	11.91	2.74	17.71	8.05
5	12.35	2.81	18.90	7.80
Mean ± SE	12.45 ± 0.13	2.70 ± 0.11	17.72 ± 0.16	7.41 ± 0.11

Data represents mean value ± standard error (SE) with 5 replicates corresponding to 5 different areas captured on the epidermis surfaces. * Means are significantly different at p=0.05 (independent samples T-test).

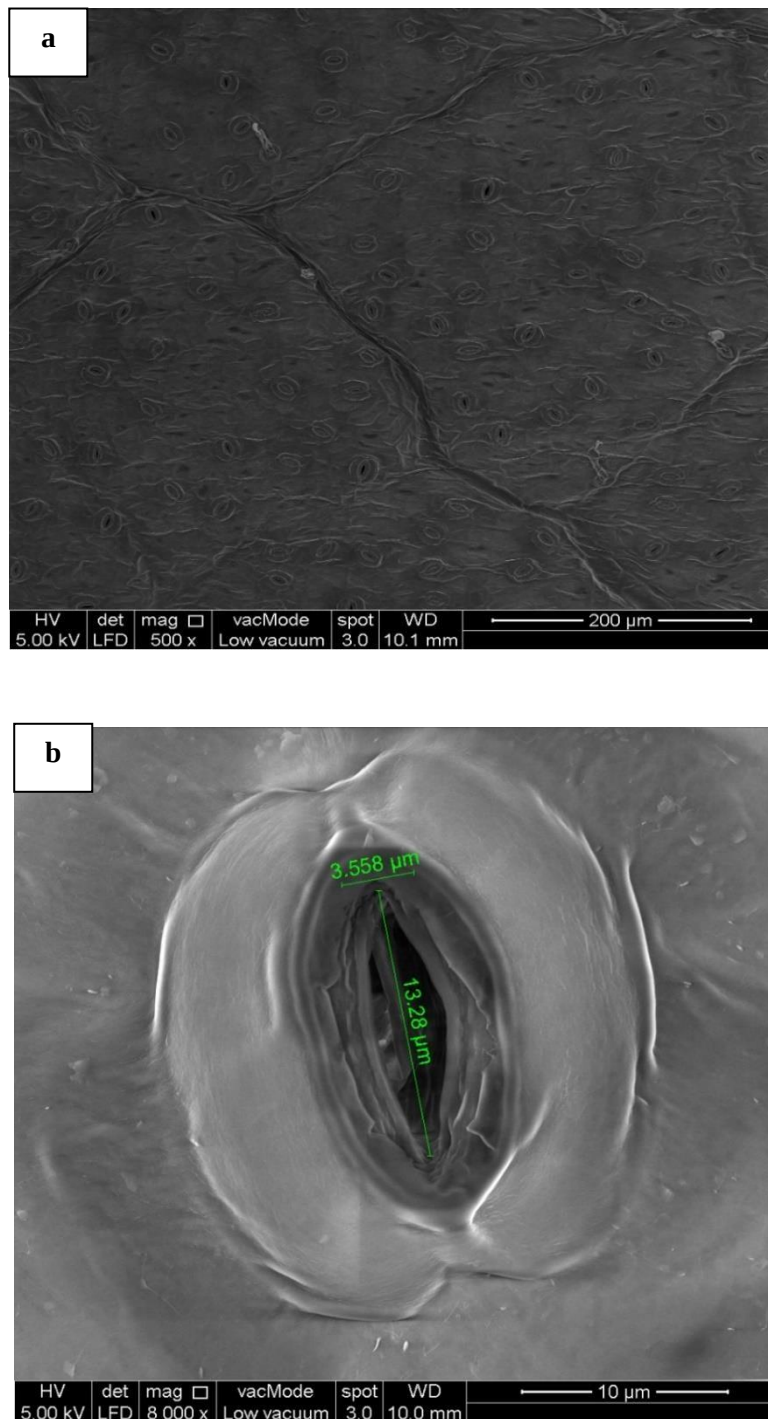


Figure 4.9: (a) FESEM micrograph (Magnification: X 500) illustrated the stomata scattered on the smooth epidermis surface (abaxial surface) of *Castanea sativa* (*in vitro* sample). (b) FESEM micrograph showed the measurement of the stoma opening (Length= 13.28 μm , Width = 3.56 μm).

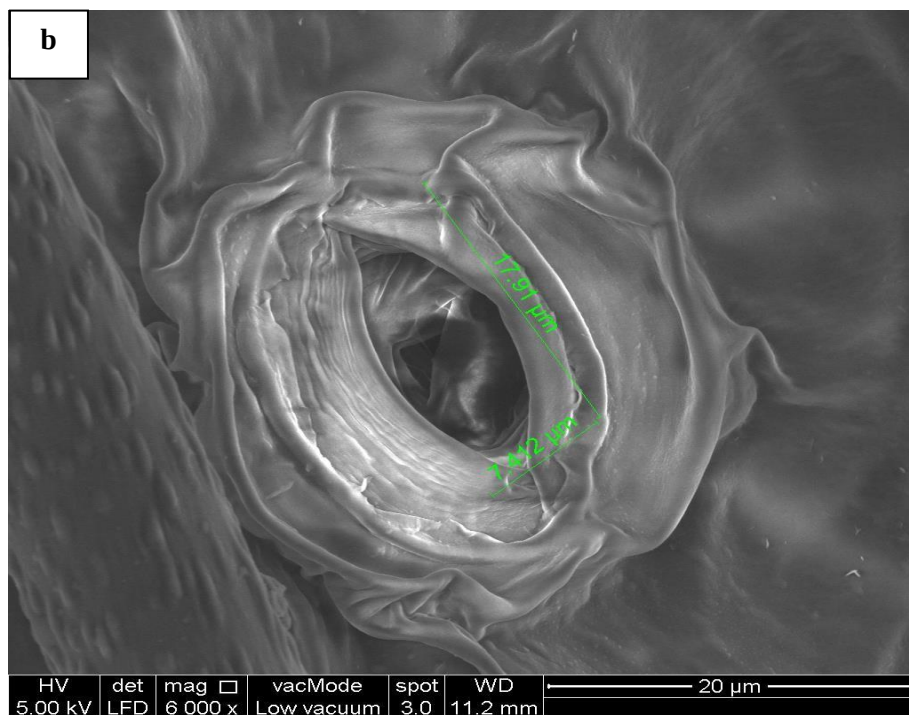
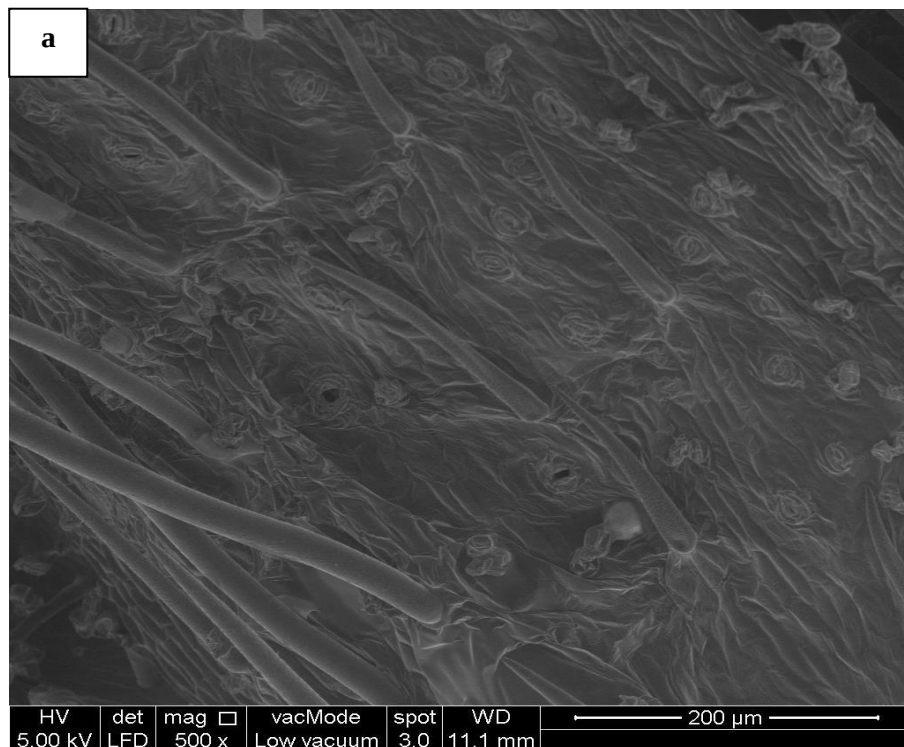


Figure 4.10: (a) FESEM micrograph (Magnification: X 500) illustrated the stomata scattered on the terminal shoot of *Castanea sativa* (*in vitro* sample). (b) FESEM micrograph showed the measurement of the stoma opening (Length= 17.91 μm , Width= 7.41 μm).

