IN VITRO REGENERTION OF TEA [*Camellia sinensis* (L.) O. Kuntze]

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CERTIFICATE

This is to certify that thesis entitled, **"*IN VITRO* REGENERTION OF TEA [*Camellia sinensis (L.)* O. Kuntze]"** *submitted to the Faculty of* **AGRICULTURE,** *Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* **MASTER OF SCIENCE** *in* **BIOTECHNOLOGY**, *embodies the result of a piece of bona fide research work carried out by* **MOKARAM HANIFA KOLY,** *Registration No.* **19-10234** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

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Dedicated to

My beloved Parents

And

My sister

ABBREVIATIONS AND ACRONYMS

Agril.	:	Agriculture
Biol.	:	Biological
cm	:	Centimeter
CRD	:	Completely Randomized Design
GA_3	:	Gibberellic acid 3
Conc.	:	Concentration
DAI	:	Days After Inoculation
et al.	:	And others (at elli)
FAO	:	Food and Agricultural Organization
WAI		Weeks After Inoculation
g/L	:	Gram per litre
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	*a*- Napthalene acetic acid
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
Int.	:	International
2-ip	:	2-isopentenyladenine
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
PVP	:	Polyvinylpyrrolidone
PLB	:	Protocorm-like bodies
CV	:	Co-efficient of Variation
$^{\circ}C$	:	Degree Celsius
etc.	:	Etcetera

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IN VITRO REGENERTION OF TEA [*Camellia sinensis* (L.) O. Kuntze]

ABSTRACT

The present research was carried out at Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207 from the period of January 2020 to September 2021 to evaluate the effect of different plant growth regulators on *in vitro* callus induction, shoot regeneration and root formation for Tea. The experiment was conducted at Completely Randomized Design (CRD) with 3 replications. Four levels of BA (1.00, 2.00, 3.00 and 4.00 mg/L) alone and in combination with four levels of NAA (1.00, 2.00, 3.00 and 4.00mg/L) were used for shoot induction. The treatment 2, 4-D was used for callus induction. Different concentration of IBA (300, 400 and 500 mg/L) was used with different dipping time for root induction of regenerated shoot in natural open condition. The highest percentage (86.51%) of callus induction was observed in combined treatment of 2.00 mg/L BA + 1.50 mg/L 2, 4- D with minimum (2.12) weeks. Highest percentage (84.39%) of shoot induction was recorded with 2.00 mg/L BA+ 2.00 mg/L NAA as well as in minimum (32.43) days. Maximum number of shoot (1.42, 2.46 and 4.49) and the highest length of shoot (1.41, 3.28 and 4.61) at 60, 90 and 120 DAI were recorded from the treatment 2.00 mg/L BA + 2.00 mg/L NAA. The highest percentage (75.32%) of root induction with minimum (6.02) weeks was recorded when regenerated shoots were dipped in 500 mg/L IBA solution for 30 minutes. The highest number of roots (4.20 and 7.80) and highest length of roots (3.31 and 5.80 cm) were recorded in 500 mg/L IBA concentration in 30 minutes dipping time. No root induction was recorded in controlled treatment. The protocol developed from the present study might be useful for large scale production of healthy, disease free planting material for Tea.

CHAPTER I

INTRODUCTION

The plant used to brew tea known as *Camellia sinensis*, which belongs to the genus *Camellia*, is classified in the order Ericales and family Theaceae. There are more than 200 species in the genus, most of which are native to the southern China, mountains of Tibet and northern and eastern India. The genus was divided into 12 sub generic divisions, one of which is Thea. Taxonomy within the genus *Camellia* has been made more difficult by the numerous interspecific crosses between its species. The plant *(Camellia sinensis* (L.) O. Kuntze) is used to produce the majority of tea beverages. Leaf morphophysiological features play a significant role in defining tea plants. From a scientific and commercial standpoint, tea is the most significant *Camellia* species. In some places of China, a number of species of *Camellia* are utilized as sources of tea-like drinks. China relies heavily on seed oil from a variety of species, including *C. japonica, C. fraterna,* and even *C. sinensis*. Numerous camellia species are also highly valuable as decorative plants. Three distinct tea varieties have been identified based on leaf characteristics including posture, size, and development pattern. These cultivars from Cambo, Assam and China respectively known as *C. assamica ssp*. Lasiocalyx, *C. assamica , Camellia sinensis* (Kumar and Loh 2012).

According to Wikipedia, In China, tea was initially made available to Western monks and merchants in the 16th century; at the time, it was known as chá. The first recorded use of the word "tea" in Europe was chiai. A European country shipped tea for the first time on record in the year 1607. The beverage was brought by the Dutch, Germany, and France as well as all over the Atlantic to New York. The Russian people first experienced tea around 1567. Russia and China signed a pact in 1679 regarding regular tea imports from China. Richard Wickham's letter has the earliest documented mention of tea in English. However, the popularity of tea in the British Isles did not really take off until the 18th century. In an effort to end China's tea monopoly, the British brought Chinese tea into India in 1836. Chinese tea seedlings were imported from the Kumaun area by Archibald Campbell, who tried his hand at growing tea in Darjeeling. When the British learned that a unique kind of tea was native to Assam and the northeastern part of India, they crossed it with a Chinese small-leaf variety of tea, and it quickly gained popularity throughout India in the

1950s. During British Empire, the cultivation of black tea was brought to Assam and Bengal, notably in the Sylhet district of Assam. In 1840, European merchants founded the very first sub continental tea gardens next to the Chittagong Club. In Sylhet's Mulnicherra Estate, commercial tea production started in 1857.

C. sinensis is now grown in tropical and subtropical areas all over the world. Tea is a small plant or evergreen shrub that is often pruned to a highest of less than 2 meters when grown for its leaves. It has a considerable root system. The flowers are white and yellow, 2.5 to 4 cm in diameter, and have 7 - 8 petals. Fruit is a three-seeded sub globule capsule. The leaves are 2 to 5 cm wide and 4 to 15 cm long. For the best tea, fresh, light-green leaves should be plucked. Their undersides are covered with small, white hairs. Aged leaves have a darker coloration.

Tea thrives in its natural habitat best in regions with warm, sunny, humid weather and moderate annual precipitation of a minimum 100 to 120 cm. For the maximum production rate, an acidic and well-drained soil condition is preferred. With these parameters, tea may grow anywhere between sea level to 2,100 m above sea level. Its particular requirements include temperatures between 10 - 30 °C, 1,250 millimeters or less of annual precipitation, acidic soils, slopes of no more than 0.5 degrees, and highests up to 2000 meters above sea level. As a result, only a few places in the world produce tea, and it is extremely sensitive to the changes in growth circumstances. Because its favorable growth conditions are incredibly susceptible, climate change is also likely to have a considerable impact on them. Tea bushes are usually planted beneath a canopy for some shade, which is a popular agricultural strategy for growing tea (Daud *et al*., 2012).

Because of the variations in the chemical composition of different leaf ages, they result in distinct tea qualities. Typically, the first 2 to 3 leaves and the tip (known as bud) are picked for processing. More or less every 1-2 weeks, this hand plucking is repeated. If left unattended, tea plants could develop into trees, although cultivated plants are cut back to waist highest to make harvesting easier.

According to Bangladesh Tea Board, there are 167 commercial tea farms and tea gardens in Bangladesh. On average, 6.74 crores kg of tea are produced annually on the 2, 79,507 acres total area. Bangladesh has broken its record for producing tea,

throughout the nation producing 96.50 million kg in 2021. The same year, Bangladesh earned $180.57 million by exporting 680,000 kg of tea worldwide. The previous-highest yearly production was 96.069 million kg in 2019.

The three most common tea kinds are completely fermented, partially fermented, and unfermented which are respectively known as black tea, oolong, and green tea. Different amounts of fermentation happen at various rates depending on the manufacturing processes used to make each type of tea.

Tea contains a variety of compounds, including catechins, theaflavins, simple polyphenols, flavonols, other polyphenols, theanine, amino acids, peptides, protein, organic acids, sugars, other carbohydrates, lipids, caffeine, other methylxanthines, potassium, and other minerals. Along with citric, isocitric, and succinic acids, tea is a substantial source of oxalic and malic acids. Both black tea and green tea have been shown to contain vitamin C. Chlorophyll and carotenoids are the primary pigments found in freshly picked tea leaves. The scent that is unique to black tea is largely created by the carotenoids. The fresh tea leaf is composed largely of lipids, terpenoids, and saponins. Potassium has been shown to be abundant in tea plants, which also contain large levels of calcium and magnesium as well as trace amounts of manganese, iron, and phosphorus, as well as copper, nickel, salt, boron, molybdenum, sulfur, and zinc. In the plant, cobalt, lead, aluminum, and cadmium have all been found. Also, tea drinks can contain a substantial amount of fluoride.

Both herbal and caffeinated teas contain polyphenols, which may help lower the risk of developing certain chronic illnesses. According to some research, tea's catechins and caffeine may aid in weight loss. Those who frequently consume black or green tea have a lower chance of developing heart disease, and green tea's catechins may help regulate blood sugar, lowering the risk of Type 2 diabetes. Some studies also claim that those who drink tea have a decreased chance of developing certain cancers. Additionally, tea polyphenols are well known for having strong antimicrobial properties. Catechins in green tea, show antibacterial action against Gram-positive and negative bacteria. According to studies, green tea inhibits oral microorganisms, preventing tooth decay. Black tea's antimicrobial properties have also sometimes been reported .According to reports, the positive benefits from tea polyphenols could be attributed to their antioxidant, hypocholesterolemic, antibacterial, anticarcinogenic,

antimutagenic, and anti-allergenic properties to a higher extent. When tea leaves are processed more, their antioxidant content decreases. The extra catechin and overall polyphenol content of green tea is what gives it its outstanding antioxidant effects. Strong quantities of the arubigins and theaflavins which have a high antioxidant potential, were found in black tea (Bancirova, 2010).

Although drinking tea regularly has many positive effects, doing it excessively might be harmful to health. The danger of caffeine overdose is one. Anxiety, restlessness, and sleep disturbance are all possible side effects of excessive caffeine use. Other digestive problems, such as loose stools, also occur in certain persons. Another possible negative effect of ingesting too much caffeine is vomiting. Other side effects include heartburn, abdominal discomfort, dizziness, and muscular pains. Caffeine shouldn't be used in excess of 400 mg per day from all sources.

Natural outcrossing of the tea plant occurs, and only the most elite genotypes are reproduced vegetatively and made available as clonal variations. Tea may be propagated vegetatively via single node cuttings, which is a relatively simple process. One cutting, however, can only produce one plant, and it needs 10-12 months to mature before planting. The industry needs between 11,000 and 15,000 plants to be planted on a hectare of land for clonal plantation, and a large quantity of clonal plants are needed every year. Producing such a massive number of plants quickly becomes very impossible. In these conditions, the necessity of micropropagation for the quick and widespread replication of clones has risen. It is also generally recognized that *in vitro* multiplication has various benefits. For woody perennials like tea and wilder species, it is highly relevant. Even though clonal propagation is still a successful model of propagation, it is constrained by a number of traits of tea and other closely related species. Numerous reasons contribute to this, including the inaccessibility of planting material because of winter dormancy, the season-dependent capacity of rooting and poor root development in some clones, slow propagation rate, low rates of nursery survival, etc. As a result, the micropropagation approach seems to be the best option for resolving the issues with traditional propagation. It also has a good multiplication rate. Virus-free plant production, quick clonal replication, and genetic resource preservation can all benefit from apical bud culture. Therefore, shoot tips

from immature plant sections and nodal segments containing dormant axillary buds are typically used as explants in tissue culture (Yu *et al*., 2022).

For the production and expansion of axillary shoots in tea, several researchers focused on the usage of indole-3-acetic acid (IAA, 0.10-2.00 mg/L) and kinetin (Kn, 0.21-8.00 mg/L). Increased 6- Benzyl Amino Purine (BAP) concentrations (0.20–2.20 mg/L) had a positive impact on the proliferation of shoots. When combined with 2,4-Dichlorophenoxy acetic acid (2, 4-D), *a*- Napthalene acetic acid (NAA) or Indole butyric acid (IBA) at doses between 1.00 and 3.00 mg/L and higher doses of Thidiazuron (TDZ) (1.10, 2.20, and 3.30 mg/L) commonly cause callusing of explants. With a combination of TDZ 1.10 mg/L and NAA 2.00 mg/L, shoot proliferation was seen to be more effective (98%) than before (Mondal *et al*., 2004).

Nakamura (1991) said ways for tea plant *in vitro* propagation that are consistent, easy and effective. When the media were combined with Benzyladenine (BA , 0.10-1.00 mg/L) + Gibberellic acid 3(GA_3,5.00 mg/L) or BA (1.00, 5.00 mg/I) + GA3 (1.00 mg/L), the development of shoots in cultures of shoot tips and axillary buds was noticeably sped up.

In Camellia, roots are often stimulated by the use of auxin, either in low quantities given in the media, by either dipping the bottom ends of the shoots in concentrated auxin before moving to an auxin free medium for the roots to form. Shoots left untreated never even try to root. For the adult *Alba Plena* cultivar, basal dipping in 1.00 g/L Indole butyric acid (IBA) for 15 min produced adequate results, just as it had for juvenile cultures throughout the rooting stage (Vieitez *et al*., 1992) .

Present study is also designed using shoot tips and nodal explants. Tissue culture technique can be utilized for its large scale production. Therefore, the research program is designed with the following objectives-

i. To study the role of phytohormone on *in vitro* regeneration in tea.

ii. Assessment of best hormonal combination for *in vitro* regeneration of tea.

iii. Establishment of *in vitro* regeneration protocol of tea

CHAPTER II
REVIEW OF LITERATURE

Growing plant cells, tissues, or organs other than the mother plant using artificial media in aseptic condition is known as plant tissue culture (Ikenganyia *et al*., 2017). It involves the development of isolated plant cells or fragments of tissue in a sterile growth medium under regulated environmental conditions. The capacity of numerous plant cells to regenerate an entire plant (totipotency) is essential for plant tissue culture. Given the necessary nutrients and plant hormones, single cell, protoplasts, fragments of leaves, stems, or roots can frequently be utilized to grow a new plant on culture media.

In vitro micro propagation of tea [*Camellia sinensis* (L.) O. Kuntze] was investigated in this present study. And some related works already performed by different institutes home and abroad have been reviewed and some of the most relevant literatures are cited below.

2.1 Explants

2.1.a Different woody plants

For woody plants like tea the growing time needed to produce micro shoots may take months rather than weeks, which would make the economics of *in vitro* cloning of woody plants less cost effective. Adventitious organ regeneration is likewise a gradual process. The length of time needed to stabilize a woody plant *in vitro* might vary greatly depending on the species, its history, as well as other elements. After 2 to 5 subcultures, certain cultures can settle. Other cultures have taken more than two years and more than 20 subcultures; other plants, including the majority of Quercus species, have never been successfully sustained as shoot cultures (Smith, 2013).

Azad *et al*., (2005) found from extruded leaf explants, it has been possible to induce shoot organogenesis and plant establishment in the small to medium-sized dioecious tree *Phellodendron amurense* Rupr. Young leaf explants were taken from established

shoot cultures created *in vitro* and utilized to stimulate callus formation, direct shoot regeneration, and shoot differentiation on MS media. After 4 weeks of culture, 1 cm 2 sections of roughly 10-day-old leaves were placed on MS medium and enhanced with 4.4 µM 6- Benzyl Amino Purine (BAP) with 1.0 µM NAA. This resulted in direct shoot regeneration. Within 3 weeks of incubation on a medium supplemented with 2.00 µM TDZ and 4.00 µM 2, 4-D or 4.00 µM NAA, the leaf explants developed callus from their cut edges.

Micropropagation procedures have been created for the cloning of mature *Balanites aegyptiaca*, *Citrus limon,* and *Syzygium cuminii* trees. As explants, fresh shoot shoots from the trees were employed. By activating axillary meristem on MS media + 0.45 µM BAP, the nodal explants generated many shoots in a laboratory setting. By repeatedly transferring the mother explants and sub culturing the nodal sections of *in vitro* differentiated shoots, shoots were further multiplied in culture. When compared to BAP, Kinetine was shown to be less efficient in activating axillary buds. BAP or kinetin at concentrations greater than 0.45 µM both produced callusing in the explants (J. S. Rathore *et al*., 2005).

Altman and Loberant (1998) stated that Tissue culture is used to grow several conifers, including the Norway spruce, radiating pine, white and black spruces, and pinus pinaster.

The desert horticulture plant *Capparis decidua* 'Ker' (Capparidaceae) serves a variety of functions. A technique for micropropagating Crappies decidua mature trees was created. Nodal explants were grown on Murashige and Skoog medium with 0.1 mg/L NAA and 5.0 mg/L BAP, and several shoots were produced. By subculturing nodal shoot segments onto MS+ 0.1 mg/L IAA + 1.0 mg/L BAP + additives and repeatedly transferring the original explant at intervals of 3 weeks, tile shoots were multiplied. When pulse-treated with 100 mg/L IBA in half-strength MS liquid medium for 4 hours, and subsequently transferred onto hormone-free half-strength MS basal salt media, 60 to 70 percent of the shoots rooted (Deora and Shekhawat, 1995) .