In the histological study, the longitudinal section (LS) of shoot tip (Figure 4.11) revealed the internal structures of apical shoot meristem (SAM), leaf primordia (LP) and young leaf primordia (YLP) whereas the transverse section (TS) of leaf and midrib (Figure 4.12) discovered the internal anatomical features such as upper epidermis layer (UE), lower epidermis layer (LE), parenchyma cells including palisade mesophyll (PM), and spongy mesophyll (SM), vascular bundle (VB), air space (AS), Stomata (St), guard cell (GC), adaxial leaf (Ad L), Abaxial leaf (Ab L), cambium, phloem (Ph) and xylem (Xy).

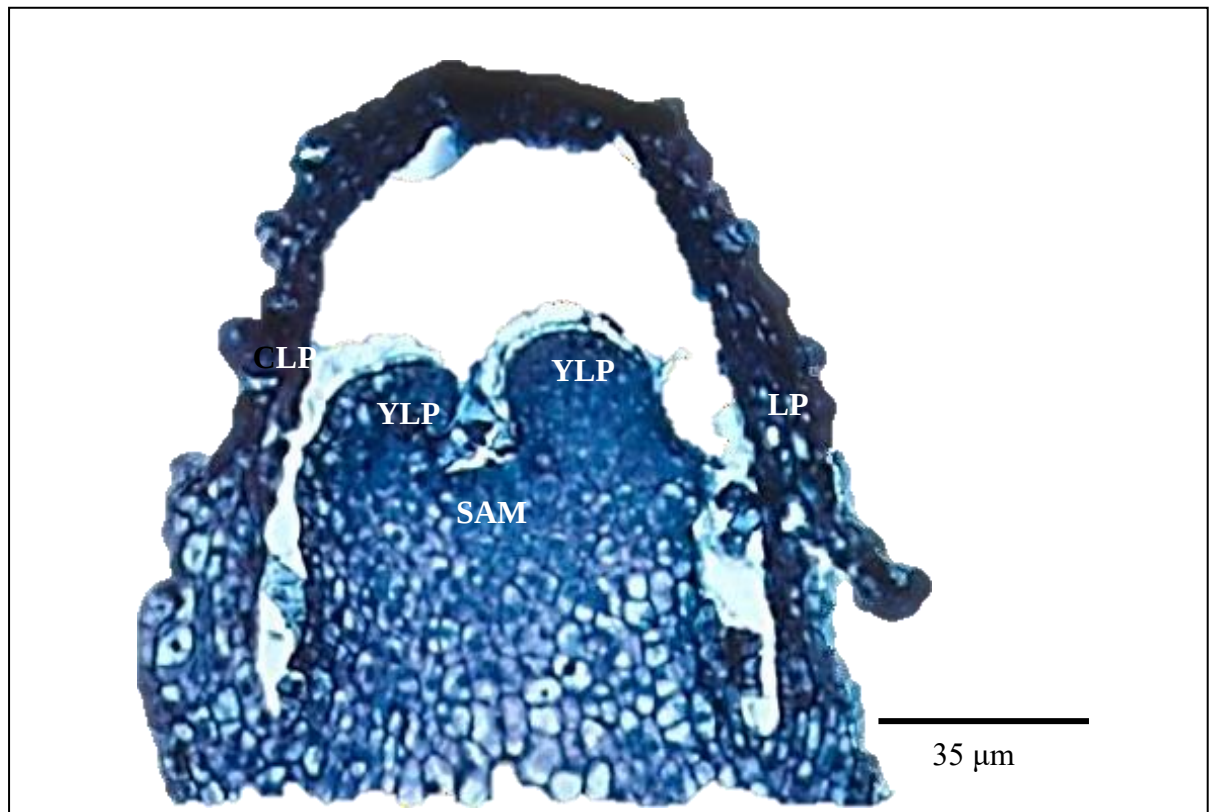


Figure 4.11: LS through the shoot tip of *Castanea sativa* regenerated *in vitro* showed the detailed structure of shoot apical meristem tissue and leaf primordia (Magnification: X 100) SAM = shoot apical meristem, LP = leaf primordia, YLP = young leaf primordia.

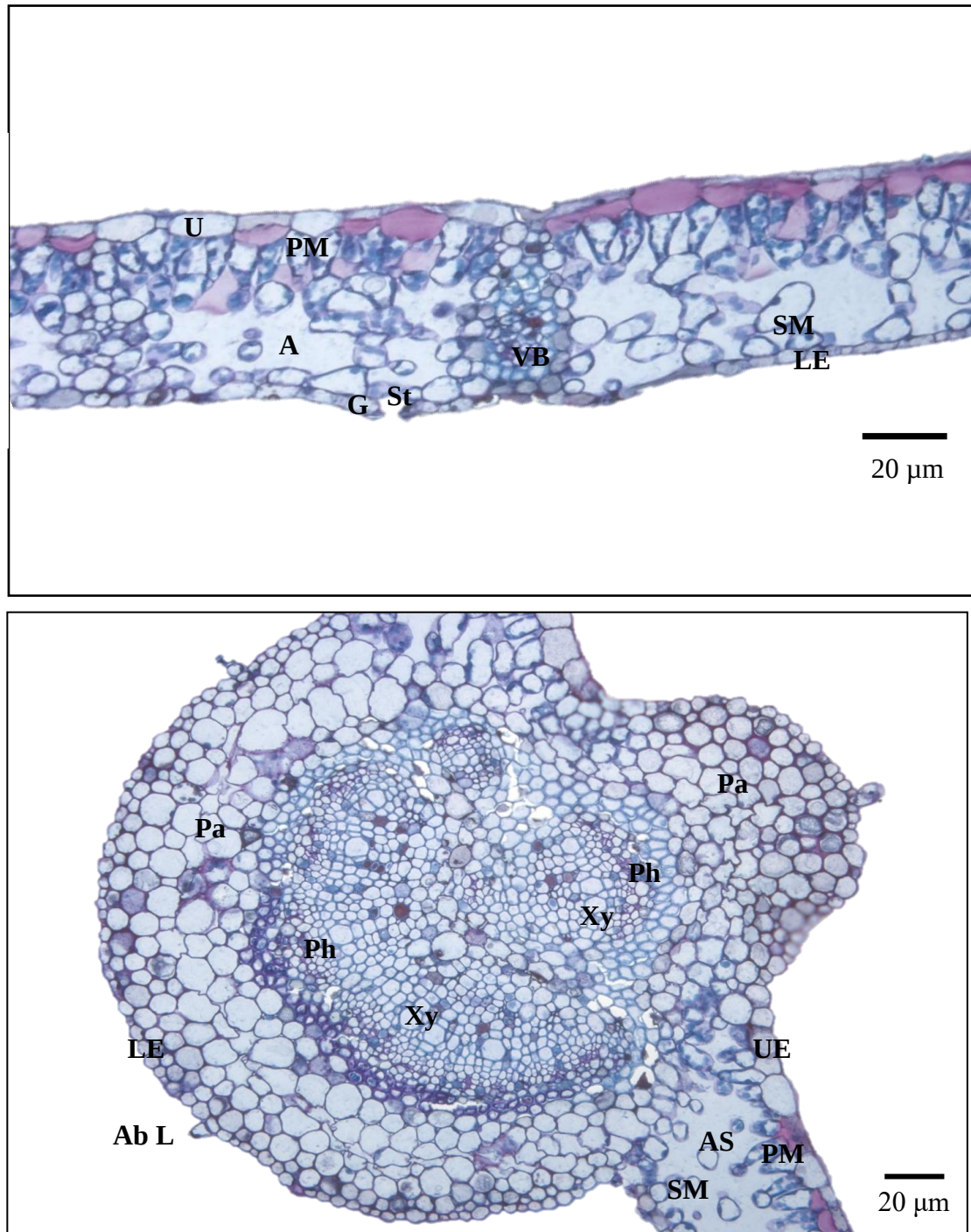


Figure 4.12: TS through the leaf and midrib of *Castanea sativa* regenerated *in vitro* showed the detailed structures of epidermis layer and vascular bundle (Magnification: X 200) UE = upper epidermis layer, LE = lower epidermis layer, PM = palisade mesophyll, SM = spongy mesophyll, VB = vascular bundle, AS = air space, St = stomata, GC = guard cell, Ad L = Adaxial leaf, Ab L = abaxial leaf, Pa = parenchyma cell, Ph = phloem, Xy = xylem.

4.5 Summary of Results

C. sativa classified as a recalcitrant seed whereby the seed rapidly perish as subjected to the long storage that employed for orthodox seeds which requires adequate moisture condition to remain viable. Instead, recalcitrant seed treatment usually needs immediate planting or specialized preservation techniques such as cryopreservation to maintain their viability for future use. In this study, germination of seeds *C. sativa* was observed after 1 week of culture, with the changes of seed colour prior to the appearance of radicles. The radicles have grown downward and continued elongation to form roots. After 1 month of culture, the shoot within the range of 1-2 cm height continues producing true leaves and roots. The shoots further grew and developed into aseptic seedlings within the range of 1-2 cm in height after 1 month of culture. *In vitro* plant regeneration study clarified most of the treatments tested producing 1 shoot per explant within 2 months of culture. The result was not significantly different from the control (≈ 1 shoot). The highest mean number of shoots was obtained in both treatments, 2 mg/l BAP alone and treatment in the combination of 3 mg/l BAP + 1 mg/l NAA which promoted 2 shoots per explant, respectively. Similarly, all treatments supplemented with Thiamine HCl either singly (BAP alone) or in combination (BAP + NAA) promoted approximately 2 cm shoot height. Nevertheless, the highest mean shoot height recorded was 2.15 cm obtained in the treatment with 3 mg/l BAP alone, which was significantly different from the control (1.17 cm). In average, 2 leaves were produced per explant after 2 months of culture and the maximum mean number of leaves was approximately 4, recorded in the treatment with 3 mg/l BAP alone. The result was significantly different from the control (≈ 2 leaves). The size of leaves produced after 2 months of culture was approximately 1 cm. The highest leaf length was 1.62 cm recorded in

the treatment with 4 mg/l BAP alone, which was significantly different from the control (0.54 cm). Ultrastructural analysis on *in vitro* regenerated samples discovered several important anatomical features including the presence of anomocytic stomata on the abaxial leaves surface. The stomata are enclosed with two guard cells that regulate the opening and closing of stomata. Besides, there were two types of anticlinal cell walls observed, sinuous on the abaxial and slightly straight on the adaxial surface of leaves. Although the abaxial leaf part has significantly higher stomata density (48.60 ± 1.57) compared to the terminal shoot tip (13.6 ± 0.24), however, they have similar sizes of stomata opening. Histological analysis on *in vitro* regenerated samples disclosed the internal anatomical features such as the epidermis layer (upper and lower), parenchyma cells (palisade and spongy mesophyll), vascular cambium (xylem and phloem).

CHAPTER 5

DISCUSSION

Current research mainly works on the investigation of direct *in vitro* propagation protocol for *C. sativa*, a noble hardwood species which offers numerous benefits and has been considered a multipurpose tree. The species is not only appreciated for timber and fruit production (Conedera et al., 2004, Conedera et al., 2016), but also for wildlife consumption, utilized in landscape conservation, rural tourism, recreation, and protection from erosion (Merkle et al., 2020). Previous study reported that the species has medicinal properties and is widely used in Mediterranean regions of Europe to treat various respiratory diseases, cough, bronchitis, cold and bronchiole infections (Chiarini, 2013).

Chestnut (*Castanea* spp.) could be propagated sexually by seeds and asexually by grafting, budding, layering and micropropagation. Asexual propagation of chestnut via conventional methods is quite difficult (Vieitez et al., 1986; Goncalves et al., 1998; Fernandez-Lorenzo et al., 2005). Thus, several attempts on asexual propagation of chestnut via micropropagation have been done. Successful *in vitro* multiplication methods have been reported for American (*C. dentata*) and European chestnut (*C. sativa*) as well as Euro-Asiatic hybrids (*C. sativa* x *C. crenata*) on the shoot multiplication, root formation and survival for *ex vitro* plantlet rates from low to high depending on the protocol used (Goncalves et al., 1998; Tetsumura & Yamashita, 2004; Vieitez et al., 2007).

Study on the propagation of the species is essential to provide sufficient and quality planting materials for commercialization. Nonetheless, the chestnut populations have been seriously threatened by ink and blight diseases (Pavese et al., 2012). Besides, conventional vegetative propagation methods via stem cutting and grafting have limited regeneration

ability (Fernandes et al., 2020). Critical issue in the traditional propagation of chestnut has been reported in the declining of rooting ability as the tree ages (Vielba et al., 2020). Consequently, the present study focuses on *in vitro* propagation to investigate and obtain the optimum medium for *in vitro* regeneration of *C. sativa*.

Micropropagation is a significant tool used to overcome the difficulties and obstacles in the adult production of chestnut species (Ballester et al., 2001; Tetsumura & Yamashita, 2004; Oakes et al., 2016). It has been widely applied as an alternative to propagate various plants species rapidly on a large scale. Besides, it is also considered a promising tool for research especially in molecular biology, biotechnology, physiology as well as crop improvement in the agriculture sector (Kumar & Loh, 2012). Furthermore, it has been introduced as an accessible method to expand the commercialization market globally and is considered among the improvised technologies besides an effective way to distribute the agricultural product to become profitable value (Baque et al., 2012).

Plant tissue culture has given an enormous contribution in many aspects including plant growth and development, plant morphology or characteristic, plant disease and resistance toward environmental stress. Nowadays, advanced studies in plant biotechnology involved the large production of disease-free plants, valuable secondary metabolites, new plant varieties etc. (Vijaya et al., 2010; Baque et al., 2012; Bhatia & Sharma, 2015).

Several factors which could be considered for the success of *in vitro* propagation are the environmental culture conditions (light and temperature), explant sources (age and culture initiation of the plant organs), nutrients including organic additive (activated charcoal, coconut water and vitamins) and plant growth regulators (BAP, NAA). Optimization of culture medium is crucial for an efficient system in plant regeneration *in*

vitro (Baque et al., 2010) whereas the ideal types and concentration of plant growth regulators are among the main components which contribute to the performance of plant cell, tissue and organ cultures (Prando et al., 2014). Similar to other plant species, chestnut requires an ideal composition or growing medium to enhance their growth and development within a short period of time (Sharma, 2017).

Nonetheless, contamination is the most critical problem that would lead to unsatisfactory findings of *in vitro* plant propagation. Generally, the contamination is closely related to the sample used besides the inefficient sterilization procedures applied. Procedures for sterilization of samples are usually proposed according to the degree of contamination of samples. In the current work, several sterilization procedures have been applied to reduce contamination of the chestnut seeds considering the plant morphological characteristics, ages, types of species and explants used (Lazo-Javalera et al., 2016). The presence of sucrose and supplementation of organic additives in plant tissue culture medium were proved readily attract the colony of contaminants (fungi and bacteria) to attach themselves on the explant and medium, which are spread faster within 2-3 days. As a result, the growth of the explants was affected and eventually destroyed by the contaminants (Putri et al., 2019). Disinfectant agents are available in various chemical products including fungicides, alcohols, mercury chloride (HgCl_2) and sodium hypochlorite (NaOCl), which are commonly used for removal of external and attachable contaminants. Mahmoud and Al-Ani (2016), proved that these disinfectant agents are found to be the most effective for sterilization techniques in micropropagation.

Several sterilant agents such as Clorox (sodium hypochlorite) and ethanol in different concentrations have been used for sterilization techniques in this research work, however,

some of them showed unsatisfactory results towards *C. sativa*. Therefore, the protocol of sterilization must be optimized to minimize the rate of contamination occurred. According to Elbasheer et al. (2019), Clorox was the most competent factor of sterilization techniques in numerous plant tissue culture research, whereby 30% and 60% concentrations of Clorox resulted in a higher number of survival explants. This conclusion was validated by Cardoso and Imthurn (2018), who indicated that sodium hypochlorite is a chlorine-based chemical sterilization agent and it enhanced the explant regeneration's most favourable results. Besides being cost-effective, sodium hypochlorite has high efficiency against contaminants and has shown no effect of toxicity toward different plant species that were used. Similarly, Teixeira da Silva et al., (2014) mentioned the use of sodium hypochlorite (Clorox) for 15 minutes had positive effects of contamination for *Anthurium andraeanum* 'Rubrun' species as the *Anthurium* fruits were soaked in 1% of sodium hypochlorite (NaOCl). Similarly, Viegas et al. (2007) reported that sodium hypochlorite was used to sterilize 100% of the leaves explant.