A technique for mass cloning and reproduction of *Maytenus emarginata,* a tree native to the Indian Desert that is extremely drought tolerant, has been devised *in vitro* (T. S. Rathore *et al*., 1992).

In vitro propagation of *P. cineraria* was significantly impacted by the age of the tree, type and size of explant, the season of explant collection, position of explant on media, plant growth regulators, and specific (Shekhawat *et al*., 1993).

The stately hardwood *Anogeissus acuminata* is a member of the Combretaceae family. On Murashige and Skoog's (MS) medium with IAA 0.10 mg/L + BAP 1.50 mg/L and ascorbic acid 50 mg/L, citric acid 25 mg/L, arginine 25 mg/L, adenine sulphate 25 mg/L, multiple shoots (16–20 shoots per explant) of Anogeissus acuminate were induced. For subsequent multiplication of shoots up to 16 consecutive cultures at an interval of 4 weeks were employed as explants. Shoots generated *in vitro* were planted on MS medium at half strength with 0.5 mg/L IBA and root formed (Rathore *et al*., 1993).

Syzygium cuminii L.is a tropical fruit tree of great economic importance. It was micro propagated On Murashige & Skoog (MS) modified media supplemented with (BA) (0.23-8.90 µM) separately or in a combination with (NAA), (IAA), multiple shoots were produced from nodal and shoot tip segments. Extracted shoots were put on MS medium with NAA and/or IBA for root induction before being moved to MS basal media to create full plantlets. The shoots on the growth regulator-free media failed to develop roots. However, following 4 weeks of translocation, shoots produced 1-2 roots when NAA and IBA were present (Yadav *et al*., 1990).

Kärkönen *et al*.,(1999) stated that axillary buds from 43 species of broad-leaved Japanese plants were gathered and utilized to start micropropagation cultures. Various sets of growth regulators (which were BAP or 2iP alone or together with IBA or IAA) were examined in four nutritional medium (MS, DKW, WPM, and A3). Shoot cultures from ten species were developed and could be utilized to micropropagate many plantlets. The shoots then rooted on medium with IBA supplements before being transferred to a peat-sand combination, where the majority of them thrived in the greenhouse environment.

At 50, 75, 90, and 105 days, leaf discs from *in vitro*-grown olives and immature zygotic embryos were obtained. The embryos were developed in MS medium that was half as strong as the original and in MS medium that had BAP mixed with either 2,4-D or NAA. Either 16 hours of continuous sunshine or an initial dark phase were used for incubation. Only in a medium with low cytokinin and auxin concentrations

was somatic embryogenesis seen. 2,4-D prevented differentiation but NAA did not. Only callus and root development were seen in leaf discs and younger and older zygotic embryos. After germinating, somatic embryos were planted in soil-filled pots (Rugini, 1988).

2.1.b Tea plants

According to Kumari *et al*.,(1984), [*Camellia sinensis* (L.) O. Kuntze] is typically reproduced vegetatively by cutting grafting, budding, and rooting. The regenerated cuttings frequently progressively lose their vigor over time, and if the mother plant is diseased, the cuttings may also transmit the illness.

Despite being well-established and having made significant contributions to tea improvement over the past few decades, conventional tea breeding has some drawbacks due to the following factors: perennial nature, long generation cycle, self-incompatibility, high inbreeding depression lack of availability of distinct mutants of various biotic and abiotic stress, absence of appropriate selection criteria, a low success rate of hand pollination, and lengthy seed maturation (Parathiraj and Kumar, 2005).

Ranaweera *et al*., (2013) noted that it takes 10–12 months for a plant to emerge from a single nodal incision used for vegetative growth of tea. Therefore, it takes 6-7 years to produce enough clone materials from a hybrid seed to test in a breeding program. Therefore, by multiplying them from hybrid embryos, micropropagation techniques are appealing possibilities to shorten the period necessary for introducing a new variety.

The mineral media that has been most often utilized for *Camellia* cultures, whether for callus culture for secondary product biosynthesis studies or for plant regeneration via embryogenesis, initiation of adventitious buds, or shoot tip and bud culture, has been Murashige and Skoog (MS) medium (1962) or certain adaptations of it (Vieirez *et al*., 1992) .

MS consistently produced the greatest outcomes for shoot multiplication phase of *C. japonica* regenerated from buds in terms of both growth and vigor. However, in a

series of shoot multiplication studies, MS was unable to provide good results for regeneration from mature *C. japonica* cv. Alba Plena material (Vieitez *et al*., 1989).

When combined with 2.00-4.00 mg/L of BA, auxin dosages of 2.00-4.00 mg/L of IBA were utilized by Kato (1985) to start the callus from which *C. sinensis* buds later differentiated. While 2, 4-D appears to be necessary for callus induction in tea cotyledons, it proved ineffectual for the regeneration of plantlets.

When applied at dosages of 10 mg/L for *C. sinensis* and 0.10–0.50 mg/L for the hybrid *C. japonica* x *C. chrysantha*, kinetin has also been demonstrated to stimulate the differentiation of plantlets from cotyledon tissues (Yamaguchi *et al*., 1987).

Initiating axillary bud development both in initial explant cultures as well as later shoot multiplication phases has often been successful with one or two mg/L of BA (Creze and MG, 1980).

While Beretta *et al*., (1987) assert that agar media is unquestionably better to liquid medium, Tian-Ling (1982) and Kato (1986) stimulated root development on a filter paper bridge over liquid medium. Vieitez *et al*., (1989) shown that neither the rooting percentage nor the quantity of roots per shoot were substantially impacted by the support type (agar or paper bridges).

Since the chemical makeup of the callus in the tea plant was examined, callus initiation and morphogenesis have been documented in a number of articles. Callus initiation has been often observed in a wide range of organs. Doi (1981) extracted roots but not buds from another callus. Depending on the source of the explant, callus from the stems displayed various capabilities for differentiation (Kato, 1985). Though plantlets were produced from the stem callus, leaf and root growth has not been noticed in the callus of the shoot tip. Slices of cotyledon were used to directly generate the embryoids (Kato, 1982). In China, haploid plants from different cultures were developed (Chen and Liao, 1983).

Cultured shoot tip explants of C. *japonica* seedlings that were 4-5 months old displayed shoot multiplication, root initiation, and later soil adaption. Additionally, in *C. japonica* and *C. chrysantha*, embryoids either formed directly from the cotyledon or through the callus, but as of now, nothing is known about how these plants regenerate other organs from callus than the cotyledon (Kato, 1989) .

For tea micropropagation, typically branch tips and nodal segments containing dormant axillary buds of either juvenile or adult origin of current year growth are employed as explants (Vieitez *et al*., 1992).

For the production of adventitious buds, Iddagoda *et al*., (1988) and Jha and Sen, (1992) utilized zygotic embryos, immature, and mature cotyledons. For the regeneration of shoots, Kato (1985) used stem segments with epidermal layers, stem segments without an epidermal layer, and entire stem segments. When compared to other calluses, those from the epidermal layers formed buds more quickly. Tea has also observed adventitious shoot buds from leaf explants (Kato, 1996).

The culture medium was suitable for both shoot initiation as well as multiplication when plant growth regulators (PGR) such BAP, 1.00-6.00 mg/L and (IBA, 0.01-2.00 mg/L) were added (Mondal, 2014).

For the induction of calluses, PGR such as 2, 4-D and NAA were effective. On the other hand, these PGRs failed to start the development and growth of tea shoots. Nakamura, (1988) when combined BAP with NAA proved particularly effective at promoting callus formation and shoot growth (Bag *et al*.,1997). For the successful shoot elongation, another PGR (2,4,5-T) was also reported (Jain *et al*., 1991).

It is generally known that growth adjuvants play a vital role in the micropropagation of tea. These include casein acid hydrolysate (Jha and Sen, 1992), yeast extract (Sarwar, 1985), coconut milk (Phukan and Mitra, 1984) , serine and glutamine (Chen and Liao, 1982) etc.

Utilizing shoot-tip explants, shoot cultures of *Camellia japonica* L. seedlings and *C. japonica* 'Alba plena' were developed *in vitro*. The greatest growth rates were supported in cultures using the Murashige and Skoog medium with BAP and kinetin (Samartin, 1989).

Again, according to Vieitez, *et al*., (1989), *Camellia japonica* cv. Alba Plena shoot cultures have been grown and propagated *in vitro*. For accomplishing shoot multiplication, the best growth regulator combination was 2.00 mg/L of BA, 2.00 mg/L of zeatin, 2.00 mg/L of 2iP, and 0.01 mg /L of IBA.

Several roots experiments were conducted in order to provide ideal circumstances for *Camellia japonica* in its juvenile development phase for *in vitro* multiplication. As opposed to incorporating auxin in the rooting media, dipping the basal ends of shoots in concentrated IBA solutions (0.50 and 1.00 g/L) for brief periods produced significantly superior outcomes. IBA caused roots to develop more effectively than NAA did. The 1 g/L IBA level performed better than 0.50 g/L in terms of rooting rates and the number of roots per rooted shoot. For this dipping intervals of 20 to 30 minutes were sufficient. More roots were produced than in the undiluted MS medium when the macronutrient content of MS medium was cut in half (Samartin *et al*., 1986).

2.2 Sterilization of Explant

According to the Webster and Mitchell (2003), external factors as plant type, age, explant source, and current meteorological conditions all have a role in explant contamination. It is quite hard to completely eradicate contamination from *in vitro* produced plants, no matter how careful one is with timing and selection.

Explant, personnel, instruments, laminar air flow, media, insects, and room air handling systems are only a few examples of possible sources of microbial contamination. Pre-wash the explants in warm and soapy water to eliminate surface pollutants such soil and dust and to improve the impact of the disinfectant. Use a wetting agent like Tween-20 or even a detergent like dishwashing soap, which functions as a surfactant, to improve disinfestation. Usually, 1-2 drops of the surfactant are sufficient for every 100 ml of the disinfectant (Smith, 2013).

Fungi, yeasts, and bacteria could be transferred into *in vitro* cultures with plant materials if the first surface sterilization is ineffective (Leifert *et al*., 1991).

Explants are often acquired from nurseries or their native habitats to establish *in vitro* investigations. After that, the explants are surface-sterilized. Different compounds are employed for this purpose. Explants are sterilized using a variety of disinfecting agents, including hydrogen peroxide, calcium hypochlorite, ethanol, silver nitrate, bromine water (BW), sodium hypochlorite, mercuric chloride, fungicides, as well as anti-infection agents (Tariq *et al*., 2020). When exposed to NaOCl, bacteria may be

more susceptible than fungus (Yu *et al*., 2022). In general, Tween-20, $HgCl_2$, NaOCl, and Bacteriocidal soap are employed for sterilization in tissue culture of Axillary Buds and Apical Buds, respectively (Davey & Anthony, 2010).

According to Ikenganyia *et al*., (2017), Plant tissue culture is carried out using equipment such a delicate weighing balance, pH meter, autoclave, camera, and microscope. Equipment that has come into touch with hazardous materials has to be autoclaved for 40 minutes. The wet heat process makes use of an autoclave that is pressurized steam-operated. This technique is typically utilized for media, liquids, and occasionally glasses as well. It is typical to utilize a time set of 15 minutes at 121°C and 15 PSI pressure; this environment is great for killing bacteria and fungus spores. It is crucial to remember that overusing the autoclave might cause undesirable chemical changes in medium, which can inhibit plant development.

Efficient explant sterilization is the first step in any effective *in vitro* culture process. The sterilization method selected for an experiment depends on the explant type, sterilization method, and genotype of the plant. There are many different sterilization techniques used to sterilize tissues, however these disinfectants are harmful to explant tissues as well, thus it is important to choose the right dose and exposure intervals to minimize plant damage. These sterilizing agents (ethanol, sodium hypochlorite, hydrogen peroxide, fungicides, mercuric chloride, and antibiotics) have been effectively employed by several researchers (Al Ghasheem *et al*., 2018).

Azad *et al*., (2005) carefully cleaned the explants with washed with tap water for 20 minutes after washing them with detergents for 15 minutes. The twigs were moved in a laminar-air-flow chamber after being surface-sterilized using 70% ethanol for 3 min. After that, surfaces were disinfected for 20 minutes using a 3% sodium hypochlorite solution. The explants were then rinsed at least three times with sterile distilled water to get rid of any remaining sterilant.

According to a methodology used by Rathore *et al*., (2005) to sterilize woody plants, nodal explants were washed and exposed to 0.1-0.2 percent Bavistin and 0.1 percent Tetracylin for 10-15 minutes. These were completely cleaned with sterile water after being surface sterilized with 0.1 percent $HgCl_2$ for 5 to 6 minutes, followed by 90 percent ethanol for 60 seconds. Finally, they were subjected to a 15–20 minute

treatment with a cold, sterile antioxidant solution containing 0.1 percent ascorbic acid, 0.05 percent citric acid, and 0.1 percent PVP.

According to Vieitez *et al*., (1992), *C. japonica* seeds treated with 70% alcohol for 90 seconds, followed by commercial bleach (20% chlorine) for 20 min, had a residual contamination rate of 12%.

When extremely small (0.20-0.50 mm) branch tips are to be utilized as explants, it is not required to disinfect the buds prior to dissection, according to Creze and MG, (1980), as these parts of plants are protected by their scales and the ultimate infection rate is less than 10%. A field-grown shoot of *C. sinensis* and *C. japonica* often have combined fungal and bacterial contamination rates of at least 90%, which makes sterilizing the material from these plants a little more challenging.

According to Khaliq *et al*., (2002), tea seeds were sterilized for five minutes using commercial bleach at a concentration of 2.26 percent, which contains 5.25 percent sodium hypochlorite. After that, the seeds were rinsed 3 times in sterilized distilled water. With commercial detergent, tea anthers were cleaned. Unopened flower buds were sterilized in a laminar flow cabinet using ethanol at a concentration of 70 percent for 2 seconds and sodium hypochlorite at concentrations of 1.0 and 1.5 percent for 10 and 15 minutes, respectively. Excess ethanol and sodium hypochlorite were then removed with three rinses in sterile water.

Tea shoot tips were cleaned in running water for 15 to 30 minutes by Arulpragasam and Latiff (1986). Then, they were surface sterilized by stirring in a 10–15 percent chlorox solution for 15 minutes while it included 0.01 percent Tween 20. Following three rounds of sterile distilled water rinsing, the shoot tips were cut to a length of 10 to 15 mm in order to prepare them for explanting. Once more, they were surface sterilized for 10 minutes in 10% chlorox, followed by three rinses in sterile distilled water.

Tea leaves were sterilized in a drop of laundry soap for one minute, rinsed with tap water, and then given 3 washes in sterile distilled water. They were then re-sterilized using 70% ethanol for 30 seconds followed by 20% sodium hypochlorite for 20 minutes, 3 rinses in sterile distilled water, and all of this was done under laminar flow (Ghanati and Ishka, 2009).

Sandal *et al*., (2001) used tea nodal segments in his work. These were then extensively cleaned in Tween-20 for fifteen minutes, and then for 5 minutes, they were surface sterilized with 0.04 percent mercuric chloride. The remaining mercuric chloride was then thoroughly removed using sterile distilled water.

According to Daud *et al*., (2012), Pre-sterilization using 0.2 percent Benomyl for 15 minutes, increased the proportion of "clean and alive" explants, particularly when it was followed by surface sterilization with mercury chloride. For leaf as well as nodal explants, treatments with 0.1 percent $HgCl_2$ for 15 and 30 seconds, respectively, had the greatest outcomes. The least toxic treatment for nodal explants seems to be 0.1 percent $HgCl_2$ for 30 seconds.

Because mercury chloride is extremely toxic, the dose and length of the treatment should be chosen to limit tissue death in the explants brought on by over-sterilization (Kataky and Handique, 2010)

Successfully surface-sterilized explants sometimes started to produce a dark-brown secretion, and the development of any axillary buds that remained alive was restricted. In order to obtain explants for direct inoculation, seedlings were grown in an aseptic environment. The seeds were thoroughly washed with sterile water after being rinsed with tap water 5-7 times. The seeds were then surface-sterilized with 0.1 percent diluted mercuric chloride solution for five minutes (Kataky and Handique, 2010).

2.3 Callus induction

According to Smith (2013), In micro culture, the callus development rates of woody perennials are frequently slower than those seen with herbaceous plants and Auxin 2,4-D is frequently used to induce calluses.

Khaliq *et al*., (2002) discovered that callus from nodal segments could be generated on MS media containing 2.00 mg/L NAA and 5.00 mg/L BAP, whereas callus from leaf tissue was achieved on MS medium containing 0.10 mg/L IBA and 2.50 mg/L BAP.

According to Tahardi and Shing (1992), callus production happened often in all cultures exposed to 10–20 mg/L 2,4-D. After six weeks, embryogenic calli were seen

in modified MS basal medium that also included 20 mg/L 2, 4-D. A somatic embryo formed from around 30% of the calli. After sub-culturing to a regeneration medium devoid of 2, 4-D, plantlet regeneration was accomplished.

An investigation was started by Frisch and Camper (1987) to create a tea *in vitro* cultivation methodology. The highest callus proliferation was seen when auxin combinations of (2,4-D; 2,4,5-T; NAA and Picloram) 10^{-7} M were utilized in MS medium plus sucrose (30 g/L), inositol (100 mg/L), thiamine-HC1 (1.30 mg/L), and kinetin. The concentrations for 2,4-D, 2,4,5-T, picloram, and NAA that caused the highest callus development were 10^{-7} M, $l0^{-6}$ M, 10^{-7} M, and 10^{-8} M, respectively, at a constant rate of kinetin (10^{-5}M). The optimal concentrations for callus development, 2,4,5-T, and kinetin, were determined to be 10^{-7} M and 10^{-5} M, respectively.