Disclaiming that the result might differ with other species that have been examined in tissue culture since every type of plant species has different tendency of growth and development. Yildiz et al. (2012) declared that sodium hypochlorite (NaOCl) has a higher capability of resistance towards all types of contaminants (fungi, bacteria and viruses) and it increased the successful rate of *in vitro* propagation in terms of germination of seeds and the establishment of explants.

According to Hesami et al. (2017), the higher concentration of sodium hypochlorite along with the increased time of immersion could reduce the percentage of contamination. In correspond to this finding, an earlier study by Maina et al. (2010), suggested the explants

immersed in 10-20 min of sodium hypochlorite resulted in the lowest rate of contamination and the growth of the explant is successfully achieved without any external and internal damages. According to Parasharami et al. (2014), the explants were immersed in 2% sodium hypochlorite for 30 minutes had a greater percentage of survival rate, 80.33%. Sodium hypochlorite (NaOCl) is known as the most productive and efficient tool rather than mercury chloride (HgCl₂), as it promoted more survived explants when immersed for 20 minutes in 5% Clorox concentration (Assareh & Sardabi, 2005; Mihaljevic et al., 2013). The previous finding revealed 70% survival rate of explants sterilized using sodium hypochlorite and, minimized the rate of contamination from fungal and bacterial infection. This, led to a positive growth of shoot induction (Gavilan et al., 2018). Clorox acts as an oxidizing agent which can kill the pathogen on a large scale, thus minimizing the rate of contamination of explants cultured (Mng'omba et al., 2012)

In the present study, not only sodium hypochlorite but also ethanol contributed to the success of *in vitro* plant regeneration and removed the contaminants (fungi and bacterial contamination). This statement was supported by Elbasheer et al. (2019), as the sterilization of explants could be enhanced with the application of 70% ethanol. On the other hand, a study by Tiwari et al. (2012), reported that the application of ethanol fortified with sodium hypochlorite showed satisfactory result. The same finding was reported by Zinabu et al. (2018), and they recommended the application of 70% ethanol along with 2% of sodium hypochlorite in explant sterilization. The earlier study also claimed that 70% of ethanol applied along with 30 seconds of immersion was good enough for the explant to completely wet and react together with the sterilizing agent (Chawla, 2002). As mentioned by Oyeibanji et al. (2009), besides being an active compound and phytotoxic chemical, ethanol can be

powerful sterilizing agent along with sodium hypochlorite to get a better result of survival rate since the combination (70% ethanol + 3.5% NaOCl) gave higher effectiveness towards the growth and development of explant. Other researchers stated that the duo combination of 25% sodium hypochlorite with 70% ethanol gave a successful result when an explant of wild strawberry was well cleaned in the medium and grow better in the culture medium (Parades & Lavin, 2005; Jan et al., 2013). Ethanol was chosen as the best wetting agent as it has a wetting property that easily passes through the surface of leaves hair (Lizarraga et al., 1986). This finding was also supported by Kunneman and Faajj-Groenen (1988), as they reported ethanol can act as removal of hydrophobic waxes and resins from the microorganisms to get contact with the explants.

Moreover, other than Clorox and ethanol, the present study also involved the application of Dettol to wash the explant aimed to get rid of the contaminants. With the increasing concentration of Dettol applied in the sterilization technique, the rate of contamination in *in vitro* regeneration could be decreased (Hammond et al., 2014). Other than that, Betadine was also used in the current research work, along with Dettol before the explant was washed under running tap water. Betadine has an antimicrobial substance, povidone-iodine that prohibits the growth of contaminants (fungi, bacteria, protozoa and virus), and enhance the growth of explants, environmental adaptation and stress tolerance in plants (Medrano-Macias, 2016; Widiastuti et al., 2018). Kusuma et al. (2016), stated that the usage of Betadine in the sterilization technique is generally to clean the surface of the explants. Thus, the combination of Betadine with Dettol would ensure the explant is completely clean with the lowest rate of contamination.

Application of Tween 20 in the present study also plays a key role in the sterilization technique as it acts as a wetting agent to improve the contact between the explants and sterilizing agents (Fay, 1992). In order to complete the sterilization method, a few drops of Tween 20 are needed to provide efficient treatment, thus washing away the attached impurities on the explants (Widiastuti et al., 2018). As mentioned by Brandl and Huynh (2014), a bacterial infection of the plant samples can be minimized using Tween 20 due to its hydrophobic compound which affects the hydrophobic interaction between the plant itself and the contaminants.

Rahman et al. (2018) stated 80% of *Lepicanthes fruticosa* explants performed well after being treated with Clorox for 15 minutes and a few drops of Tween 20 in the *in vitro* propagation and produced uncontaminated explants with only 10% of browning symptoms. Browning symptoms and phenolic exudation are also reported in *in vitro* plant regeneration. The browning and exudation of the phenolic content of the explants could be minimized by washing the explants under running tap water (Lakshmi & Raghava, 1993; Muralidharan, 1997). These problems always appear in the woody plant species due to the leaking of phenolic content and secondary metabolites from the cut surface of explants which led to hamper further response of growing and rooting of explants (Aliyu, 2005; Krishna et al, 2008; Pirttila et al, 2008). Phenolic exudation become a major problem when it comes to the establishment of *in vitro* propagation as it may inhibit the growth of the explant. Usually, the phenolic content was noticed from the cut surface of the explants and the oxidation occurred due to polyphenol and air reaction, thus gradually diffused into the culture medium turning the explant into browning and eventually dying (Compton & Preece, 1986; Aliyu, 2005; Unouha et al., 2011; Pushpraj & Patel, 2016). In contrast, the production of

polyphenols appeared to be higher in older plants compared to new and young aged plants. As a result, older plants might exhibit more browning symptoms in the *in vitro* propagation (Pati et al., 2008).

Other factors that may be utilized into consideration in the *in vitro* plant propagation, besides contamination, are the types of explants used, the medium types and the age of explants. The explant is any plant parts such as the shoot tips, nodes, internodes, leaf tissues, petioles, root tips, anther, embryo etc. that were used to regenerate plant tissue culture (Sharma & Vashistha, 2015). Different types of explants were being used in *in vitro* propagation of *C. sativa* like nodal, root and internodal segments (Tafazoli et al., 2013). In the current study, the nodal explant of *C. sativa* was recommended for *in vitro* regeneration (Tafazoli et al. (2013). They clarified that the nodal segment was the best explant to generate callus because it is more responsive for indirect organogenesis. Therefore, the *in vitro* propagation of *C. sativa* was done using nodes as an initial explant to regenerate shoots. Nodal segments showed the earliest signs of callus formation on the cut edges as the explants cultured on MS medium supplemented with 1 mgL⁻¹ TDZ after one week (Tafazoli et al., 2013). As shown in the present study, the nodal segments presented positive results toward shoot regeneration and multiplication. Almost all the treatments including control recorded 100% of explant producing shoots. This finding corresponds to the previous study by Hou et al. (2010), whereby the single nodal segments of *C. mollissima* cv. 'yanshanhong' induced shoot on MS medium with a half concentration of NO₃ supplemented with 0.5 mg/L⁻¹ BA. However, there are some findings that use another type of explants such as shoot segments for plantlet regeneration on *C. sativa* works well in the previous experiment. Rodriguez (1982) stated that the chestnut shoot segment was used in the previous experiment and

promoted 80% germination rate as the explants were cultured on basal medium (control) and 0.9 mg/L BA.

The controlled conditions include types of medium composition, pH of medium, favourable environment and temperature are among the crucial factors for the growth and development of explants (Kumar & Jakhar, 2018). The selection of media composition or types of media used have a significant impact on the effect of the progress of micropropagation. Culture medium provides the energy source for explant to supply their needs and sufficient nutrition for regeneration (Read & Preece, 2014). Iliev (2015) stated that the success of shoot regeneration and shoot induction is determined by the selection of culture medium used.

According to Yerbolova et al (2013), one of the limiting factors of woody plant species in micropropagation is due to concentration of plant growth regulators. The differences in BAP concentration used in different cultivars determine the reaction between internal and external plant growth regulators (Verma et al., 2009). The plant growth regulator that is commonly used for *in vitro* proliferation study of chestnuts is Benzyl adenine (BA) (Giovannelli & Giannini, 2000; Vieitez et al., 2007). According to previous studies, BA is the most efficient plant growth regulator in shoot induction and node production of chestnuts (Tetsumura & Yamashita, 2004; Fernandez-Lorenzo et al., 2005;). Rodriguez (1982) proposed that the addition of BAP hormone in the media might improve the *in vitro* initiation and multiplication of shoot buds. High concentration of BAP induced shoot formation however inhibited subsequent shoot development. For stimulation of axillary shoot proliferation and production of high-quality shoots, BAP was considered the most effective cytokinin compared to kinetin and glycine. In the present study for shoot induction, the

sterilized nodal segment from aseptic seedlings of chestnut (2-month-old) were cultured in MS medium fortified with different concentrations of single BAP and in combination hormone of BAP and NAA along with coconut water and 0.1% activated charcoal. Observation made after 1 month revealed the highest mean number of shoots (2 shoots per explant) for single BAP observed in treatment 5 (MS + CW + A.C + 2.0 BAP) while for combination hormone of BAP and NAA, observed in treatment 10 (MS + CW + A.C + 3.0 BAP + 1.0 NAA). BAP has a significant effect on the tissue culture of woody plant species and found to be the suitable and efficient hormone in shoot multiplication of shoot of explant (Gonbad et al., 2014). The axillary buds of mangosteen plants recorded the highest number of shoots formed in the medium containing 3 mg/L of BA (Rostika et al., 2008). Harahap et al. (2014) mentioned that the mangosteen shoots become swell and the initial buds produced were stunted. The buds were not formed into shoots due to high concentrations of BAP hormones. In fact, the mangosteen shoots were increased in size by about 2 cm when cultured in 2 mg/L of BAP. According to another research by Ling et al. (2018), axillary shoot tips of *Ficus carica* cv Japanese explants produced the most shoots with an average of 1.67 shoots per explant in MS media supplemented with 2.0 mg/L BAP. This result (1.67 shoots per explant) was significantly higher if compared to the results of other treatments (control, 0.5 mg/L BAP and 1.5 mg/L BAP). The finding concluded that a higher concentration of BAP could lead to a higher proliferation rate meanwhile a lower concentration of BAP could be used to induce axillary shoot tips (Ling et al., 2018).

Excess application of plant growth regulators in *in vitro* plant propagation could also harm the explant itself. This might result in stunted plant growth, reduction of leaves size, internodes shortening and hyperhydricity which can lead to a major problem in the later

phase (Radmann et al., 2011). In *Fagaceae* family, the most commonly used of cytokinins are BA and zeatin hormone for shoot proliferation. The same finding was observed in American oak species as it gets along with the BA and zeatin hormone well in the *in vitro* propagation (Vieitez et al., 2009). Previous research by Vieitez et al. (2003) also reported that the rate of shoot proliferation of beech species (woody plant) was improved with the use of BA and zeatin. Previous study by Ibanez et al. (2005) stated that medium supplemented with 1 and 2 mg/L BAP promoted well grown *Vitis* (grapevine cultivar) explants in terms of axillary bud and shoot tip development which parallel the study by Mukherjee et al. (2010). The silver birch (*Betula pendula*) contributed to the largest number of shoots, with an average of 3.1 as the explants cultured in 1.0 mg/L BAP. Thus, clarified that BAP as synthetic cytokinin promoted far better than zeatin in case of shoot proliferation (Girgze & Samsone, 2017). The supplementation of cytokinin in the plant tissue culture medium is well known for the proliferation of shoot tips of woody plant species. Woody plant species such as tea plant in various species viz. *Camellia sinensis*, *Camellia japonica*, *Camellia oleifera* and *Camelia sasanqua* recorded the best shoot proliferation in the medium fortified with BAP (1– 4 mg/L) (Mondal, 2014). As a result, BAP ranging from 1– 4 mg/L is considered as among the optimum concentrations for proliferation with the highest number of shoots produced (Rakrawee et al., 2018). The finding was also supported by Panda and Hazra (2010), which mentioned that BAP applied singly produced a higher number of multiple shoots compared to the combination BAP, and the result recorded was about 7-8 buds/explant after 3 months of culture.

The concentrations of BAP ranged from 0.5-2.0 mg/L, considered to be the most suitable concentrations for *B. pendula* culture without any addition of auxin (Vitamvas et

al., 2020). On the other hand, along with higher concentrations of BAP hormone, the mean number of shoots per explant recorded showed a fluctuating pattern (Kamal et al., 2018) because the explants already achieved their optimum level of growth. As the regime of BAP hormones getting higher until 10 mg/L of BAP, the competition among the shoots become intense as the shoots struggle in absorbing the hormone and nutrient in the culture medium, thus producing shorter shoots (Kamal et al., 2018). For the majority of genotypes, BAP was claimed as the most effective yet responsive cytokinin among the others (ZEA, ZiP and KIN) which, resulted in about 88% of shoot formation with soft and green buds (Ali et al., 2012). Other studies in *in vitro* propagation of *D. sissoo* also mentioned that BAP has a better response compared to kinetin (Kavyashree, 2007; Nayak et al., 2007; Behera et al., 2008).

In general, the present study clarified the highest mean height of shoot for *C. sativa* was 2.48 after 2 months of culture on MS medium supplemented with MS + CW + A.C + 3.0 mg/L BAP. Similarly, the highest total number of leaves (≈ 4) was reported after 2 months of culture in the same treatment (MS + CW + A.C + 3.0 mg/L BAP) (Table 4.3).

The addition of organic additives in the medium for *in vitro* propagation of woody tree species can be used to produce uniform explants in the global market. Activated charcoal is one of the agents used to overcome browning symptoms of explants as it can delay the decomposition of auxin into the culture medium and adsorb the inhibitor such as phenolic substance which can affect the growth of plants (Thomas, 2008). Media supplemented with activated charcoal usually could increase the ratio of rooting, thus significantly increasing the length of shoot produced (Simsek et al., 2017). Addition of activated charcoal for woody plant species seemed to be a good idea as it can generate the proliferation of shoots (Hassan & Zayed., 2018). Activated charcoal not only plays a role in adsorbing inhibitory compounds

such as toxic metabolites and phenolic exudation, but it can also enhance the growth of explants by releasing the natural compound that important and essential for explants (Hesami et al., 2018). It is a challenging endeavour for woody species to handle root formation in tissue culture, hence activated charcoal was chosen as a wise solution to curb the browning problem, phenolic leakage and hyperhydricity (Oakes & Powell, 2013). Previous studies by Sharma et al. (2012) mentioned that releasing natural substances in the culture medium which involves inhibitory and stimulatory actions, actually aids in the growth of explants and adsorption of important elements such as metal ions, vitamins and plant growth regulators (PGRs). These elements play an important role in keeping the explants to form the best rooting response based on the number of roots produced.

On the other hand, coconut water also gave an impact on the current research of *C. sativa*. Coconut water was not assumed as an essential compound of the tissue culture medium. Although the application of coconut water is not crucial in tissue culture, it can be used as a growth accelerator of cultures and it helps in building up the growth of explants (Mondal et al., 2012). The frequency of explants in terms of regeneration of shoots, multiplication of shoots and elongation of shoots showed a positive effect toward the presence of coconut water in the medium. Coconut water is an organic additive that supplies nutrients mainly consisting of vitamins, zeatin, myo-inositol and reduced nitrogen compounds (Kalpona et al., 2000; Aktar et al, 2008). Organic additive like coconut water is required in the culture medium because they act as natural carbon source as they have natural vitamins, phenols, fiber, hormones and protein (Gnasekaran et al., 2010). Coconut water has been widely applied to the micropropagation of orchids and contains 1,3 – diphenylurea, an active ingredient that showed cytokinin-like activity. Green coconut water has an anti-fungal

effect and is recommended as a selected agent to treat the growth of *C. albicans* (Cholan et al., 2017).