According to Kato (1989), fresh clones produced from cotyledon callus have been effectively obtained. Although stem callus was used to regenerate plantlets, leaf and root bud development is not seen in the callus of shoot tips.

After 42 days of incubation, Sarwar (1985) discovered the initiation of a callus. Zero callus developed, explant swelled, little callus formed, callus established enough not to be detached, and abundant callus formed were the five classes for callus formation.

Sarathchan *et al*., (1988) noted on nodal cuttings cultivated in a medium containing a high quantity of sucrose, kinetin, and 2, 4-D, calluses formed and multiplied quickly. At the cut ends of the nodal explants and the leaf edges, IBA and 2, 4-D both individually induced callus. In contrast to IBA, callus development was more active in 2, 4-D with a high concentration of sucrose.

Scran *et al*., (2007) tried to create an *in vitro* callus from tea leaves. To find the ideal concentration and exposure duration for sterilizing field-grown leaves, unfolded leaves were gathered and surface sterilized in a range of CloroxTM concentrations (15 percent to 75 percent) for a range of exposure times (15 to 60 minutes). Results showed that after 30 minutes of soaking in 60% and 75% solutions of Cloroxm, respectively, 50% and 58% aseptic callus cultures were produced. Additionally, this author noted the initiation of shoot and leaves from callus subculture.

The epidermal layers, the intact stem segment, as well as the stripped segment were the 3 kinds of explants that Kato (1986) injected. After two weeks of growth on the

medium supplemented with 2.00 mg/L IBA+ 4.00 mg/L BA, and 4.00 mg/L IBA+2.00 mg/L BA, callus initiation from these explants was noticed. On the callus induction medium, callus tissues were grown for eight weeks. After that, they were put in a medium with 0.5 mg/L IBA and 10 mg/L BA. The callus from the layers of the epidermis started to generate adventitious buds eight weeks later. With 1 or 0.5 mg/L IBA, these buds that had grown in a portion of the calluses, were subcultured. 2 weeks later inoculated on the rooting media, roots began to emerge.

2.4. Shoot initiation and elongation

Minocha (1987) said Different plants are affected physiologically in a variety of ways by different types of plant growth regulators. The kind of growth regulator, its concentration, the existence or exclusion of other growth regulators, the genetic make-up, and the physiological state of the target tissue all have an impact on these effects. Various growth regulator(s) or different combinations of growth regulator(s) may be necessary to achieve the same physiological response in different tissues, even within the same plant.

The inclusion of BA (1.00-6.00 mg/L) and IBA (0.01-2.00 mg/L) in the culture media have worked very well for shoot induction and subsequent multiplication. Although it was discovered that 2,4-D and NAA caused callus, they were ineffectual for the induction, growth, and maturation of tea shoots (Nakamura, 1988). Within 8–12 weeks, auxins like NAA and BA either formed callus or stimulated 4-5 shoot buds per explant (Bag *et al*., 1997).

According to Sandal *et al*.,(2005), rhizogenic callus followed by adventitious shoot buds were produced by extended cultivation of explant on a medium containing 7.50 and 10.0 mg/L 2,4-D for 12 to 16 weeks. These morphogenic processes took place when the 2, 4-D supplemented medium gradually depleted over time (12–16 weeks), resulting in an apparent concentration of 2,4-D being obtained at various phases of morphogenesis. Thus, it appears that 2,4-D concentrations and various phases of adventitious shoot morphogenesis are related.

According to Nissen and Sutter (2019), IBA was much more stable against autoclaving than IAA. Under liquid Murashige and Skoog (MS) medium, IBA was

likewise discovered to be more stable than IAA in growth chamber conditions. In agar-solidified MS, IBA and IAA stabilities were comparable. The reduction in IAA and IBA content, especially in agar-solidified medium, has significant effects on the preservation of media.

George and Sherrington (1984) discovered that when several concentrations of cytokinins (Kn, BAP, 2ip, and TDZ) alone were investigated, 2.00 mg/L BAP demonstrated the greatest response towards regeneration for both explants. When BAP tried alone, multiple shoot formations did not happen. It shows that cytokinin is insufficient on its own to support multiple shoot growth from both explants.

Jain *et al*.,(1993) and Arulpragasam and Latiff (1986) also reported on the appropriateness of GA_3 for proliferation and shoot growth. In order to encourage homogeneous shoot elongation, Tahardi and Shing (1992) grew shootlets singly or in clusters in WP medium supplemented with either GA_3 (2-10 µM) or IAA (2-10 µM). IAA had a substantially stronger positive effect on micropropagated tea shoot elongation than GA_3.

In accordance with Agarwal *et al*., (1992), the development of tea explant, as well as creation of many shoots were both shown to benefit most from a combination of IAA and BA. The greatest results were achieved on medium containing 1.40 µM IAA and 17.80 µM BA for the initiation of development of both terminal and axillary buds. Regardless of the kind of explant, it began within 10 to 15 days of inoculation and consisted mostly of a small amount of explant lengthening, leaf growth, and, in some cases, a maximum of two shoots per explant.

However, according to Sharma *et al*., (1999), Maximum shoot multiplication result was seen on 1/2 MS+ IAA (0.20 mg/L)+ BA (2.50 mg/L) including sucrose(20 g/L) and agar (8 g/L).

According to Kumari *et al*., (1984), In every nodal segment, 3–4 shoot-buds were generated after 2 months without callusing when culture medium supplemented with 2.00 mg/L IAA + 8.00 mg/L Kn. The explants' initial shoot-buds did not develop into shoots with lower levels of IAA (l.00 mg/L) and Kn (3.00 mg/L).

Sandal *et al*., (2001) describes how well TDZ causes fast tea shoot multiplication in liquid media. While BA caused hyperhydricity, necrosis, and low rates of shoot

proliferation, TDZ caused significant rates of shoot proliferation. Additionally, the introduction of liquid culture stages with longer subculture times has a significant impact on the reduction of labor costs and costs associated with the volume of medium agar, and subculture times.

Tahardi and Shing (1992) explained Single leaf-bud cuttings were cultured in woody plant (WP) basal medium enriched with BAP or TDZ, both in conjunction with IAA, to produce tea shoots *in vitro*. In medium supplemented with 10.00 µM BAP, shoot multiplication at a 4.1 fold increase every 6 weeks was achieved. In comparison to BAP treatments, the inclusion of TDZ at 5 µg/L significantly increased shoot multiplication.

Sandal *et al*., (2001) standardized an effective liquid culture technique for tea shoot proliferation. They discovered that the optimal combination for shoot proliferation in liquid medium was MS and 2.50–5.00 M TDZ.

Sandal *et al*., (2001) also used a productive liquid culture method for the growth of tea shoots. As opposed to TDZ, which has the inductive ability to cause a roughly 2-fold increase in tea shoot multiplication on solid media, BA must combine with auxins in tea in order to provide the same effect. However, it is possible that the uniform access and better use of TDZ to the whole explant in liquid medium as opposed to solid medium containing 5 µM TDZ is the cause of the higher development and multiplication of shoots. This is further confirmed by the observation that even after four weeks at 8.88 µM BA, there is a rise in the number of shoots in liquid media from 2.0 to 7.6 whereas there is virtually no response in solid medium supplemented with BA.

The growth in 20 ml liquid media was the best when the various liquid media volumes were examined in terms of the number of shoots (2–25), shoot length (4–16mm), leaf size (0.20–0.10 cm to 1.1–0.5 cm), number of nodes (2–6).

2.5 Multiple roots induction

Using very modest amounts of IBA (0.39-0.74 µM), Wynne and McDonald (2002) reports that maximum rooting efficiencies and rooting densities (root number) were

reached in woody plants. Both the dark and the light regimes generated roots, while the latter produced larger yields. After 30 days of development, the light-treated cultures had attained their maximum rooting percentage. IBA is often utilized, despite the fact that IAA is thought to be the main auxin responsible in the rooting process.

Tahardi and Shing (1992) discovered Rhizogenesis was induced by cultivating the shootlets for 1-2 weeks in the dark in WP medium that included 1.00-5.00 mg/L NAA and 1.50-3.00 percent sucrose. After transferring the shootlets to an auxin free medium in the light, root initiation took place around 10 days later. After exposure to 3.00 mg/L NAA for 7 days, the best rooting response (69–83%) was attained.

Shimazaki (1988) accused of rooting IBA was employed in the basic MS medium at doses of 0-1.00, 0-2.00, 1.00, and 2.00 mg/L without the addition of any cytokinins.

Bag *et al*., (2000) discovered other than auxins, a number of parameters, such as mineral nutrients, a carbon source, the pH of the food, gelling agents, light intensity/duration, and temperature, have been observed to affect *in vitro* rooting.

According to Caton (2008), benzyladenine (BA), a cytokinin that is frequently utilized in micropropagation systems, can hinder root growth. Good multiplication rates are obtained *in vitro* using the novel BA analog meta-topolin (mT), which also does not interfere with post-vitro or *in vitro* root development.

In general, IBA (0.50-8.00 mg/L) was favored more than NAA in tea and in C. *japonica*, *C. sasanqua*, and *C. reticulata*, according to several investigations. Because of the shorter, thicker, and associated calli, NAA-induced roots make future transplantation challenging. If treated with IBA, rooting took place significantly later, but the roots were large and fibrous (Mondal *et al*., 2004).

Tea was successfully rooted *in vitro* using a liquid medium and filter paper bridge (Kato, 1985 ; Tian-Ling, 1982 and Nakamura, 1987)

Ex vitro rooting, according to Jain *et al*., (1993), outperformed all other forms of *in vitro* rooting techniques, including liquid shake culture, agar-solidified media, and filter paper bridges. When clipped shoot ends were immersed in IBA (50 mg /L) for two hours before to transplantation into potting mixes, they were able to achieve 97 percent roots.

Another significant finding by Banerjee and Agarwal, (1990) was that root induction in tea benefited from low light and a low pH (4.5-4.6). These results support Nakamura (1987) assertion that auxin-dipped shoots received a dark treatment to encourage roots in tea.

The genotype affects *in vitro* rooting of tea as well. Murali *et al*., (1996) showed that when IBA treatment (1.00 mg/L) was applied for 30 minutes to the UPASI-26 and UPASI-27 cultivars, compared to UPASI-3 and BSB-1, the maximum rooting was attained. In general, tea micro-shoots were successfully *in vitro* rooted either by being cultured on auxin free media for a longer period of time or by being subjected to a "auxin shock" treatment at a high concentration.

Shoots placed on root induction media and then transferred to hormone free medium may improve *in vitro* root induction in Darjeeling tea clones (Jha and Sen, 1992). It is well recognized that shoots that have successfully navigated a number of subcultures are easier to root than those that have not (Arulpragasam and Latiff, 1986).

According to estimates, the cost of producing one micropropagated plant is seven times greater than the cost of producing a plant using vegetative means (Mondal *et al*., 2004). Plants produced using micropropagation may cost between 30 and 75 percent more when *in vitro* root initiation is included in (Debergh *et al*., 1992). *In vitro* rooted tea plants are difficult to harden, and their outdoor survival percentage is quite poor (Sharma *et al*., 1999). *Ex vitro* rooting is therefore a desirable choice for micropropagation as it reduces the expenses associated with manipulation as well as the time needed for acclimation and rooted (Rajasekaran and Mohankumar, 1992)

For rooting in tea, liquid medium was also discovered to be effective. *Ex vitro* rooting material outperformed *in vitro* rooting in terms of performance (Jain *et al*., 1993).

Tea shoot cut ends were pretreated by immersing them in an IBA (50.00 mg/L) solution for two hours prior to the hardening phase, which demonstrated a 97 percent rooting effectiveness (Banerjee and Agarwal, 1990).

E*x vitro* rooting and simultaneous hardening happen of tea microshoots treated with 500 mg/L IBA cultivated on a soil mixture consisting of soil, sand, and farm yard at ratio 5:4:1. However, when the medium's pH was increased to 5.4, the study's greatest root initiation rate was found at 71.6%. The use of plant growth regulators, the kind,

the age of the stock plant, and the grafting season all had an impact on the success rate, which ranged from 80 to 90% (Ranaweera *et al*., 2013).

Nakamura (1991) discovered that a decrease in the medium's salt content had a significant impact on root initiation and root elongation in tea. As opposed to micro shoots grown on half-strength MS media without growth regulators, micro shoots grown on full-strength MS medium treated with 3.00 mg/L BAP and 0.50 mg/L IBA developed more roots after being transferred to an *ex vitro* rooting mix.

The optimal *in vitro* circumstances for *ex vitro* root initiation may be thought of as cultivating tea micro shoots on a complete MS medium supplemented with 3.00 mg/L BAP, 0.50 mg/L IBA, 30.00 g/L sucrose, and 8.00 g/L agar during 8 weeks prior transfer into the *ex vitro* media (Ranaweera *et al*., 2013.)

Ex vitro rooting often results in the formation of adventitious root systems that are longer, thicker, flexible, and branching (McCulloch *et al*., 1994). *Ex vitro* rooting has one major benefit over *in vitro* rooting which is less root damage likely to happen upon transplantation to soil. When rooting takes place *ex vitro*, root production rates are frequently higher and root condition is maximized (Davey and Anthony, 2010).

Agarwal *et al*., (1992) stated the single most crucial ingredient for the initiation of rooting was discovered to be dipping the plants in IBA solution prior to inoculation on rooting media.

Sand that has been autoclaved in *ex vitro* was put to the soil to increase its texture. Within two to three weeks of being transferred to the rooting media, the shoots began to grow, and approximately a month later, the roots began to develop. The roots that developed were branching rootlets and resemble a tap root system. The bottom of the shoot had developed a callus due to higher IBA concentrations in the medium. The quantity of IBA needed for rooting varies depending on the clone (Arulpragasam and Latiff, 1986).

In comparison to *in vitro* roots, *ex vitro* rooting of tea micro-shoots with concurrent acclimation decreased the production costs of micropropagated plants by 71% (Mukhopadhyay *et al*., 2016).

By Agarwal *et al*., (1992) Shoots between 90 and 105 days following the first inoculation were moved to rooting medium, where rooting was best started with 34.5 µM of IBA. Within a month, rooting began. The frequency of roots initiation increased when the cutting ends of the shoots were immersed in an aqueous solution containing 493 µM IBA for 30 minutes prior to inoculation. The plants were moved to 1/2 strength MS medium without growth regulators as soon as rooting began, where abundant development of the root and shoot was seen. Over 60% of rooted plants have successfully been transplanted to soil.

CHAPTER III
MATERIALS AND METHODS

3.1 Time and location of the experiment

The present research was carried out in Biotechnology Laboratory in the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of January 2020 to September 2021.

3.2 Experimental materials

3.2.1 Source of material

The planting materials (Plate 1) of tea were collected from Tea garden, Atwari Upazila, Panchagarh and Regional Office of Bangladesh Tea Research Institute, Panchagarh.

Plate 1. Plant material of Tea for micropropagation

3.2.2 Explant

The shoot tips and nodal segments of *Camellia sinensis* (L.) O. Kuntze was used as experimental materials in the present investigation (Plate 2).

Plate 2. Nodal segment of tea as explants

3.2.3 Instruments

Metal instruments as forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^0C for 45 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glass ware

The Borosil glassware was used for all the experiments. Oven dried erlenmeyer flasks, culture bottles, flat bottom flasks, pipettes, Petri dishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware's were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water, until the detergent was removed completely. Finally, they were washed with distilled water before being sterilized for 3–4 hours in an oven set to 160–180°C.

3.2.5 Culture Medium

Plant growth regulators are essential for any kind of plant to regenerate successfully. All of the major and minor minerals, vitamins, and growth regulators necessary for optimal plantlet development should be present in tissue culture media. Explants were inoculated into media containing the plant growth regulators as well as the basic MS (Murashige and Skoog, 1962) medium. In appendix I, the MS media composition was mentioned. Depending on the needs, various media received separate additions of hormones. To do this, hormone stock solutions were made ahead of time and kept at a temperature of 4°C.

Treatments

1. BA (1.00, 2.00, 3.00 and 4.00 mg/L) was used for shoot proliferation.
2. 2, 4-D (1.00, 1.50, 2.00 and 2.50 mg/L) was used for callus induction.
3. BA (2.00 mg/L) with 2, 4-D (1.00, 1.50, 2.00 and 2.50 mg/L) were used for callus and shoot induction.
4. BA (2.0 mg/L) with NAA (1.00, 2.00, 3.00 and 4.00 mg/L) were used for shoot and leaves formation.
5. IBA (300, 400 and 500 mg/ L) was used in natural open condition for root formation.

3.3 The preparation of hormonal stock solutions

In order to prepare hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Cytokinins were dissolved in few drops of 1N NaOH solution and auxins were dissolved in 100% pure ethyl alcohol.