Coconut water was proven scientifically to have organic compounds and mineral nutrients that play a main role in plant regeneration in tissue culture, due to its richness in magnesium, phosphate and high amount of sugar. Thus, it can enhance the development and growth of olive embryos (de Souza et al., 2013). Previous research by Nasib et al. (2008) also supported our current finding which indicated the optimum length of shoots and number of leaves were due to the supplementation of coconut water with BAP. Hence, it can be concluded that the supplementation of coconut water is significant in the tissue culture system. Not only because of the mineral contents but it could be applied instead of using zeatin, the expensive growth regulators required for plant growth and development *in vitro*. Prando et al. (2014) also mentioned that the multiplication of explant did not fully achieve if coconut water was applied alone. In fact, the addition of plant growth regulators especially cytokinin helps in increasing the rates of woody plant multiplication. Therefore, the combination of BAP and CW was more effective for shoot induction and multiplication in woody plant species (Sharma, 2017). In the previous research, coconut water elevated a healthy growth of plant growth due to its cytokinin-like substances which help in the multiplication of shoot formation (Inpeuy et al., 2011). Hence, minimized the browning symptoms of embryo cultures (Te-Chato et al., 2009). Moreover, coconut water is recognized as the best organic additive for *in vitro* germination compared to other additives like pineapple juice, tomato juice and palm fruit water as it achieved the success of the embryo germination which reached the maximum shoot production (Thejaswini & Narasimhan, 2017). Most of the additives like olive, almond and pomegranate together with

coconut water fortified in the medium promoted the best multiplication and induction of shoots (Hassan & Zayed, 2018).

In the present study, *in vitro* regenerated plantlets were subjected to FESEM. Different plant parts such as the abaxial leaf, adaxial leaf and terminal shoot were selected for morphology and anatomy study. Ahmad et al. (2014) suggested the morphology and anatomical characteristics of the chestnut tree are mostly influenced by external factors (development, environmental condition and types of cultivation). The present findings revealed interesting external and internal structures of *C. sativa*'s leaf and shoot. The morphology study revealed *C. sativa* has a unique structure of hypostomatic leaf, anomocytic stomata, polygonal shape of the epidermal cell, sinuous (abaxial) and straight (adaxial) anticlinal wall. Previous morphological study done by Perveen et al. (2007) using FESEM justified that the structure of stomata was anomocytic, whereby the guard cells of stomata are not surrounded by any subsidiary cells. Pinto et al. (2011) stated the stomata were irregularly celled and surrounded by an indefinite number of subsidiary cells which are indistinguishable from neighbouring cells. Earlier study done by Van Cotthem (1970) also clarified that *C. sativa* featured stomata on its lower surface, and the stomatal complex was categorized as the anomocytic. According to Liu et al. (2009), *Castanea*'s leaf cuticular characteristics revealed that most of the anticlinal walls on the adaxial surface are straight. On the other hand, anticlinal walls on the abaxial surface of *Castanea*'s leaf are sinuous.

The term stoma typically refers to the epidermal opening through which gas exchange occurs between the intracellular spaces of the subepidermal cells and the atmosphere (Abid et al., 2007). Stomata are important organelles that can affect a plant's capacity to adapt by regulating transpiration, photosynthesis, and conveying details about

the ideal circumstances for plant growth (Salisbury & Ross, 1992; Brownlee, 2001). According to Shekhawat et al. (2017) stomata development are of utmost significance in controlling transpiration from an *in vitro* to a field setting.

Further details on the morphology of *C. sativa* indicated that different parts of the plant showed slight differences in the mean number of stomata per area (μm), the mean size of stomata pores aperture in terms of length and width of opening stomata. Nevertheless, based on statistical analysis, there was no significant difference between the samples tested. The mean number of stomata per area (μm) recorded in the abaxial leaf part of *C. sativa* was higher than the terminal shoot tip of *C. sativa*. Pinto et al. (2011) stated that there is variation in the cultivar and part of the leaf tested based on the number of stomata per unit area. Current study suggested the mean of stomata pores aperture of the terminal shoot part is higher than in the abaxial leaf part. Our findings are remarkably similar to a prior work by Pinto et al. (2011) that found stomata on the bottom leaf surfaces (abaxial) of three Portuguese cultivars of *C. sativa* Mill. with the stomata openings observed around 13 mm length and 6 mm width.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

In vitro propagation is an alternative to conventional plant breeding for the production of a great number of plantlets rapidly in a just short time period. It presents an opportunity to regenerate high quality or elite plants (plants with desirable characteristics, free-disease plants, resistant-disease plants, uniform planting stock, etc.). *In vitro* propagation is also a preferable technique to produce a high amount of natural products specifically for plants that have slow growth production. Hence, this technique is considered in the present study that mainly focuses on the *in vitro* propagation of *C. sativa* considering it might assist in investigating or obtaining the ideal medium for production of *C. sativa* Mill. (chestnut) in industrial scale in Malaysia specifically, as it has high potential in food, timber, pharmaceutical industry etc. The present study has discovered that direct *in vitro* propagation of *C. sativa* was influenced by either internal or external factors. Among the main factors are plant growth regulators and plant additives. In general, most of the treatments producing 100% shoot whereby the explants could produce approximately 1-2 shoots per explant ($\approx$ 1 shoot per explant) with 1.0-2.0 cm shoot height ($\approx$ 2.0 cm) besides producing 1-4 leaves (2 leaves) and the leaves within the range of 0.3-2.0 cm (1.0 cm) in 2 months of culture. Analysis of the results discovered that medium containing MS supplemented with CW + AC + 3.0 mg/L BAP + 1.0 mg/L NAA is highly recommended for regeneration of the plant species as 100% explants producing shoot within 2 months, with the average of 2 shoots per explant besides 2.0 cm shoot height and 4 leaves with 1.0 cm length. Furthermore, the present study has also established a sterilization protocol with an optimum percentage of seed germination about 80%. In addition, morphological and

anatomical studies revealed unique external and internal structures of the leave and shoot tips of the regenerated plantlets *in vitro*.

In view of the fact that there is still a lack of study on the *in vitro* propagation of *C. sativa* Mill. in Malaysia. Further research on micropropagation and histological studies of *C. sativa* is greatly encouraging. More parameters such as seed germination and acclimatization should be considered for data analysis as well as the significant factors that could influence the growth and performance of *C. sativa* Mill. Further research is necessary to provide more information on the effect of growing medium towards every different condition besides understanding on cultivation of the species, especially in Malaysia.

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APPENDICES

Appendix 1

MS medium (Murashige and Skoog, 1962) (basic medium)

Macronutrient		concentration (Mg/l)
MgSO ₄ .7H ₂ O	Macronutrient	370
KH ₂ PO ₄		170
KNO ₃		1900
NH ₄ NO ₃		1650
CaCl ₂ .2H ₂ O		440
Micronutrient		
H ₂ BO ₃	Micronutrient	6.2
MnSO ₄ .4H ₂ O		15.6
ZnSO ₄ .7H ₂ O		8.6
NaMoO ₄ .2H ₂ O		0.25
CuSO ₄ .5H ₂ O		0.025
CoCl ₂ .6H ₂ O		0.025
KI	Iron	0.83
FeSO ₄ .7H ₂ O		27.8
Na ₂ EDTA		37.3
Vitamins		
Thiamine HCl	Vitamin	0.5
Pyridoxine HCl		0.5
Nicotinic acid		0.05
Myo-inositol		100

Sucrose

30 000

Agar

8000

Appendix 2

FIXATION METHOD

SAMPLE PREPARATION FOR FESEM (FIELD EMISSION SCANNING ELECTRON MICROSCOPE)

Fixation reagent methods for preparation

1. 4% Glutaraldehyde (100 ml)
 - a) 0.2 M Phosphate (PO_4^{3-}) buffer.....
50ml
 - b) 25% Glutaraldehyde ($\text{C}_5\text{H}_8\text{O}_2$) solution.....
16ml
 - c) Distilled Water (H_2O)
34ml
 - 4% Glutaraldehyde in 0.1 M Phosphate buffer

2. McDowell and Trump's Fixative (1976) suitable for **plants**
 - a) 0.2M buffer
solution... 50ml
 - b) 37%
formaldehyde.....
.... 11ml
 - c) 25%
glutaraldehyde.....
.. 4 ml
 - d) Distilled water to make 100 ml

SEM TISSUE PREPARATION SCHEDULE
(CPD METHODS)

1. The fixative of McDowell-Trump was fix together (alternatives: Karnovsky's fixative or 5% Gluteraldehyde) and 0.1M phosphate buffer or cacodylate buffer (pH 7.2) was prepared at 4 °C for 2-24 hours. The fixative was done to maintain the shape of cell and kept it from shrinkage or denatured.
2. The fixative was washed in buffer (step 1) for three times, each in 10 minutes.
3. Then, postfix in 1% Osmium tetroxide that prepared in the same buffer with room temperature for about 1-2 hours.
4. The tissue specimen was washed in distilled water for two times, each in 10 minutes.
5. Dehydrate as follows:
 - 50% ethanol (15 minutes)
 - 75% ethanol (15 minutes)
 - 95% ethanol (15 minutes x 2)
 - 100% ethanol (20 minutes x 3) – done in the CPD holder.
6. The tissue specimen was transferred into the CPD specimen holder and immersed in ethanol solution to avoid hydration. The specimen holder was immersed enough in the 100% ethanol solution to cover the tissue specimen for about 15-30 minutes.

7. After that, the tissue specimen undergoes the Critical Point Drying (CPD).
8. Then, the tissue specimen was put inside dessicator.
9. The dried specimen mounted onto SEM specimen stub with a double-sided sticky tape and was coated with gold, palladium, chromium or carbon.

Last but not least, the specimen went through the view in the SE

Appendix 3

Descriptives

						95% Confidence Interval for			
				Std.		Mean			
N			Mean	Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Noofshoot	1	20	1.3000	.97872	.21885	.8419	1.7581	1.00	5.00
	2	20	1.0500	.51042	.11413	.8111	1.2889	.00	2.00
	3	20	.8500	.48936	.10942	.6210	1.0790	.00	2.00
	4	20	1.0500	.22361	.05000	.9453	1.1547	1.00	2.00
	5	20	1.4500	.94451	.21120	1.0080	1.8920	1.00	4.00
	6	20	1.1000	.55251	.12354	.8414	1.3586	.00	2.00
	7	20	1.3000	1.17429	.26258	.7504	1.8496	.00	6.00
	8	20	.9500	.82558	.18460	.5636	1.3364	.00	3.00
	9	20	1.2000	1.00525	.22478	.7295	1.6705	.00	4.00
	10	20	1.4500	.68633	.15347	1.1288	1.7712	1.00	3.00
	11	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	12	20	1.1500	.36635	.08192	.9785	1.3215	1.00	2.00
	13	20	1.4000	.59824	.13377	1.1200	1.6800	1.00	3.00
	14	20	1.1500	.36635	.08192	.9785	1.3215	1.00	2.00
	15	20	1.1500	.48936	.10942	.9210	1.3790	1.00	3.00
	16	20	1.0500	.22361	.05000	.9453	1.1547	1.00	2.00
	17	20	1.1500	.36635	.08192	.9785	1.3215	1.00	2.00
	18	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	19	20	1.3000	.57124	.12773	1.0327	1.5673	1.00	3.00
	Total	380	1.1605	.63994	.03283	1.0960	1.2251	.00	6.00
	1	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00

Percentageofshootperex plants	2	20	.9000	.30779	.06882	.7559	1.0441	.00	1.00
	3	20	.8000	.41039	.09177	.6079	.9921	.00	1.00
	4	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	5	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	6	20	.9000	.30779	.06882	.7559	1.0441	.00	1.00
	7	20	.9500	.22361	.05000	.8453	1.0547	.00	1.00
	8	20	.7000	.47016	.10513	.4800	.9200	.00	1.00
	9	20	.8000	.41039	.09177	.6079	.9921	.00	1.00
	10	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	11	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	12	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	13	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	14	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	15	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	16	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	17	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	18	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	19	20	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	Total	380	.9500	.21823	.01120	.9280	.9720	.00	1.00
Percentageofleavesperex plant	1	20	.4000	.50262	.11239	.1648	.6352	.00	1.00
	2	20	.4000	.50262	.11239	.1648	.6352	.00	1.00
	3	20	.4000	.50262	.11239	.1648	.6352	.00	1.00
	4	20	.3000	.47016	.10513	.0800	.5200	.00	1.00
	5	20	.6500	.48936	.10942	.4210	.8790	.00	1.00
	6	20	.6500	.48936	.10942	.4210	.8790	.00	1.00
	7	20	.8000	.41039	.09177	.6079	.9921	.00	1.00

	8	20	.6000	.50262	.11239	.3648	.8352	.00	1.00
	9	20	.8000	.41039	.09177	.6079	.9921	.00	1.00
	10	20	.8500	.36635	.08192	.6785	1.0215	.00	1.00
	11	20	.4000	.50262	.11239	.1648	.6352	.00	1.00
	12	20	.5000	.51299	.11471	.2599	.7401	.00	1.00
	13	20	.7000	.47016	.10513	.4800	.9200	.00	1.00
	14	20	.4000	.50262	.11239	.1648	.6352	.00	1.00
	15	20	.6000	.50262	.11239	.3648	.8352	.00	1.00
	16	20	.5500	.51042	.11413	.3111	.7889	.00	1.00
	17	20	.5500	.51042	.11413	.3111	.7889	.00	1.00
	18	20	.3000	.47016	.10513	.0800	.5200	.00	1.00
	19	20	.6500	.48936	.10942	.4210	.8790	.00	1.00
	Total	380	.5526	.49788	.02554	.5024	.6029	.00	1.00
Meanheighttofexplant	1	20	1.1705	.28690	.06415	1.0362	1.3048	.70	1.60
	2	20	1.0625	.55934	.12507	.8007	1.3243	.00	2.00
	3	20	.7125	.56937	.12731	.4460	.9790	.00	1.90
	4	20	1.0000	.56754	.12691	.7344	1.2656	.50	2.40
	5	20	1.4200	.70550	.15776	1.0898	1.7502	.75	4.00
	6	20	2.1500	1.75687	.39285	1.3278	2.9722	.00	7.00
	7	20	1.8625	1.78715	.39962	1.0261	2.6989	.00	8.50
	8	20	.7600	.59330	.13266	.4823	1.0377	.00	2.00
	9	20	1.2000	.83351	.18638	.8099	1.5901	.00	2.50
	10	20	1.8575	.76799	.17173	1.4981	2.2169	.50	3.00
	11	20	1.8500	.72729	.16263	1.5096	2.1904	1.00	3.50
	12	20	1.6875	.61170	.13678	1.4012	1.9738	.50	3.00
	13	20	1.6000	.96450	.21567	1.1486	2.0514	.50	4.00

	14	20	1.6250	.72321	.16171	1.2865	1.9635	.50	3.00
	15	20	1.9500	1.22367	.27362	1.3773	2.5227	.50	5.00
	16	20	1.8375	.75339	.16846	1.4849	2.1901	.50	3.00
	17	20	1.8975	.74929	.16755	1.5468	2.2482	.50	3.50
	18	20	1.5500	.80948	.18101	1.1712	1.9288	.50	3.00
	19	20	1.8525	.86351	.19309	1.4484	2.2566	.50	4.00
	Total	380	1.5287	.98304	.05043	1.4296	1.6279	.00	8.50
Meanlengthofleaves	1	20	.5363	1.04893	.23455	.0453	1.0272	.00	3.58
	2	20	.3417	.56714	.12682	.0763	.6072	.00	1.70
	3	20	.5963	.95167	.21280	.1509	1.0416	.00	3.20
	4	20	.3725	.60076	.13433	.0913	.6537	.00	1.60
	5	20	.9305	.92409	.20663	.4980	1.3630	.00	2.38
	6	20	1.1109	1.08250	.24206	.6043	1.6175	.00	3.00
	7	20	1.6175	1.39050	.31093	.9667	2.2683	.00	4.00
	8	20	.5738	1.05156	.23514	.0816	1.0659	.00	3.58
	9	20	.3467	.56974	.12740	.0801	.6134	.00	1.70
	10	20	.5453	.88907	.19880	.1292	.9613	.00	3.00
	11	20	.3725	.60076	.13433	.0913	.6537	.00	1.60
	12	20	.9303	.92368	.20654	.4980	1.3625	.00	2.38
	13	20	1.3110	1.21529	.27175	.7422	1.8798	.00	3.83
	14	20	1.5060	1.41468	.31633	.8439	2.1681	.00	4.00
	15	20	.8050	.84044	.18793	.4117	1.1983	.00	2.57
	16	20	1.2050	.97596	.21823	.7482	1.6618	.00	3.00
	17	20	1.1575	.66192	.14801	.8477	1.4673	.00	2.13
	18	20	.6210	.97949	.21902	.1626	1.0794	.00	3.00
	19	20	.6750	.81151	.18146	.2952	1.0548	.00	2.50