Hormones (Solute)	Solvents used
BA	1 N NaOH
NAA	100% ethyl alcohol
2,4-D	1 N NaOH
IBA	100% ethyl alcohol

The stock solution of hormones was prepared by following a general procedure. Ten ml of 100% ethyl alcohol or 1 (N) NaOH solvent and 100 mg of powder hormone was

placed in a small beaker and then dissolved with the addition of sterile distilled water using a measuring cylinder and the volume was made up to 100 ml. The prepared hormone solution was then labeled and stored at 4±1°C for use up to two month.

3.4 Preparation of different stock solution

3.4.1 Preparation of IN NaOH

40 g of NaOH pellets were dissolved in one liter of distilled water to prepare IN NaOH stock solution. Prepared solution was stored in cool and dry places in a glass bottle. This solution was used for adjusting pH of the final culture media preparation.

3.4.2 Preparation of 0.2% $HgCl_2$

To prepare 0.2% solution of $HgCl_2$,200 mg of the substances was dissolved in 100of H_2O with the help of a hot plate magnetic stirrer.

3.4.3 Preparation of 70% ethanol

In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured. Then 30ml double distilled water was added to make 100 ml solution. It was stored in sterilized glass bottle. This solution was made fresh each time before use.

3.5 Experiments

Four sub-experiments were conducted to assess the effect of different concentrations of BA and IBA on shoot proliferation and subsequent rooting of the regenerated shoot.

3.5.1 Sub-experiment 1

Effect of BA on shoot induction potentiality in Tea

Four levels of BA (1.00, 2.00, 3.00 and 4.00 mg/L) and control treatment (0.00 mg/L) were used. The experiments were arranged in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 5 culture vials.

3.5.2 Sub-experiment 2

Callus induction potentiality in Tea

Four levels of 2, 4-D (1.00, 1.50, 2.00 and 2.50 mg/L) and control treatment (0.00 mg/L) were used. The experiments were also arranged in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 5 culture vials.

3.5.3 Sub-experiment 3

Combined Effect of BA and 2, 4-D on callus induction potentiality in Tea

Four levels of 2, 4-D (1.00, 1.50, 2.00 and 2.50 mg/L) in a combination of 2.00 mg/L BA and control treatment (0.00 mg/L) were used. The experiments were also arranged

in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 5 culture vials.

3.5.4 Sub-experiment 4.

Combined Effect of BA and NAA on shoot induction and leaf development potentiality in Tea

Four levels of NAA (1.00, 2.00, 3.00 and 4.00 mg/L) in a combination of 2.00 mg/L BA and control (0.00 mg/L) were used. The experiments were arranged in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 5 culture vials.

3.5.5 Sub-experiment 5: Root induction of regenerated shoot through dipping in rooting hormone under natural condition

Three levels of IBA (300, 400 and 500 mg/L) with different dipping times (10, 20, 30, 40 and 50 min) and control (0.00 mg/L) were practiced. Each concentration in each dipping time was considered as an individual treatment. The combination of IBA concentration and dipping times are as follows:

T1= 300 mg/L IBA + Dipping time 10 mins
T2= 300 mg/L IBA + Dipping time 20 mins
T3= 300 mg/L IBA + Dipping time 30 mins
T4= 300 mg/L IBA + Dipping time 40 mins
T5= 300 mg/L IBA + Dipping time 50 mins
T6= 400 mg/L IBA + Dipping time 10 mins
T7= 400 mg/L IBA + Dipping time 20 mins
T8= 400 mg/L IBA + Dipping time 30 mins
T9= 400 mg/L IBA + Dipping time 40 mins
T10= 400 mg/L IBA + Dipping time 50 mins
T11= 500 mg/L IBA + Dipping time 10 mins
T12= 500 mg/L IBA + Dipping time 20 mins
T13= 500 mg/L IBA + Dipping time 30 mins
T14= 500 mg/L IBA + Dipping time 40 mins
T15= 500 mg/L IBA + Dipping time 50 mins

The experiments were also arranged in Completely Randomized Design (CRD) with 3 replications.

3.6 The preparation of culture media

To prepare 1000 ml of culture media the following steps were followed:

Step-1. Seven hundred ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. Five g of MS media and 30 g of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

Step-3. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

Step-4. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 g agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

3.7 Sterilization

3.7.1 Sterilization of Glassware and instruments

All type of glassware and instruments were first rinsed with liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Finally they were rinsed with distilled water and sterilized in autoclave at 121 °C for 15 psi for 30 minutes.

3.7.2 Sterilization of culture medium

For sterilization 30 mL culture medium was poured in 200 ml culture bottles and then autoclaving was done at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.7.3 Sterilization of culture room and transfer area

Before using culture room it was carefully cleansed with detergent and wiped with 70% ethanol.

3.7.4 Sterilization of Laminar Air Flow Cabinet

One and a half hours before to working, the laminar air flow cabinet was turned on. Cotton soaked in 70% ethanol was used to clean the air flow cabinet surface. Except for culture medium, all glassware was stored on the cabinet to prevent contamination.

The cabinet's lid was securely fastened, and UV was turned on for 30 minutes with the airflow turned off. When the specified amount of time had passed, UV was turned off, the door was opened, and the air flow was turned on. Work began in less than five minutes. Before beginning work, the hands and forearms were sterilized by rubbing with 70% ethanol. All of the equipment was repeatedly flamed during the culture after being dipped in 95 percent ethanol.

3.8 Culture method

3.8.1 Preparation of explants

Prior to surface sterilization, explants shoot tips and nodal segments were washed four or five times with distilled water after being rinsed with tap water and detergent. After that, the explants were stored in a 250 ml sterile beaker in a laminar airflow cabinet. Throughout sterilization, the beaker containing the explants was repeatedly shaken. After being exposed to 70% ethanol for one minute, they were washed with sterile distilled water. After the explants were submerged in 0.2% $HgCl_2$ in a beaker, 3–4 drops of Tween–20 were added, and the beaker was continuously shaken in both a clockwise and counterclockwise orientation for about 4-5 minutes. The explants were then cleaned three to four times with autoclaved distilled water to remove any chemicals and then it was ready for inoculation in culture medium.

3.8.2 Inoculation of culture

Following the correct sterilization procedure in a laminar airflow cabinet, the sterilized explants were properly inoculated (Plate 3). The inside of the laminar flow bench was sprayed with 70% ethyl alcohol before usage, and the surface was cleaned with the same alcohol before use. In an autoclave, all glassware, tools, and media were steam-sterilized. Throughout the process, working instruments were immersed in a beaker filled with ethanol at a 70 percent concentration and repeatedly flamed with a spirit burner. Throughout the time, the worker's hands and forearms were frequently sprayed with 70% alcohol and thoroughly cleansed with soap and water. Before and after the explant was placed on the medium, the edge of each culture vial was flamed.

Plate 3. Inoculation of explants in the MS culture medium

Explants were moved to big sterile glass Petri dishes or glass plates for inoculation using sterile forceps and under strictly aseptic circumstances. Here, the explants were further cut to the proper size using a sterile scalpel blade. Explants between 0.5 to 1 cm in length can be inoculated in MS medium using 25 mL of culture media. Explants are vertically inoculated in a culture bottle, and then the opening of the container is swiftly flamed and firmly sealed. The bottles were moved to the growing chamber after being properly labeled with the medium code, date of inoculation, and other information

3.8.3 Growth chamber

The bottles were maintained on the culture racks and given a controlled environment to develop the inoculated explants properly. The cultures were kept at a temperature of 21±2 ^{0}C, a photoperiod of 14 hours, with a light intensity of 3000–3500 lux (25W white bulbs). The culture was developed using white fluorescent bulbs. Typically, the photoperiod was 14 hours of light and 10 hours of darkness with 70% relative humidity (RH).

3.8.4 Sub-culture

3.8.4.a Sub-culture of the callus

After 2-4 weeks of incubation, the explants started to produce callus in the medium containing 2, 4-D. So regular subculturing at 4 weeks interval was practiced for the proliferation of callus. Callus were removed aseptically from the vial in the laminar air flow cabinet and put on the sterilized petri dishes. Then they were cut into small pieces and sub cultured into the vial containing 25 ml shooting hormone in each as well as in the same medium. For shoot induction and development, BA in combination with 2,4-D or NAA were used. The cultured vials were kept in 16 hour photoperiod at 25±2°C. Observation of explants growth and data collection were noted regularly. Contaminated vial were discarded carefully from culture room.

3.8.4.b Sub-culturing and maintaining proliferating shoots

The explants were cultured on MS nutrient medium supplemented with different concentration of BA alone or in combination of NAA. Percentage of explants showing shoot proliferation, days for shoot induction, number shoots and leaves per explants and average length of shoots were considered as parameter for evaluating this experiment. After successful shoot proliferation, subculture was done with newly form shoots. Shoots ware excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase shoot and leaves number. The observations on development pattern of shoots and leaves were made throughout the entire culture period. Data were recorded from 60, 90 and 120 days after first inoculation.

3.8.5 Root induction in regenerated shoot

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to *ex vitro* or natural open rooting media. Before that, different concentrations of IBA with different dipping time were used for root induction followed by the direct transformation in the soil (mixture of autoclaved garden soil, compost and sand in the ratio 2:1:1). The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 10 WAI and 14 WAI.

3.9 Data recording

The observations on development pattern of shoots and roots were made throughout the entire culture period. 3 replicates each of them containing 5 bottles (single shoot per culture bottle) were used per treatment. Data were recorded at 6, 10 and 14 WAI of culture, starting from day of inoculation on culture media in case of callus proliferation. In event of shoot formation on the medium containing BA and NAA, it was done after 60, 90 and 120 days of inoculation, and the leaves data were taken at 90 and 120 DAI. For rooting information data was taken at 10 WAI and 14 WAI. The following observations were recorded in cases of callus, shoot and root formation under *in vitro* condition.

1. Days for callus induction
2. Percentage (%) of callus response
3. Weight. of callus (g)
4. Days to shoot induction
5. Percentage (%) of shoot response
6. Number of shoot
7. Length of shoot (cm)
8. Number of leaves/shoot
9. Days to root induction
10. Percentage (%) of root response
11. Number of roots per shoot
12. Length of root/shoot (cm)

3.9.1 Calculation of days/weeks to shoots and roots induction

Days/weeks to shoots and roots induction was calculated by counting the days/weeks from explants inoculation to the first induction of shoots/roots.

3.9.2 Calculation of Percentage of callus induction from culture

Number of explants formed callus were recorded and the percentage of callus induction was calculated as

$$\text{Percent of callus induction} = \frac{\text{Number of explants induced callus}}{\text{Number of explants inoculated}} \times 100$$

3.9.3 Calculation of weight of callus (g)

The fresh weight of callus was measured in (g) using analytical balance.

3.9.4 Calculation of number of shoots per explant

Number of shoots per explants was calculated by counting the total numbers to shoots induced then the mean was calculated.

3.9.5 Calculation of number of leaves per explant

Number of leaves per explants was calculated by counting the total numbers of leaves induced then the mean was calculated.

3.9.6 Calculation of number of roots per explant

Number of roots per explants was calculated by counting the total numbers to roots induced then the mean was calculated.

3.9.7 Calculation of percent of shoot induction from culture

Number of shoot was recorded and the percentage of shoot induction was calculated as:

$$\text{Percent (\%) of shoot induction} = \frac{\text{Number of explant induced shoot}}{\text{Number of explant incubated}} \times 100$$

3.9.8 Calculation of percent of root induction from culture

Numbers of root were recorded and the percentage of root induction was calculated as:

$$\text{Percent (\%) of root induction} = \frac{\text{Number of explant induced root}}{\text{Number of explant incubated}} \times 100$$

3.9.9 Calculation of shoots length (cm)

Shoot length was measured in centimeter(cm) from the base to the top of the explants by a measuring scale. Then the mean was then calculated.

3.9.10 Calculation of root length (cm)

Root length was measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the mean was then calculated.

3.10 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with 3 replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using LSD (least significant difference) at 5% probability level using Statistix 10 program.

CHAPTER IV

RESULTS AND DISCUSSION

Different investigations were made on this experiment to evaluate the effect of different plant growth regulators for regeneration of tea. The results obtained from the experiment were described and discussed here with plates (4-11), Figures (1-8) and tables (1-9). Analysis of variance (ANOVA) presented in Appendix II-XXXII.

4.1 Sub-experiment 1.

Effect of BA on shoot induction potentiality in Tea [*Camellia sinensis* (L.) O. Kuntze]

The result of the effect of different concentrations of BA has been presented under following headings with Figure (1-2), Table 1 and Plate 4.

4.1.1 Days to shoot induction

The minimum 39.17 days to shoot induction was observed in the treatment 2.00 mg/L of BA followed by 3.00 mg/L BA (42.75 days) and 1.00 mg/L BA (47.43 days). Then again, maximum 50.34 days was required in 4.00 mg/L BA. There was no response found in control treatment for the days of shoot induction (Figure 1). The different concentration of BA showed significant variation on days to shoot induction. Nakamura (1991) reported that more than 2 weeks needed for shot induction when treated with BA.

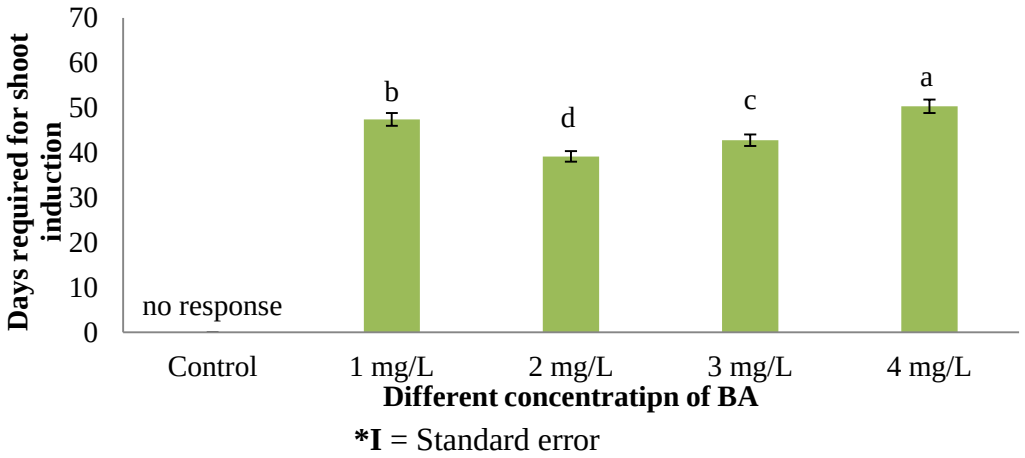
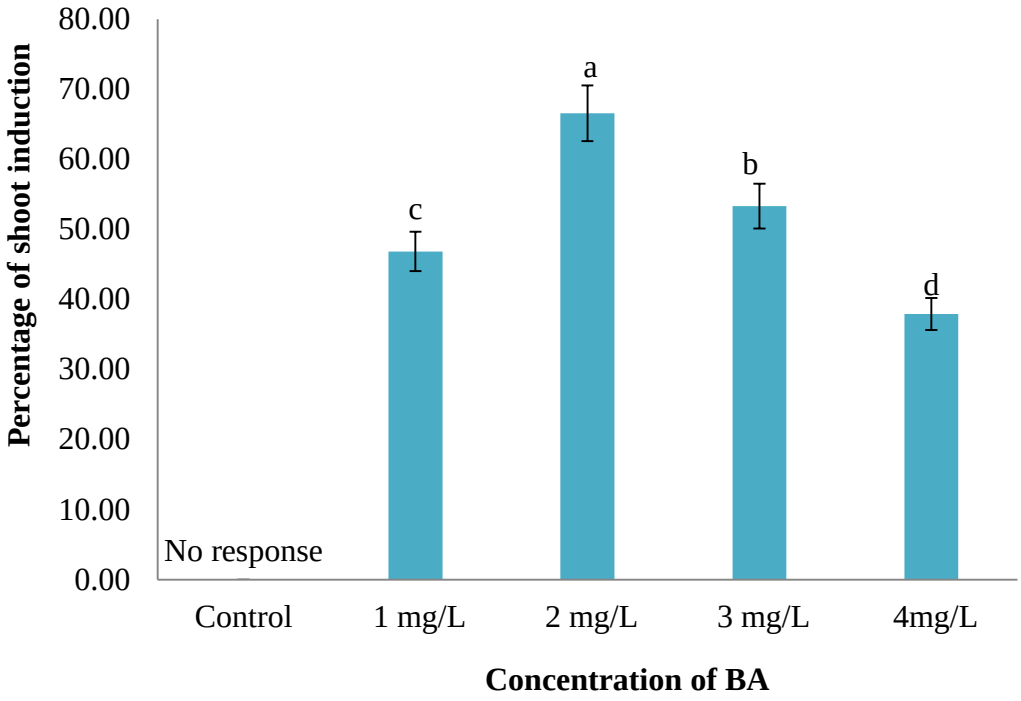


Figure 1. Days to shoot induction in Tea with different concentration of BA

4.1.2 Percentage of response of explant

The highest frequency (66.58%) of shoot induction was recorded at 2.00 mg/L BA followed by 3.00 mg/L BA (53.32%) treatment. While the lowest frequency of shoot induction (32.94%) was required in 4.00 mg/L BA treatment followed by 1.00 mg/L BA (46.84%). There is no response found in control treatment for the percentage of shoot induction (Figure 2). From figure 2 we can conclude that a significant variation among different concentration of BA was found for percentage of shoot induction. Here 2.00 mg/L concentration of BA showed the best response. When several types of cytokinins such Kn, BAP, 2-isopentenyladenine (2ip), and TDZ alone were investigated, Boonerjee *et al*., (2013) observed that 2.00 mg/L BAP demonstrated the greatest response towards regeneration.



***I** = Standard error

Figure 2. Percentage of shoot induction at different concentration of BA in Tea

4.1.3 Length of shoot (cm)

The treatment 2.00 mg/L BA had produced the highest length of shoots (0.53, 1.35 and 1.91 cm) at 60, 75 and 90 DAI respectively (Plate 4). The second highest shoot length was founded in 3.00 mg/L BA concentration which was 0.49, 1.08 and 1.47 cm respectively at 60, 75 and 90 DAI. On the other hand, treatment 1.00 mg/L BA had produced the lowest length of shoots (0.38, 0.64 and 1.03 cm) followed by 4.00 mg/L BA (0.43, 0.82 and 1.14 cm) at 60, 75 and 90 DAI respectively (table 1). There was no response founded in controlled treatment for the length of shoot at different DAI. Significant influence of different concentrations of BA on the length of shoot at different DAI was founded. Though BA reasoned very low for shoot elongation, there is no response for multiple shoot production in this study. Boonerjee *et al*., (2013) mentions that when BAP tried alone, multiple shoot formation did not happen. It suggests that multiple shoot growth from tea explants is not suited for cytokinin on its own. George and Sherrington (1984) detected a similar observation.

Table 1. Effect of different concentration of BA on length of shoot at different DAI

Con of BA (mg/L)	**Length of Shoot (cm)**		
	60 DAI	**75 DAI**	**90 DAI**
Control	–	–	–
1.00 mg/L	0.38 d	0.64 d	1.03 d
2.00 mg/L	0.53 a	1.35 a	1.91 a
3.00 mg/L	0.49 b	1.08 b	1.47 b
4.00 mg/L	0.43 c	0.82 c	1.14 c
CV (%)	4.11	4.76	2.43
$LSD_{(0.05)}$	0.0355	0.0873	0.0636

*DAI=Days after inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, $LSD_{(0.05)}$ = Least significant difference.

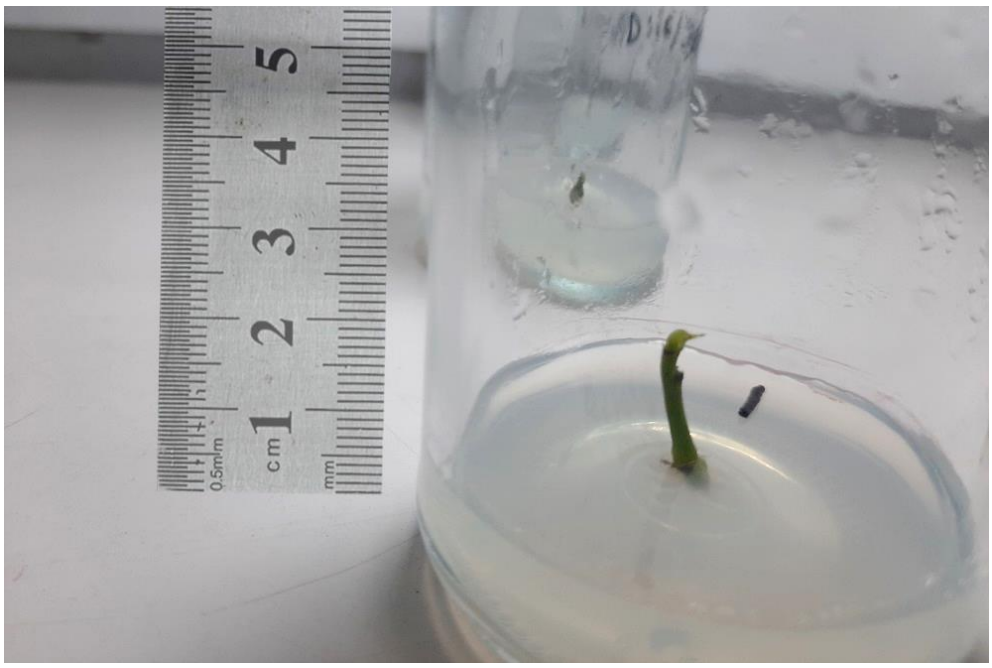


Plate 4. Length of shoot in the treatment 2.00 mg/L of BA at 90 DAI

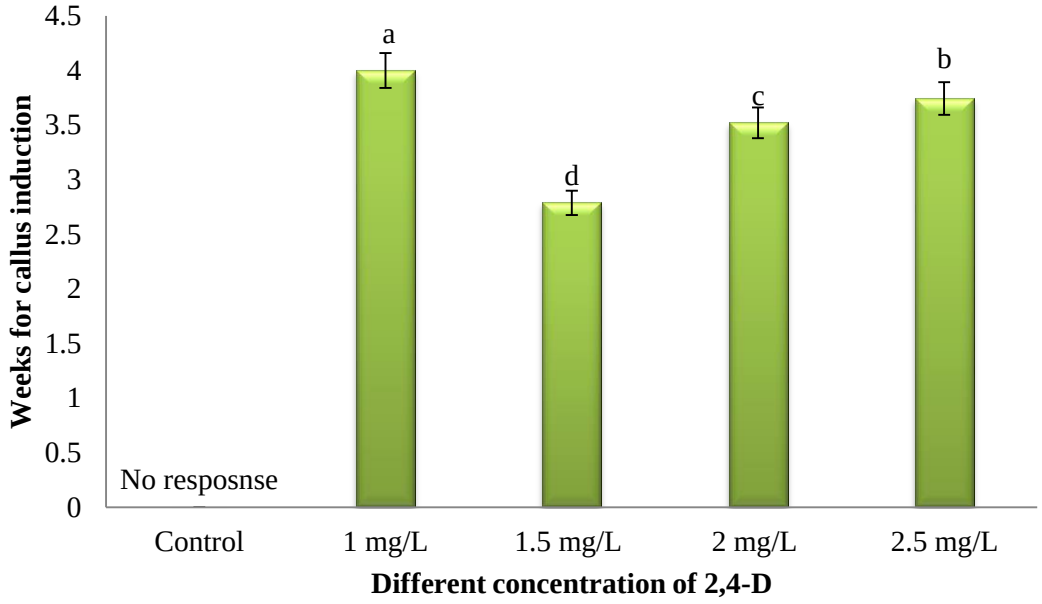
4.2 Sub-experiment 2.

Callus induction potentiality in Tea [*Camellia sinensis* (L.) O. Kuntze]

To develop callus, the shoot tip and nodal segments were took and transferred into callus induction media supplemented with different concentration 2, 4-D. The results of the experiment have been presented under following headings with Figure 3-4, Table 2 and Plate 5-6.

4.2.1 Weeks to callus induction

The minimum 2.78 weeks were required for callus induction in the treatment 1.50 mg/L of 2, 4-D and it was followed by 2.00 mg/L of 2, 4-D which was 3.52 weeks. Maximum 4 weeks was required in the treatment 1.00 mg/L of 2, 4-D. At the end there is no response found in control treatment for the days of callus induction. Different concentrations of 2, 4-D on callus induction are statistically different from one another (Figure 3). According to Sandal *et al*., (2005), after four weeks of culture on different 2,4-D doses (2.50, 5.00, 7.50, and 10.00 mg/L), callus was induced.

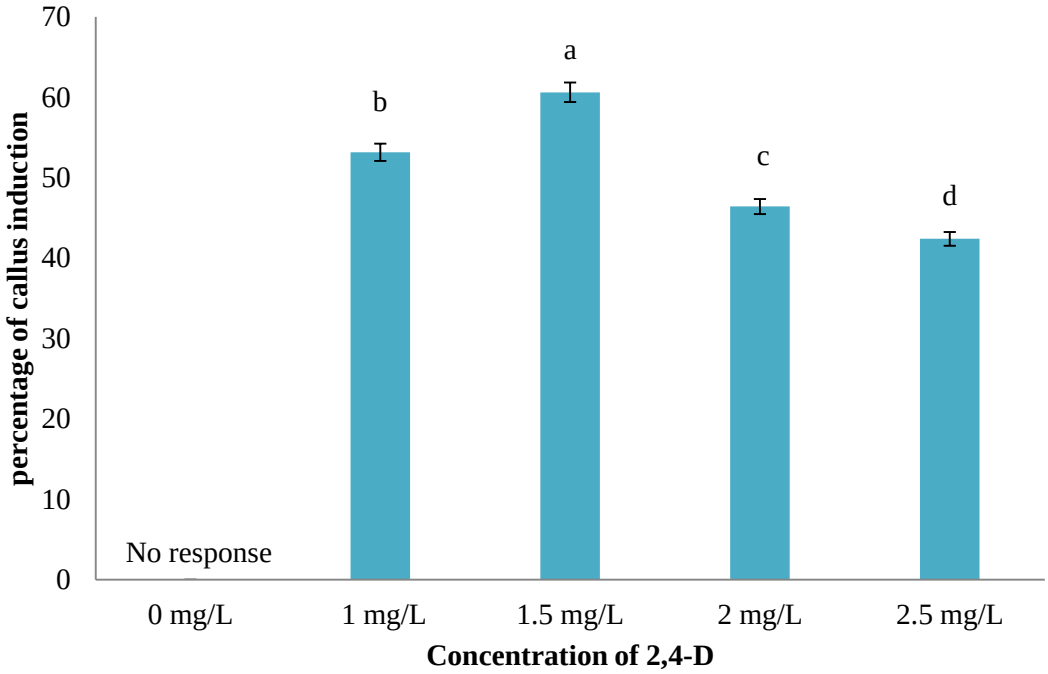


***I** = Standard error

Figure 3. Weeks to callus induction in Tea with different concentration of 2, 4-D

4.2.2 Percentage of callus induction

The highest percentage of callus induction was recorded at 1.50 mg/L of 2, 4-D (60.61%) followed by the treatment 1.00 mg/L of 2,4-D (53.17%). On the other hand the lowest percentage of callus induction (42.39%) was required in the treatment 2.50 mg/L of 2, 4-D followed by 2.00 mg/L (46.42%). There is no response found in control treatment for the percentage of callus induction. Significant variation among different concentration of 2, 4-D on percentage of callus induction was recorded (Figure 4).



***I** = Standard error

Figure 4. Percentage of callus induction at different concentration of 2, 4-D in Tea

4.2.3 Fresh weight of callus (g)

At 6 WAI the treatment 1.00 mg/L 2, 4-D showed significant difference from other treatments but At 10 WAI each treatment was significant difference from others. On the other hand, at 14 WAI 1.50 mg/L 2, 4-D was statistically different form 1.00, 2.00 and 2.50 mg/L treatments. At 6 WAI callus weight of 1.50 mg/L and 2.00 mg/L 2, 4-D were same (0.15 g). But at 10 and 14 WAI 1.50 mg/L 2, 4-D had produced the highest weight of callus (0.49 and 0.84 g) (Plate 5, 6) followed by 2.00 mg/L (0.42 and 0.70 g). On the other hand, 1.00 mg/L 2, 4-D had produced the lowest weight of callus (0.10 g) at 6 WAI. But in case of 10 and 14 WAI the lowest weight was found with the treatment 2.50 mg/L 2,4-D which were 0.33 and 0.63 g respectively .There was no response founded in controlled treatment for the weight of callus at different WAI (Table 2). There was no further responses like shoot or root production from these treatments. Sandal *et al*., (2005) made similar discoveries. On the same medium or when sub-cultured onto new media, they observed that even though callus was produced by 2, 4-D, the callus did not exhibit any morphogenic response.

Table 2. Effect of 2,4-D on fresh weight of callus at different WAI

Concentration of 2,4-D (mg/L)	**Weight of callus (g)**		
	6 WAI	**10 WAI**	**14 WAI**
Controlled	-	-	-
1.00 mg/L	0.10 b	0.38 c	0.67 bc
1.50 mg/L	0.15 a	0.49 a	0.84 a
2.00 mg/L	0.15 a	0.42 b	0.70 b
2.50 mg/L	0.13 a	0.33 d	0.63 c
CV (%)	10.78	2.87	5.01
$LSD_{(0.05)}$	0.0272	0.0221	0.0671

*WAI=Weeks after Inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, $LSD_{(0.05)}$ = Least significant difference.

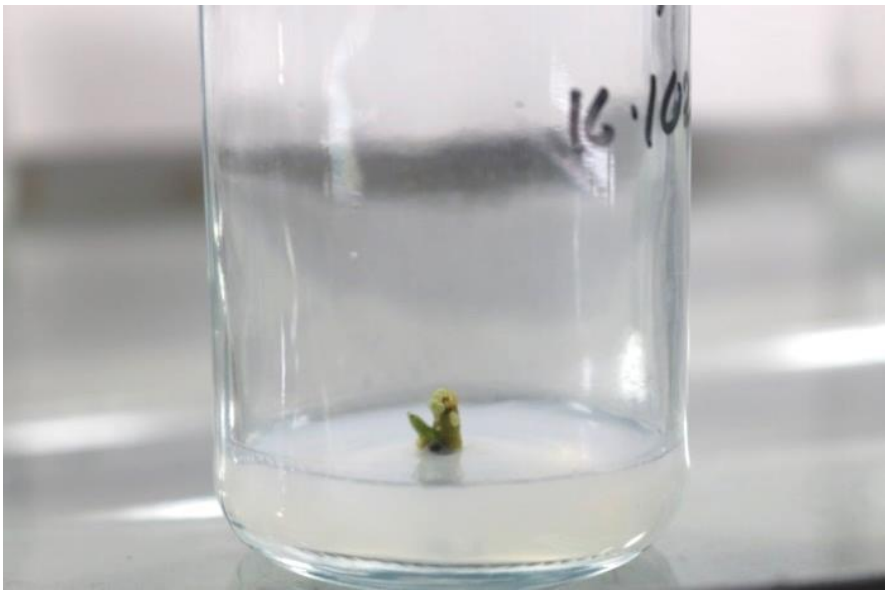


Plate 5. Callus induction at 6 WAI in the treatment 1.50 mg/L 2, 4-D

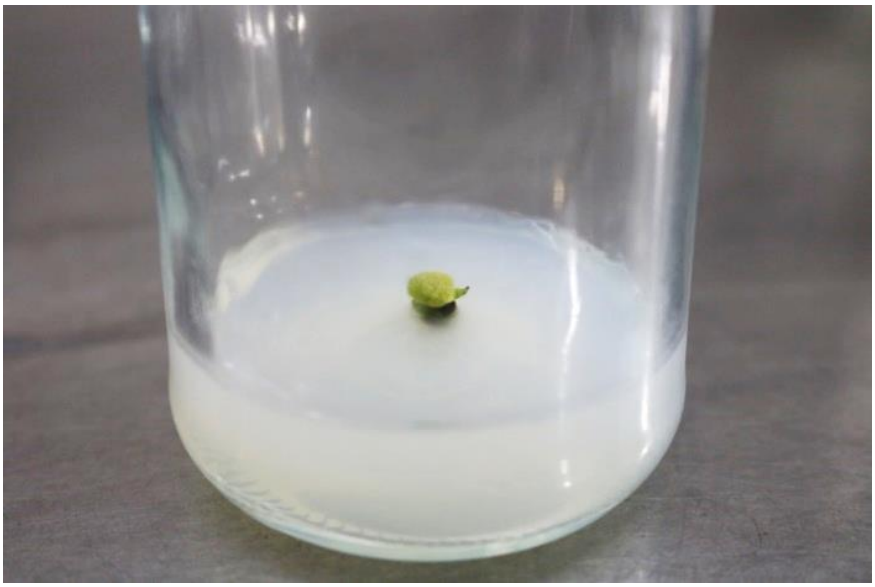


Plate 6. Callus induction at 10 WAI in the treatment 1.50 mg/L 2, 4-D

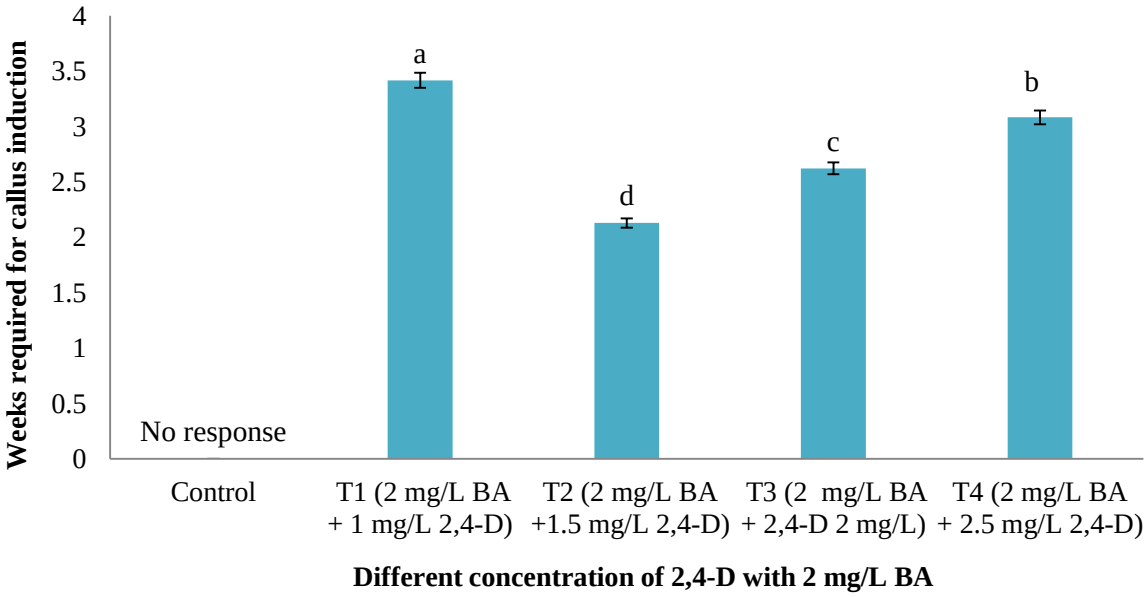
4.3 Sub-experiment 3.