	Total	380	.8187	1.00949	.05179	.7168	.9205	.00	4.00
Noofleaves	1	20	1.5000	2.46021	.55012	.3486	2.6514	.00	9.00
	2	20	1.2000	1.82382	.40782	.3464	2.0536	.00	5.00
	3	20	1.0500	1.57196	.35150	.3143	1.7857	.00	5.00
	4	20	1.2000	2.23842	.50053	.1524	2.2476	.00	7.00
	5	20	2.0360	2.44957	.54774	.8896	3.1824	.00	8.00
	6	20	4.1500	4.65974	1.04195	1.9692	6.3308	.00	13.00
	7	20	3.4500	4.05845	.90750	1.5506	5.3494	.00	18.00
	8	20	1.8000	1.85245	.41422	.9330	2.6670	.00	6.00
	9	20	2.5500	1.84890	.41343	1.6847	3.4153	.00	6.00
	10	20	3.6500	2.71981	.60817	2.3771	4.9229	.00	8.00
	11	20	.9500	1.31689	.29447	.3337	1.5663	.00	4.00
	12	20	1.2500	1.55174	.34698	.5238	1.9762	.00	4.00
	13	20	2.2000	3.20526	.71672	.6999	3.7001	.00	14.00
	14	20	1.2500	1.77334	.39653	.4201	2.0799	.00	6.00
	15	20	2.2000	2.56700	.57400	.9986	3.4014	.00	8.00
	16	20	1.6500	1.87153	.41849	.7741	2.5259	.00	6.00
	17	20	1.6000	1.90291	.42550	.7094	2.4906	.00	7.00
	18	20	.5500	.94451	.21120	.1080	.9920	.00	3.00
	19	20	2.4000	2.83586	.63412	1.0728	3.7272	.00	10.00
	Total	380	1.9282	2.58577	.13265	1.6674	2.1890	.00	18.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
No of shoot	Between Groups	10.358	18	.575	1.434	.112
	Within Groups	144.850	361	.401		
	Total	155.208	379			
Percentage of shoot per explants	Between Groups	2.900	18	.161	3.839	.000
	Within Groups	15.150	361	.042		
	Total	18.050	379			
Percentage of leaves per explant	Between Groups	10.247	18	.569	2.455	.001
	Within Groups	83.700	361	.232		
	Total	93.947	379			
Mean height of explant	Between Groups	65.293	18	3.627	4.351	.000
	Within Groups	300.959	361	.834		
	Total	366.252	379			
Mean length of leaves	Between Groups	57.991	18	3.222	3.543	.000
	Within Groups	328.241	361	.909		
	Total	386.231	379			
No of leaves	Between Groups	339.568	18	18.865	3.103	.000

Within Groups		2194.507	361	6.079		
Total		2534.075	379			

Post Hoc Tests

No of shoot

Duncan^a

Treatment	N	Subset for alpha = 0.05		
		1	2	3
3	20	.8500		
8	20	.9500	.9500	
11	20	1.0000	1.0000	1.0000
18	20	1.0000	1.0000	1.0000
2	20	1.0500	1.0500	1.0500
4	20	1.0500	1.0500	1.0500
16	20	1.0500	1.0500	1.0500
6	20	1.1000	1.1000	1.1000
12	20	1.1500	1.1500	1.1500
14	20	1.1500	1.1500	1.1500
15	20	1.1500	1.1500	1.1500
17	20	1.1500	1.1500	1.1500
9	20	1.2000	1.2000	1.2000
1	20	1.3000	1.3000	1.3000
7	20	1.3000	1.3000	1.3000
19	20	1.3000	1.3000	1.3000
13	20		1.4000	1.4000

5	20			1.4500
10	20			1.4500
Sig.		.070	.070	.071

Means for groups in homogeneous subsets are displayed.

Percentage of shoot per explants

Duncan^a

Treatment	N	Subset for alpha = 0.05		
		1	2	3
8	20	.7000		
3	20	.8000	.8000	
9	20	.8000	.8000	
2	20		.9000	.9000
6	20		.9000	.9000
7	20			.9500
1	20			1.0000
4	20			1.0000
5	20			1.0000
10	20			1.0000
11	20			1.0000
12	20			1.0000
13	20			1.0000
14	20			1.0000
15	20			1.0000
16	20			1.0000
17	20			1.0000

18	20			1.0000
19	20			1.0000
Sig.		.147	.162	.221

Means for groups in homogeneous subsets are displayed.

Percentage of leaves per explant

Duncan^a

Treatment	N	Subset for alpha = 0.05		
		1	2	3
4	20	.3000		
18	20	.3000		
1	20	.4000	.4000	
2	20	.4000	.4000	
3	20	.4000	.4000	
11	20	.4000	.4000	
14	20	.4000	.4000	
12	20	.5000	.5000	.5000
16	20	.5500	.5500	.5500
17	20	.5500	.5500	.5500
8	20	.6000	.6000	.6000
15	20	.6000	.6000	.6000
5	20	.6500	.6500	.6500
6	20	.6500	.6500	.6500
19	20	.6500	.6500	.6500
13	20		.7000	.7000
7	20			.8000

9	20			.8000
10	20			.8500
Sig.		.062	.112	.058

Means for groups in homogeneous subsets are displayed.

Mean height of explant

Duncan^a

Treatment	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
3	20	.7125					
8	20	.7600					
4	20	1.0000	1.0000				
2	20	1.0625	1.0625	1.0625			
1	20	1.1705	1.1705	1.1705			
9	20	1.2000	1.2000	1.2000	1.2000		
5	20		1.4200	1.4200	1.4200	1.4200	
18	20		1.5500	1.5500	1.5500	1.5500	1.5500
13	20		1.6000	1.6000	1.6000	1.6000	1.6000
14	20		1.6250	1.6250	1.6250	1.6250	1.6250
12	20			1.6875	1.6875	1.6875	1.6875
16	20				1.8375	1.8375	1.8375
11	20				1.8500	1.8500	1.8500
19	20				1.8525	1.8525	1.8525
10	20				1.8575	1.8575	1.8575
7	20				1.8625	1.8625	1.8625
17	20					1.8975	1.8975

15	20					1.9500	1.9500
6	20						2.1500
Sig.		.144	.065	.065	.056	.135	.088

Means for groups in homogeneous subsets are displayed.

Mean length of leaves

Duncan^a

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
2	20	.3417			
9	20	.3467			
4	20	.3725			
11	20	.3725			
1	20	.5363	.5363		
10	20	.5453	.5453		
8	20	.5738	.5738		
3	20	.5963	.5963		
18	20	.6210	.6210	.6210	
19	20	.6750	.6750	.6750	
15	20	.8050	.8050	.8050	
12	20	.9303	.9303	.9303	.9303
5	20	.9305	.9305	.9305	.9305
6	20		1.1109	1.1109	1.1109
17	20		1.1575	1.1575	1.1575
16	20		1.2050	1.2050	1.2050

13	20			1.3110	1.3110
14	20				1.5060
7	20				1.6175
Sig.		.113	.068	.053	.051

No of leaves

Duncan^a

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
18	20	.5500				
11	20	.9500	.9500			
3	20	1.0500	1.0500			
2	20	1.2000	1.2000			
4	20	1.2000	1.2000			
12	20	1.2500	1.2500			
14	20	1.2500	1.2500			
1	20	1.5000	1.5000			
17	20	1.6000	1.6000			
16	20	1.6500	1.6500			
8	20	1.8000	1.8000	1.8000		
5	20	2.0360	2.0360	2.0360	2.0360	
13	20	2.2000	2.2000	2.2000	2.2000	
15	20	2.2000	2.2000	2.2000	2.2000	
19	20	2.4000	2.4000	2.4000	2.4000	
9	20		2.5500	2.5500	2.5500	2.5500
7	20			3.4500	3.4500	3.4500

10	20				3.6500	3.6500
6	20					4.1500
Sig.		.053	.098	.068	.075	.061

Means for groups in homogeneous subsets are displayed.