Combined Effect of BA and 2, 4-D on callus induction potentiality in Tea [*Camellia sinensis* (L.) O. Kuntze]

To develop callus, shoot tip and nodal segments were took and transferred into callus induction media supplemented with 2.00 mg/L BA with different concentration 2, 4-D. The results of the experiment have been presented on Table 3, figure 5-6 and plate 7.

4.3.1 Weeks to callus induction

The minimum 2.12 weeks was required for callus initiation at treatment-2 (2.00 mg/L BA + 1.50 mg/L 2,4-D) and it was followed by treatment-3 (2.00 mg/L BA + 2.00 mg/L 2,4-D) with 2.62 weeks. Again, maximum 3.41 weeks required in treatment -1 (2.00 mg/L BA + 1.00 mg/L 2, 4-D) .Treatment-3 (2.00 mg/L BA + 2.50 mg/L 2,4-D) needed 3.08 weeks to respond. Towards the end there is no response found in control treatment for the days of callus initiation (figure 5). Also, statistical analysis for different concentration of 2,4-D with 2.00 mg/L BA showed significant difference from each other in case of weeks to callus induction.

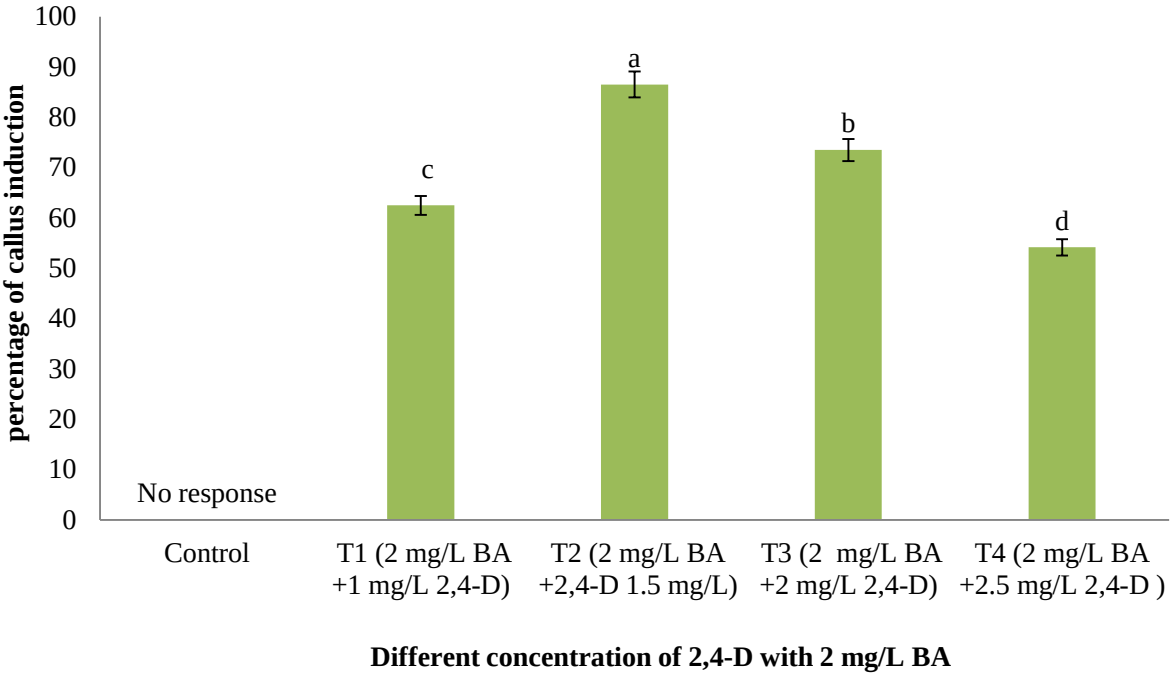


***I** = Standard error

Figure 5. Weeks to callus induction in Tea with different concentration of 2, 4-D with 2.00 mg/L BA

4.3.2 Percentage of callus induction

The treatment-2 (2.00 mg/L BA + 1.50 mg/L 2, 4-D) had showed the highest percentage of callus induction (86.51%) followed by treatment-3 (2.00 mg/L BA + 2.00 mg/L 2,4-D) (73.48%). Then again treatment-4 (2.00 mg/L BA + 2.50 mg/L 2,4-D) had the lowest frequency of callus induction (54.14%), which was followed by treatment-1 (2.00 mg/L BA + 1.00 mg/L 2,4-D) which was 62.48% (figure 6). Also a significant variation among different concentration of 2, 4-D with 2.00 mg/L BA found regarding percentage of callus induction.



***I** = Standard error

Figure 6. Combined effect of BA and 2, 4-D on percentage of callus induction in Tea

4.3.3 Fresh weight of callus (g)

At 6 WAI, the treatment-2 (2.00 mg/L BA + 1.50 mg/L 2, 4-D) and treatment-3 (2.00 mg/L BA + 2.00 mg/L 2,4-D) were given statistically similar result and they were significantly different from treatment-1 (2.00 mg/L BA + 1.00 mg/L 2,4-D) and treatment-4 (2.00 mg/L BA + 2.50 mg/L 2,4-D). At 10 WAI, treatment-2 (2.00 mg/L BA + 1.50 mg/L 2,4-D) showed significant difference from other treatments and at 14 WAI, treatment-1 (2.00 mg/L BA + 1.00 mg/L 2,4-D) and treatment-4 (2.00 mg/L BA + 2.50 mg/L 2,4-D) were statistically same but different from treatment-2 (2.00 mg/L BA + 1.50 mg/L 2,4-D) and treatment-3 (2.00 mg/L BA + 2.00 mg/L 2,4-D). Treatment-2 (2.00 mg/L BA + 1.50 mg/L 2, 4-D) had produced the highest weight of callus (0.18, 0.56 and 1.11 g) followed by treatment-3 (2.00 mg/L BA + 2.00 mg/L 2,4-D) with callus weight (0.16, 0.45 and 0.82 g) at 6, 10 and 14 WAI respectively. On the contrary, Treatment-1 (2.00 mg/L BA + 1.00 mg/L 2,4-D) had produced the lowest weight of callus (0.11 and 0.69 g) followed by treatment-4 (2.00 mg/L BA + 2.50 mg/L 2,4-D) where callus weight were 0.14 g and 0.72 g at 6 WAI and 14 WAI respectively. But at 10 WAI, treatment-4 (2.00 mg/L BA + 2.50 mg/L 2, 4-D) produced the lowest weight of callus (0.38 g) followed by treatment-1 (2.00 mg/L BA + 1.00 mg/L 2,4-D) ,where callus weight was 0.42 g. No response founded in controlled treatment (Table 3). Callus at 6 WAI and 14 WAI was shown in plate 7. Though callus formed and weight increased, no further shoot or root formation was found in this experiment. Similar result found by some researchers. According to Nakamura (1991), BA and auxins together caused callus development while inhibiting organogenesis. But organogenesis from callus also reported in several studies. According to Sarathchan *et al*., (1988), callus tissue sub-cultured in a basal medium mixed with 2.00 mg/L IBA and 10 mg/L BAP showed organogenesis.

Table 3. Effect of BA and 2, 4-D on fresh weight of callus at different WAI

Treatment (BA+ 2,4-D) mg/L	weight of callus (g)		
	6 WAI	10 WAI	14 WAI
Controlled	-	-	-
T1(2.00 + 1.00) mg/L	0.11 c	0.42 bc	0.69 c
T2(2.00 + 1.50) mg/L	0.18 a	0.56 a	1.11 a
T3 (2.00 + 2.00) mg/L	0.16 a	0.45 b	0.82 b
T4 (2.00 + 2.50) mg/L	0.14 b	0.38 c	0.72 c
CV (%)	4	4.62	2.77
$LSD_{(0.05)}$	0.0111	0.0393	0.0435

*WAI=Weeks after Inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, $LSD_{(0.05)}$ = Least significant difference.

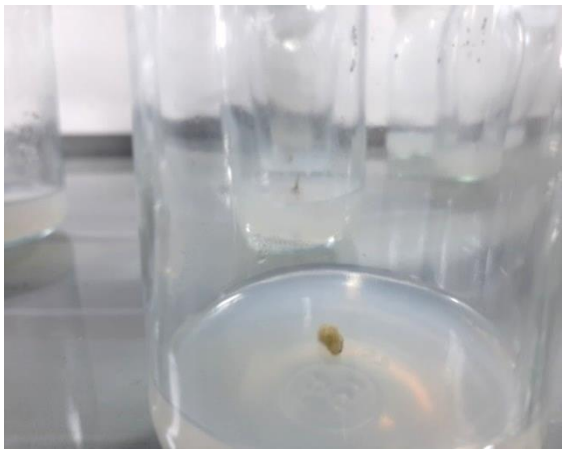


Plate 7(A). Callus at 6 WAI

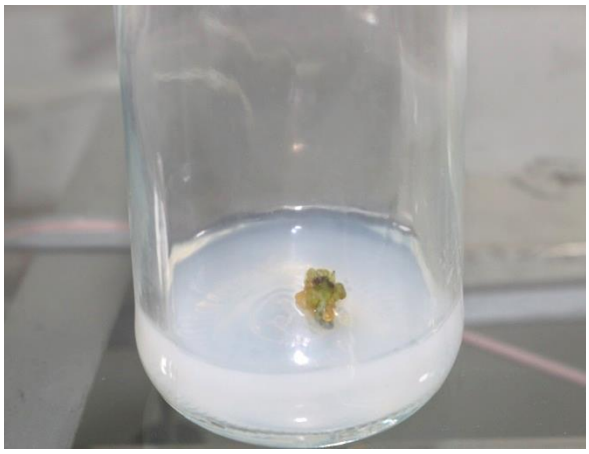


Plate 7(B). Callus at 14 WAI

Plate 7. Callus at 6 WAI (A) and 14 WAI (B) in the treatment 2.00 mg/L BA + 1.50 mg/L 2, 4-D

4.4 Sub-experiment 4.

Combined Effect of BA and NAA on shoot induction and leaf development potentiality in Tea [*Camellia sinensis* (L.) O. Kuntze]

The results of the combined effect of combined concentrations of BA and NAA have been presented under following headings with figure 7, Table 4-7 and plate 8-10.

4.4.1 Days to shoot induction

The minimum 32.43 days to shoot induction was noticed in the treatment 2.00 mg/L BA + 2.00 mg/L NAA and followed by 2.00 mg/L BA + 3.00 mg/L NAA which was 36.83 days. The maximum 45.77 days to shoot initiation was recorded in treatment 2.00 mg/L BA + 4.00 mg/L NAA followed by 2.00 mg/L BA + 1.00 mg/L NAA which was 40.45 days (Table 4). The different concentration of NAA with 2.00 mg/L BA gives an idea about significant variation on days to shoot induction. All the treatments showed statistically different results from each other. According to Nakamura (1991), the ideal concentration of BA for *Camellia spp*. shoot tips is between 0.50 and 3.00 mg/L. According to Mondal *et al*., (2004), the shoot tips' axillary buds start to develop about the fourth week after explanting, which is nearly similar to our experiment. Several reporters reported 30-50 days required for shoot initiation in different concentration of auxin and cytokinine.

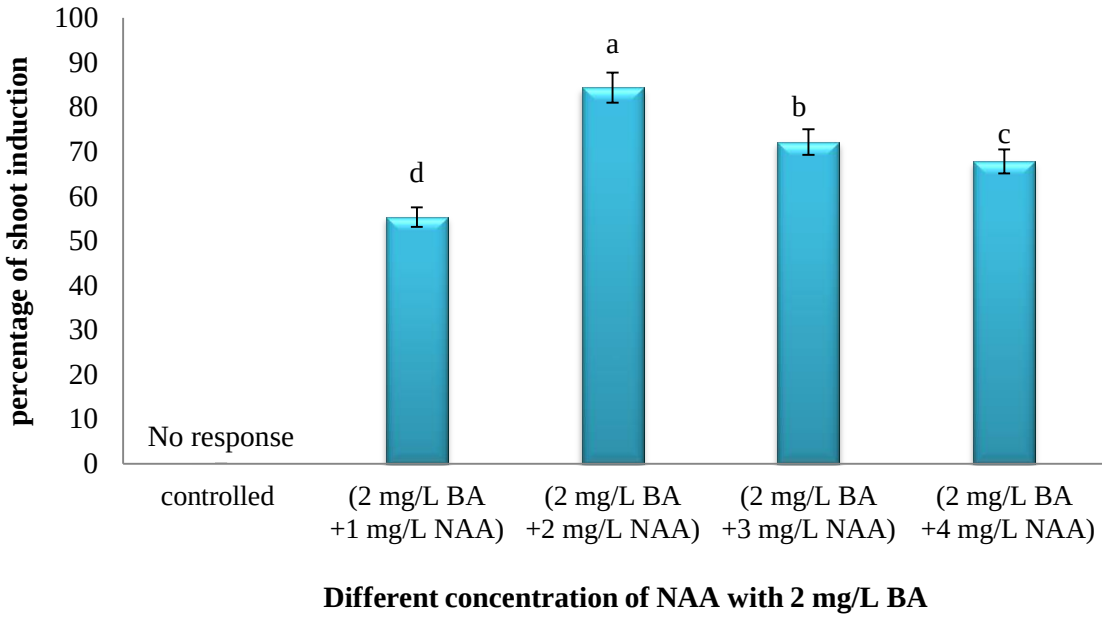
Table 4. Combined Effect of BA and NAA on days of shoot induction in Tea

Treatment (BA + NAA) mg/L	**Days of shoot induction**
(Controlled)	_
2.00 + 1.00	40.45 b
2.00 + 2.00	32.43 d
2.00 + 3.00	36.83 c
2.00 + 4.00	45.77 a
CV (%)	0.47
$LSD_{(0.05)}$	0.34

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, $LSD_{(0.05)}$ = Least significant difference**.**

4.4.2 Percentage of shoot induction

The treatment 2.00 mg/L BA + 2.00 mg/L NAA had produced the highest percentage of shoot (84.39%) followed by 72.15% at the treatment 2.00 mg/L BA + 3.00 mg/L NAA and 67.82% at 2.00 mg/L BA + 4.00 mg/L NAA treatment. Lowest percentage was found at 2.00 mg/L BA + 1.00 mg/L NAA (55.32%). On the other hand controlled treatment had no response on shoot induction (Figure 7). A significant variation among different concentration of NAA with 2.00 mg/L BA was found for percentage of shoot induction. Begum *et al*., (2015) found highest response 75% for shoot tip and 66.67% for nodal segments response in the combined treatment of 3.00 mg/L BAP + 0.05 mg/L IAA when the lowest 10% were found in 3.00 mg/L BAP + 0.10 mg/L Kn. Which were nearly similar to our experiment. Different studies reported different success percentage of shoot induction depends on the type of explant, hormones and their concentrations.



***I** = Standard error

Figure 7. Combined Effect of BA and NAA on percentage of shoot induction in Tea

4.4.3 Number of shoot per explant

At 60 and 90 DAI all treatments are significantly different from each other, but at 120 DAI, the treatment 2.00 mg/L BA + 1.00 mg/L NAA showed significantly similar result with 2.00 mg/L BA + 4.00 mg/L NAA but they were significantly different from rest of the treatments. The treatment 2.00 mg/L BA + 2.00 mg/L NAA had produced the highest number of shoots (1.42, 2.46 and 4.49) (plate 8) and followed by the treatment 2.00 mg/L BA + 3.00 mg/L NAA where number of shoots were (1.16, 1.98 and 3.16) at 60, 90 and 120 DAI respectively. On the contrary, 2.00 mg/L BA + 1.00 mg/L NAA had produced the lowest number of shoots (0.86 and 1.43) at the 60 and 90 DAI respectively. The treatment 2.00 mg/L BA + 4.00 mg/L NAA produced 1.05 shoots at 60 DAI and 1.62 shoots at 90 DAI. But at 120 DAI, 2 mg/L BA + 4.00 mg/L NAA had produced the lowest number of shoot (2.46) followed by 2.00 mg/L BA + 1.00 mg/L NAA (2.63) (Table 5). These results showed that there was a significant influence of different concentrations of BA and NAA on number of shoot and the multiplication in controlled treatment was not found even for long period of time and numbers of subculture. Similar results were found by Jha and Sen (1992). They discovered that hormone-free basal media produced no reaction. According to Khan *et al*., (1997), a medium containing both BA and auxins generated more shoots than a media simply having cytokinins. According to Azad *et al*., (2005), the MS medium supplemented with 4.40 µM BAP and 1.00 µM NAA had the most total no of shoots (35.5 ± 0.6) which is different from this study. According to Boonerjee *et al*., (2013), MS media supplemented with 2.00 mg/L BAP and 1.00 mg/L NAA produced mean number of shoots/explant 4.60 and 5.00 for shoot tip and nodal segment explants respectively after 120 days which is nearly similar to this study.

Table 5. Combined effect of BA and NAA on Number of shoot at different DAI

Treatment (BA + NAA) mg/L	Number of shoot		
	60 DAI	**90 DAI**	**120 DAI**
(Controlled)	0	0	0
2.00 + 1.00	0.86 d	1.43 d	2.63 c
2.00 + 2.00	1.42 a	2.46 a	4.49 a
2.00 + 3.00	1.163 b	1.98 b	3.16 b
2.00 + 4.00	1.05 c	1.62 c	2.46 c
CV (%)	3.9	4.19	3.82
$LSD_{(0.05)}$	0.0826	0.1478	0.2294

*DAI=Days after inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, $LSD_{(0.05)}$ = Least significant difference.

Plate 8(A). No. of Shoot at 90 DAI

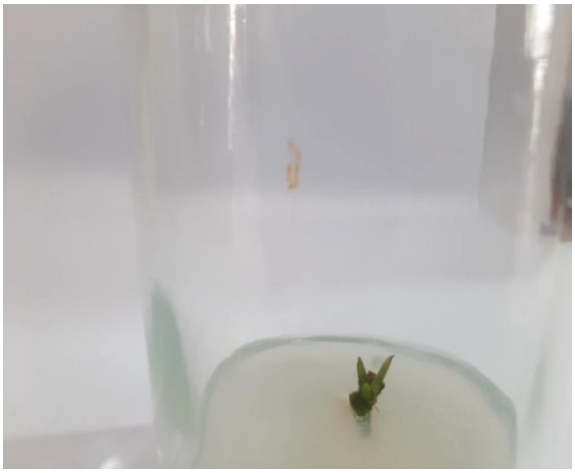


Plate 8(B). No. of Shoot at 120 DAI

Plate 8. Number of Shoot at 90 DAI (A) and 120 DAI (B) with the treatment 2.00 mg/L BA + 2.00 mg/L NAA

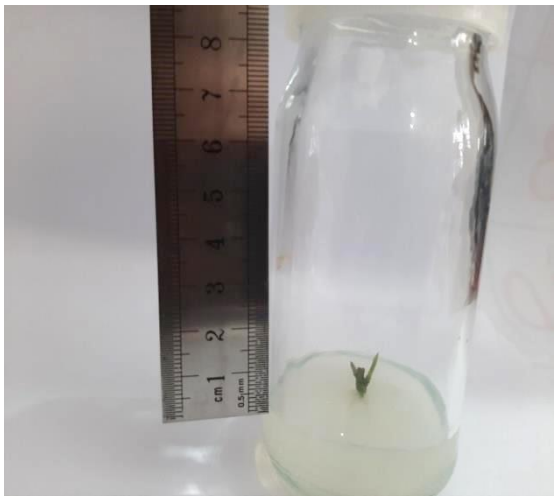
4.4.4 Length of shoot (cm)

The treatment 2.00 mg/L BA + 2.00 mg/L NAA had produced the highest length of shoot (1.41, 3.28 and 4.61 cm) at 60, 90 and 120 DAI respectively. In case of 60 DAI, the second highest result was found in the treatment 2.00 mg/L BA + 4.00 mg/L NAA (1.12 cm) followed by 2.00 mg/L BA + 1.00 mg/L NAA (0.97 cm) . But at 90 and 120 DAI, the second highest result was found in 2.00 mg/L BA + 1.00 mg/L NAA (2.70 and 3.97 cm respectively) followed by 2.00 mg/L BA + 4.00 mg/L NAA (2.31 and 3.36 cm). On the contrary, 2.00 mg/L BA + 3.00 mg/L NAA had produced the lowest number of shoot (0.80, 1.85 and 2.85 cm) at 60, 90 and 120 DAI where no response found at controlled (Table 6). Length of shoot at 90 and 120 days with the treatment 2.00 mg/L BA + 2.00 mg/L NAA was shown in plate 9. At 60, 90 and 120 DAI, each treatment showed significantly different results from each other. According to different reports, BA combined with either auxins or gibberellin at different dosages was administered to increase the development of shoot tips. According to Azad *et al*., (2005), The longest shoots were grown from MS media containing 4.40 µM BAP + 2.00 µM NAA. Begum *et al*., (2015) found the highest length of shoot for shoot tips and nodal segments were 3.40 cm and 3.30 cm respectively in the concentration of 3.00 mg/L BAP + 1.50 mg/L NAA after 3 months. Boonerjee *et al*., (2013) found after 120 days, the combined concentration of 3.00 mg/ L BAP + 1.50 mg/L NAA produced 4.00-4.30 cm long shoot.

Table 6. Combined effect of BA and NAA on length of shoot at different DAI

Treatment (BA + NAA) mg/L	**length of shoot (cm)**		
	60 DAI	**90 DAI**	**120 DAI**
(Controlled)	-	-	-
2.00 + 1.00	0.97 c	2.70 b	3.97 b
2.00 + 2.00	1.41 a	3.28 a	4.61 a
2.00 + 3.00	0.80 d	1.85 d	2.85 d
2.00 + 4.00	1.12 b	2.31 c	3.36 c
CV (%)	5.3	1.61	1.53
$LSD_{(0.05)}$	0.1078	0.0768	0.1067

DAI=Days after inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, $LSD_{(0.05)}$ = Least significant difference.



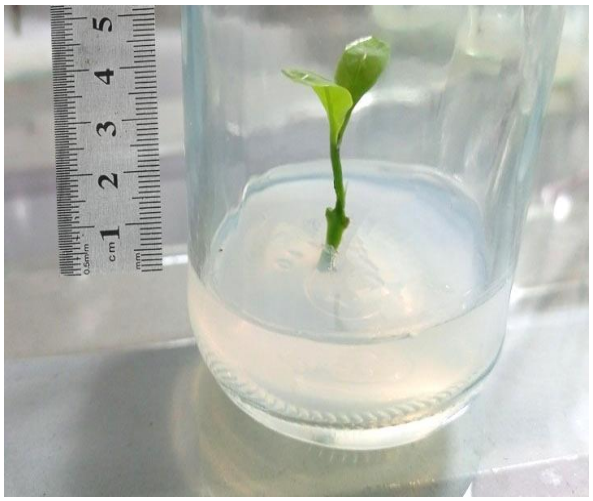


Plate 9(A). Length of shoot at 60 DAI **Plate 9(B).** Length of shoot at 120 DAI

Plate 9. Length of shoot at 60 DAI (A) and 120 DAI (B) with the treatment 2.00 mg/L BA + 2.00 mg/L NAA

4.4.5 Number of leaves

At 90 DAI, the treatment 2.00 mg/L BA + 3.00 mg/L NAA showed significant variation from the treatment 2.00 mg/L BA + 1.00 mg/L NAA and 2.00 mg/L BA + 2.00 mg/L NAA. At 120 DAI each treatment was significant difference from others. 2.00 mg/L BA + 3.00 mg/L NAA had produced the highest number of leaves (3.36 and 5.53) (Plate 10) followed by 2.00 mg/L BA + 4.00 mg/L NAA (3.04 and 4.74) and 2.00 mg/L BA + 2.00 mg/L NAA (2.67 and 4.28) at 90 and 120 DAI respectively. On the contrary, at 90 and 120 DAI, 2.00 mg/L BA + 1.00 mg/L NAA had produced the lowest number of leaves (2.13 and 3.85 respectively). There was no response founded in controlled treatment for the number of leaves at different DAI (Table 7). As per Nakamura (1991), BA (3.00 mg/L) produced the most leaves. After culturing and sub-culturing explants for 4-6 weeks, Prakash *et al.*, (1999) reported four to six leaves.

Table 7. Combined effect of BA and NAA on number of leaves at different DAI

Treatment (BA + NAA) mg/L	Number of leaves	
	90 DAI	**120 DAI**
(Controlled)	-	-
2.00 + 1.00	2.13 c	3.85 d
2.00 + 2.00	2.67 b	4.28 c
2.00 + 3.00	3.36 a	5.53 a
2.00 + 4.00	3.034 ab	4.74 b
CV (%)	7.46	4.8
$LSD_{(0.05)}$	0.3932	0.4161

*DAI=Days after inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV = Coefficient of variation, $LSD_{(0.05)}$= Least significant difference**.**

Plate 10(A). No. of leaves at 90 DAI

Plate 10(B). No. of leaves at 120 DAI

Plate 10. Number of leaves at 90 DAI (A) and 120 DAI (B) in the treatment

2.00 mg/L BA and 3.00 mg/L NAA

4.5 Sub-experiment 5.

Root induction of regenerated shoot through dipping in rooting hormone [*Camellia sinensis* (L.) O. Kuntze]

Different concentrations of IBA and its dipping times significant effect on root initiation. Several studies showed that *ex vitro* rooting is more effective and less costly then *in vitro*. Moreover, *in vitro* rooting in tea as well as other woody plants is difficult. We tried several months for *in vitro* rooting but there was no response. Different concentrations on BA individually and along with NAA were practiced for both shooting and rooting. We found shoot initiation and multiple shoot but no root initiation occurred. Then we decided to practice *ex vitro* rooting by dipping the basal part of regenerated shoots in high concentration of IBA with different time followed by direct transplanting on soil (sand: Garden soil: Compost in ratio 1:2:1).The results have been presented under following headings with figure 8 , Table 8-9 and plate 11.

4.5.1 Weeks to root induction :

The minimum weeks to root induction was recorded when the regenerated shoots were dipped in different IBA concentration for 30 mins. Lowest weeks (6.02) were recorded with 500 mg/L IBA for 30 mins followed by 400 mg/L IBA for 30 min (6.82). The regenerated shoots when treated for 50 mins, they showed necrosis phenotype in shoot and when treated for 10 mins with IBA 300 mg/L as well as 400 mg/L concentration did not show any responses for root initiation (Table 8). But dipping in 500 mg/L IBA for 10 mins showed response for rooting (8.62 weeks). Rooting responses depend on auxin concentration and also depend on dipping time. Response increased with the increasing concentration and time. But after 50 mins the result turned into necrosis. The significant variations were observed among different concentrations of IBA and its dipping time on weeks to root induction at 5% level of significance in laboratory. *Ex vitro* rooting (rooting in natural open pot condition) was found to be superior to all other *in vitro* rooting techniques by Jain *et al*., (1993). They also reported that when micro shoots were treated for 30 minutes with 500 mg/L IBA before being directly transplanted into the soil mixture, root formation began to occur after 45 days. Begum *et al*., (2015) said that dipping in IBA 300 mg/L for various periods of time resulted in a 62–68 day root induction period.

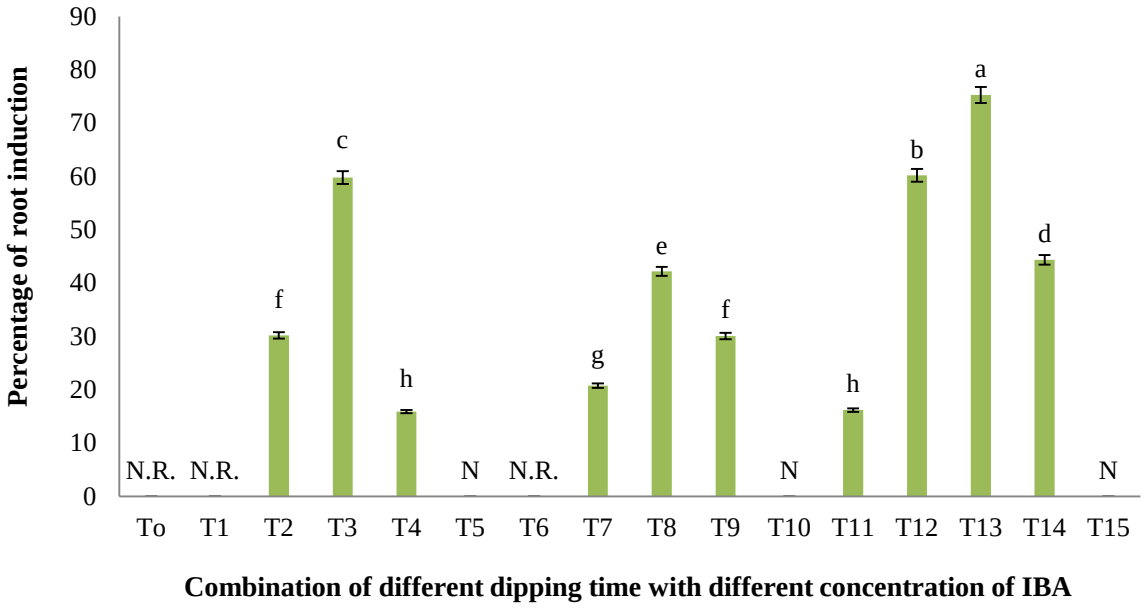
Table 8. Effect of different concentration and dipping time (min) of IBA on Weeks of root induction

IBA concentration (mg/L)	Dipping time (min)	Weeks of root induction
Controlled	_	No response
IBA 300 mg/L	10	No response
	20	8.26 d
	30	6.94 g
	40	9.24 ab
	50	Necrosis
IBA 400 mg/L	10	No response
	20	7.69 e
	30	6.82 g
	40	9.37 a
	50	Necrosis
IBA 500 mg/L	10	8.62 c
	20	7.49 f
	30	6.02 h
	40	9.11 b
	50	Narcosis
CV (%)	1.25	
LSD $_{(0.05)}$	0.1697	

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, LSD $_{(0.05)}$ = Least significant difference.

4.5.2 Percentage of root induction

The highest percentage (75.32%) of root induction was found when shoots treated in 500 mg/L IBA for 30 mins (T13) followed by 500 mg/L IBA when dipping time was 20 mins (T12) which was 60.24%. No responses were found in controlled treatment (T0) as well as 10 mins dipping time for 300 mg/L (T1) and 400 mg/L of IBA (T6) concentrations. Necrosis found in each concentration when dipping time was 50 mins (Figure 8). The significant variations were observed among different concentration of IBA. In accordance with Boonerjee *et al*., (2013) , when the separated micro shoots were pretreated with a high concentration of IBA (500 mg/L; 30 mins) and then transferred straight to the soil, 85 percent rooting was accomplished**.** *Ex vitro* rooting was used by Ranaweera *et al*., (2013) to produce a low-cost technique of micropropagation and achieve 100% rooted plantlet. Sharma *et al*., (1999) obtained similar results. After treating the micro shoots in 500 mg/L IBA for 30 minutes prior to transferring them into the soil, they achieved a maximum rooting of 71.6 percent. These results are nearly similar to our results.



***I** = Standard error, N.R. = No response; N= Necrosis

Figure 8. Percentage of root induction at different concentration of IBA and different dipping time in Tea

Here, T0= Controlled treatment T1= 300 mg/L IBA + Dipping time 10 mins

T2= 300 mg/L IBA + Dipping time 20 mins T3= 300 mg/L IBA + Dipping time 30 mins

T4= 300 mg/L IBA + Dipping time 40 mins T5= 300 mg/L IBA + Dipping time 50 mins

T6= 400 mg/L IBA + Dipping time 10 mins T7= 400 mg/L IBA + Dipping time 20 mins

T8= 400 mg/L IBA + Dipping time 30 mins T9= 400 mg/L IBA + Dipping time 40 mins

T10= 400 mg/L IBA + Dipping time 50 mins T11= 500 mg/L IBA + Dipping time 10 mins

T12= 500 mg/L IBA + Dipping time 20 mins T13= 500 mg/L IBA + Dipping time 30 mins

T14= 500 mg/L IBA + Dipping time 40 mins T15= 500 mg/L IBA + Dipping time 50 mins

4.5.3 Number and length of root

The highest number of roots (4.20 and 7.80) and highest length of roots (3.31 cm and 5.80 cm) at both 10 WAI and 14 WAI of regenerated shoots were found when treated with 500 mg/L IBA for 30 min among all treatments.

The IBA concentrations of 300 mg/L, 30 minutes of dipping time had the highest results after 10 WAI and 14 WAI. The mean number of roots was 3.57 and 6.10, respectively, and the lengths were 2.60 cm and 5.35 cm in the same treatment. For 400 mg/L IBA, treatment time of 20 minutes showed the highest number of roots for 10 WAI (3.10) and 14 WAI (6.27). The lowest number (1.41 and 3.30) and length of roots (0.64 and 1.14 cm) were recorded when IBA concentration was 400 mg/L with 40 mins dipping time. Finally, every IBA concentration caused necrosis when the treatment period was 50 minutes and there was no root development. Overall, it was determined that 500 mg/L IBA with 30 minute dipping time was the optimum treatment for *ex vitro* rooting (Table 9). The significant variations were observed among different concentrations of IBA and its dipping time on number of root at 5% level of significance in laboratory. A highest number of roots (8.50) with a 500 mg/L IBA (30 mins) concentration was reported by Boonerjee *et al*., (2013). Additionally, the same treatment period produced roots with a highest mean length of 5.40 cm in the same concentration. A 50 minute treatment period also revealed necrosis on their study. Begum *et al*., (2015) discovered that 300 mg/L IBA concentration had no effect

with 10 minutes dipping time, but treatment with 30 minutes dipping time caused a highest response for shoot length (2.40 cm) for the same concentration of IBA. In both circumstances and in our investigation, extending the treatment period more than 30 mins resulted in decreasing outcomes.

Table 9. Effect of different concentration and dipping time (min) of IBA on number and length of root at different WAI

IBA concentration	Dipping time	Number of root/explant		length of root (cm)	
		10 WAI	14 WAI	10 WAI	14 WAI
Controlled	–	–	–	–	–
IBA 300 mg/L	10	–	–	–	–
	20	2.18 e	5.37 c	1.22 de	3.12 c
	30	3.57 b	6.10 b	2.60 b	5.35 a
	40	1.78 f	3.50 e	0.83 fg	1.73 e
	50	Necrosis	Necrosis	Necrosis	Necrosis
IBA 400 mg/L	10	–	–	–	–
	20	3.10 c	6.27 b	1.49 d	2.33 d
	30	2.47 de	4.80 c	2.67 b	4.23 b
	40	1.41 g	3.30 e	0.64 g	1.14 f
	50	Necrosis	Necrosis	Necrosis	Necrosis
IBA 500 mg/L	10	2.67 d	4.10 d	1.08 ef	2.28 d
	20	3.47 bc	6.06 b	2.16 c	3.42 c
	30	4.20 a	7.80 a	3.31 a	5.8 a
	40	2.10 ef	3.8 de	0.91 efg	1.35 ef
	50	Necrosis	Necrosis	Necrosis	Necrosis
CV (%)		8.1	6.81	11.81	9.29
LSD $_{(0.05)}$		0.372	0.5925	0.3403	0.4864

*WAI= Weeks after inoculation. Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other. CV= Coefficient of variation, LSD $_{(0.05)}$ = Least significant difference.

Plate 11. Root initiation of regenerated tea shoots by dipping in IBA solution in natural open condition.

CHAPTER V

SUMMARY AND CONCLUSION

The present experiment was conducted to evaluate the effect of different plant growth regulators on *in vitro* callus induction, shoot regeneration and root formation for Tea. The experiment was conducted at Completely Randomized Design (CRD) with 3 replications. BA were used as cytokinin and 2, 4-D , NAA, IBA was used as auxin group of hormone. The combined effect of hormones was also studied. Fresh, healthy and disease free shoot tips and nodal segments were used as explants.

It revealed that, the days to callus induction, weight of callus were significantly influenced with 2,4-D alone or in combination with BA. In case of 2, 4-D, the highest percentage of callus induction was recorded at 1.50 mg/L 2, 4-D (60.61%) and the lowest percentage (42.39%) was required in 2.50 mg/L. In combined treatment of BA and 2,4-D, the treatment 2.00 mg/L BA + 1.50 mg/L 2,4-D had showed the highest percentage of callus induction (86.51%) where 2.00 mg/L BA + 2.50 mg/L 2,4-D had the lowest frequency (54.14%). The minimum weeks for callus induction found 2.21 in combined effect of 2.00 mg/L BA + 1.50 mg/L 2,4-D and highest (4 weeks) was in 2,4-D 1.00 mg/L. Again, in 6, 10 and 14 WAI 2.00 mg/L BA + 1.50 mg/L 2, 4-D produced highest weight of callus which were 0.18, 0.56 and 1.1 g respectively. No callus induction was found in controlled treatment. Moreover, this experiment only produced callus.

Effect of shoot induction with BA alone or in combination with NAA was observed in tea. The results showed though highest percentage (84.39%) of shoot induction was recorded in the combined effect of 2.00 mg/L BA with 2.00 mg/L NAA. The lowest percentage (32.94%) was found with 4.00 mg/L BA concentration. In case of single BA treatments no further multiplication occurred. But 2.00 m/L BA in combination with NAA showed further multiplication.

Maximum 50.34 days to shoot induction was recorded in 4.00 mg/L BA treatment whereas minimum 32.43 days in media containing 2.00 mg/L BA + 2.00 mg/L NAA. The highest number of shoots (1.42, 2.46 and 4.49 in 60, 90 and 120 DAI) were also recorded in 2.00 mg/L BA + 2.00 mg/L NAA and the lowest number of shoots (0.86 and 1.43) at 60, 90 DAI were in 2.00 mg/L BA + 1.00 mg/L NAA treatment. Though

BA single concentrations elongated as shoot like structure, it did not result into multiple shoots even after multiple subculture up to 90 DAI. So the combined treatment 2.00 mg/L BA + 2.00 mg/L NAA considered as the best treatment for shoot induction and multiplication. Moreover, the longest shoot (4.61 cm) after 120 days was found in 2.00 mg/L BA + 2.00 mg/L NAA. And the lowest (1.03 cm) length of shoot at 90 DAI was founded in 1.00 mg/L BA concentration. Highest number of leaves (3.36 and 5.53 at 90 and 120 DAI) found in 2.00 mg/L BA + 3.00 mg/L NAA. It indicates that combination of BA and NAA performed better than only single treatment of hormone. No response was founded in controlled treatment for shoot.

No root formation was founded throughout this time in single dose of BA or in the combined dose of BA and NAA even after a long period and multiple subcultures. So we decided to go with *ex vitro* (natural open pot condition) rooting technique.

For *ex vitro* rooting three different concentration of IBA (300,400 and 500 mg/L) and five different dipping times (10, 20, 30, 40 and 50 minutes) were used. The highest percentage (75.32%) of root induction was recorded when regenerated shoots from shoot induction medium were dipped in 500 mg/L IBA solution for 30 minutes. No root induction was recorded in controlled treatment. After 30 minutes the responses started to decrease with time and at 50 minutes all three concentrations showed necrosis.

The maximum 9.37 weeks to root induction was required in 400 mg/L IBA (40 minutes dipping time) among the responsive treatments and the minimum 6.02 weeks were required in case of 500 mg/L IBA (dipping time: 30 minutes). Also the highest number of root per regenerated shoot (4.20 and 7.80) as well as highest length of root (3.31 cm and 5.80 cm) for 10 and 14 WAI respectively were recorded in 500 mg/L IBA concentration (dipping time : 30 minutes).

The protocol developed from the present study may be useful for large scale production of healthy, disease free planting material in Tea. The results showed that combinations of BA + NAA was better than single cytokinine (BA) treatment for shoot multiplication and IBA concentration 500 mg/L is better than other concentrations for rooting, also dipping time 30 minutes was showed better responses then others. This study showed that excessive dipping time caused necrosis. This protocol can be used for commercial cultivation of tea as a cost effective method

CHAPTER VI

RECOMMENDATIONS

Following recommendations could be addressed based on the present investigation:

i. Further study with large number of popular tea cultivar (leaves, anther, seed etc. could be used as explants) is suggested to reconfirm the results.

ii. Callus induction can be done with Kinetin or other callus induction hormone for large number of shoot regeneration.

iii. TDZ and BAP can be used for better results in case of shoot induction.

iv. *In vitro* rooting in combination with *in vivo* can also be practiced.

v. Semi solid media for shoot and root induction and multiplication can be used.

vi. Research should be carried out up to the field level to evaluate genetic variation between the plants derived from tissue culture as well as conventional breeding.

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APPENDICES

Appendix I. Composition of Duchefa Biochemic MS (Murashige and Skoog, 1962) medium including vitamins

Components	**Concentrations**	**Concentrations**
Micro Elements	**(mg/L)**	**(µM)**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**mg/L**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
$NH4NO_3$	1650.00	20.61
Vitamins	**mg/L**	**(µM)**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Appendix II. Analysis of variance (ANOVA) of effect of different concentration of BA on days to shoot initiation

Source	DF	SS	MS	F	P
Treatment	3	220.474	73.4915	4168.86	0
Error	8	0.141	0.0176		
Total	11	220.615			
CV%	0.3				
LSD $_{(0.05)}$	0.25				

Appendix III. Analysis of variance (ANOVA) of effect of different concentration of BA on length of shoot at 60 DAI

Source	DF	SS	MS	F	P
Treatment	3	0.03934	0.01311	36.97	0
Error	8	0.00284	0.00035		
Total	11	0.04218			
CV%	4.11				
LSD $_{(0.05)}$	0.0355				

Appendix IV. Analysis of variance (ANOVA) of effect of different concentration of BA on length of shoot at 75 DAI

Source	DF	SS	MS	F	P
Treatment	3	0.86271	0.28757	133.69	0
Error	8	0.01721	0.00215		
Total	11	0.87992			
CV%	4.76				
LSD $_{(0.05)}$	0.0873				

Appendix V. Analysis of variance (ANOVA) of effect of different concentration of BA on length of shoot at 90 DAI

Source	DF	SS	MS	F	P
Treatment	3	1.428	0.476	417.61	0
Error	8	0.00912	0.00114		
Total	11	1.43712			
CV%	2.43				
LSD $_{(0.05)}$	0.0636				

Appendix VI. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D on weeks to callus induction

Source	DF	SS	MS	F	P
Treatment	3	2.44605	0.81535	184.94	0
Error	8	0.03527	0.00441		
Total	11	2.48132			
CV%	1.89				
LSD $_{(0.05)}$	0.125				

Appendix VII. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D on fresh weight of callus at 6 WAI

Source	DF	SS	MS	F	P
Treatment	3	0.004441	0.00148	7.1	0.0121
Error	8	0.001668	0.000209		
Total	11	0.006109			
CV%	10.78				
LSD $_{(0.05)}$	0.0272				

Appendix VIII. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D on fresh weight of callus at 10 WAI

Source	DF	SS	MS	F	P
Treatment	3	0.04264	0.01421	103.3	0
Error	8	0.0011	0.00014		
Total	11	0.04374			
CV%	2.87				
LSD $_{(0.05)}$	0.0221				

Appendix IX. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D on fresh weight of callus at 14 WAI

Source	DF	SS	MS	F	P
Treatment	3	0.07678	0.02559	20.13	0.0004
Error	8	0.01017	0.00127		
Total	11	0.08695			
CV%	5.01				
LSD $_{(0.05)}$	0.0671				

Appendix X. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D with BA on weeks to callus induction

Source	DF	SS	MS	F	P
Treatment	3	2.82276	0.94092	125.12	0
Error	8	0.06016	0.00752		
Total	11	2.88292			
CV%	3.08				
LSD $_{(0.05)}$	0.1633				

Appendix XI. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D with BA on fresh weight of callus at 6 WAI

Source	DF	SS	MS	F	P
Treatment	3	0.007679	0.00256	73.31	0
Error	8	0.0002793	0.00003492		
Total	11	0.007958			
CV%	4				
LSD $_{(0.05)}$	0.0111				

Appendix XII. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D with BA on fresh weight of callus at 10 WAI

Source	DF	SS	MS	F	P
Treatment	3	0.05118	0.01706	39.17	0
Error	8	0.00348	0.00044		
Total	11	0.05466			
CV%	4.62				
LSD $_{(0.05)}$	0.0393				

Appendix XIII. Analysis of variance (ANOVA) of effect of different concentration of 2,4-D with BA on fresh weight of callus at 14 WAI

Source	DF	SS	MS	F	P
Treatment	3	0.31816	0.10605	198.97	0
Error	8	0.00426	0.00053		
Total	11	0.32242			
CV%	2.77				
LSD $_{(0.05)}$	0.0435				

Appendix XIV. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on days to shoot initiation

Source	DF	SS	MS	F	P
treatment	3	287.237	95.7455	2823.25	0
Error	8	0.271	0.0339		
Total	11	287.508			
CV	0.47				
LSD $_{(0.05)}$	0.3467				

Appendix XV. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on number of shoot at 60 DAI

Source	DF	SS	MS	F	P
Treatment	3	0.49095	0.16365	85.06	0
Error	8	0.01539	0.00192		
Total	11	0.50634			
CV%	3.9				
LSD $_{(0.05)}$	0.0826				

Appendix XVI. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on number of shoot at 90 DAI

Source	DF	SS	MS	F	P
Treatment	3	1.84838	0.61613	99.97	0
Error	8	0.0493	0.00616		
Total	11	1.89768			
CV%	4.19				
LSD $_{(0.05)}$	0.1478				

Appendix XVII. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on number of shoot at 120 DAI

Source	DF	SS	MS	F	P
Treatment	3	7.65542	2.55181	171.84	0
Error	8	0.1188	0.01485		
Total	11	7.77422			
CV%	3.82				
LSD $_{(0.05)}$	0.2294				

Appendix XVIII. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on Length of shoot at 60 DAI

Source	DF	SS	MS	F	P
Treatment	3	0.5966	0.19887	60.71	0
Error	8	0.0262	0.00328		
Total	11	0.6228			
CV%	5.3				
LSD $_{(0.05)}$	0.1078				

Appendix XIX. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on Length of shoot at 90 DAI

Source	DF	SS	MS	F	P
Treatment	3	3.33715	1.11238	668.16	0
Error	8	0.01332	0.00166		
Total	11	3.35046			
CV%	1.61				
LSD $_{(0.05)}$	0.0768				

Appendix XX. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on Length of shoot at 120 DAI

Source	DF	SS	MS	F	P
Treatment	3	5.17984	1.72661	538.14	0
Error	8	0.02567	0.00321		
Total	11	5.20551			
CV%	1.53				
LSD $_{(0.05)}$	0.1067				

Appendix XXI. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on number of leaves at 90 DAI

Source	DF	SS	MS	F	P
Treatment	3	2.49536	0.83179	19.07	0.0005
Error	8	0.34893	0.04362		
Total	11	2.84429			
CV%	7.46				
LSD $_{(0.05)}$	0.3932				

Appendix XXII. Analysis of variance (ANOVA) of effect of different concentration of BA and NAA on number of leaves at 120 DAI

Source	DF	SS	MS	F	P
Treatment	3	4.66812	1.55604	31.86	0.0001
Error	8	0.39069	0.04884		
Total	11	5.05881			
CV%	4.8				
LSD $_{(0.05)}$	0.4161				

Appendix XXIII. Analysis of variance (ANOVA) of effect of different concentration of IBA with different dipping time for weeks of root initiation

Source	DF	SS	MS	F	P
Treatment	9	35.5901	3.95445	398.32	0
Error	20	0.1986	0.00993		
Total	29	35.7886			
CV%	1.25				
LSD $_{(0.05)}$	0.1697				

Appendix XXIV. Analysis of variance (ANOVA) of effect of different concentration of IBA with different dipping time on number of root at 10 WAI

Source	DF	SS	MS	F	P
Treatment	9	20.847	2.31633	48.57	0
Error	20	0.9539	0.04769		
Total	29	21.8009			
CV%	8.1				
LSD $_{(0.05)}$	0.372				

Appendix XXV. Analysis of variance (ANOVA) of effect of different concentration of IBA with different dipping time on number of root at 14 WAI

Source	DF	SS	MS	F	P
Treatment	9	57.707	6.41189	52.99	0
Error	20	2.42	0.121		
Total	29	60.127			
CV%	6.81				
LSD $_{(0.05)}$	0.5925				

Appendix XXVI. Analysis of variance (ANOVA) of effect of different concentration of IBA with different dipping time on length of root at 10 WAI

Source	DF	SS	MS	F	P
Treatment	9	23.2432	2.58258	64.68	0
Error	20	0.7986	0.03993		
Total	29	24.0418			
CV%	11.81				
LSD $_{(0.05)}$	0.3403				

Appendix XXVII. Analysis of variance (ANOVA) of effect of different concentration of IBA with different dipping time on length of root at 14 WAI

Source	DF	SS	MS	F	P
Treatment	9	71.3064	7.92293	97.15	0
Error	20	1.631	0.08155		
Total	29	72.9374			
CV%	9.29				
LSD $_{(0.05)}$	0.4864				

Appendix XXVIII. Analysis of variance (ANOVA) of effect of different concentration of BA on percentage of shoot induction

Source	DF	SS	MS	F	P
Treatment	3	1307.8	435.934	4773.38	0
Error	8	0.73	0.091		
Total	11	1308.53			
CV%	0.59				
LSD $_{(0.05)}$	0.569				

Appendix XXIX. Analysis of variance (ANOVA) of effect of different concentration of 2, 4-D on percentage of callus induction

Source	DF	SS	MS	F	P
Treatment	3	574.653	191.551	2820.52	0
Error	8	0.543	0.068		
Total	11	575.196			
CV%	0.51				
LSD $_{(0.05)}$	0.4907				

Appendix XXX. Analysis of variance (ANOVA) of effect of different concentration of BA with 2, 4-D on percentage of callus induction

Source	DF	SS	MS	F	P
Treatment	3	1770.01	590.003	6552.12	0
Error	8	0.72	0.09		
Total	11	1770.73			
CV%	0.43				
LSD $_{(0.05)}$	0.565				

Appendix XXXI. Analysis of variance (ANOVA) of effect of different concentration of BA with NAA on percentage of shoot induction

Source	DF	SS	MS	F	P
Treatment	3	1296.02	432.008	12076.31	0
Error	8	0.29	0.036		
Total	11	1296.31			
CV%	0.27				
LSD $_{(0.05)}$	0.3561				

Appendix XXXII. Analysis of variance (ANOVA) of effect of different concentration of IBA with different dipping time on percentage of root induction

Source	DF	SS	MS	F	P
Treatment	9	11348.2	1260.91	25615.25	0
Error	20	1	0.05		
Total	29	11349.2			
CV%	0.56				
LSD $_{(0.05)}$	0.3779				