Micropropagation and antimicrobial activity of *Brucea mollis* Wall. ex Kurz - an endangered plant of Northeast India

A THESIS

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CERTIFICATE

I am pleased to forward this Ph.D. thesis entitled, **"Micropropagation and antimicrobial activity of *Brucea mollis* Wall. ex Kurz - an endangered plant of Northeast India''** submitted by Prapty Das for examination to the Gauhati University for the award of Degree of Doctor of Philosophy (Ph.D.) in Botany under the Faculty of Science.

Mrs. Prapty Das has carried out this piece of work under my supervision and guidance and fulfills all the requirements under the Ph.D. Regulation of the Gauhati University.

I also certify that the results of research work embodied in this thesis are original and has neither been submitted to any other university nor anywhere for award of Degree.

5/4/19

(S.K. Borthakur)

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Place: Guwahati

Date: 5/04/2019

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DECLARATION

I hereby declare that the subject matter of this thesis entitled **"Micropropagation and antimicrobial activity of *Brucea mollis* Wall. ex Kurz - an endangered plant of Northeast India"** has been submitted to Gauhati University in the Department of Botany for partial fulfilment for the award of the degree of Doctor of Philosophy in Botany. This is an original work carried out by me. Further, I declare that no part of this work has been reproduced elsewhere for award of any other degree.

Prapty Das

Date: 5/04/2019 (**Prapty Das)**

Place: Guwahati

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAP	6 Benzyl amino purine
B_5	Gamborg
ca	Circa (Lat.)
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CFU	Colony forming unit
cm	centimetre
$CuSO_4.5H_20$	Copper sulphate pentahydrate
DMRT	Duncan multiple range test
et al	et alia(and others) (Lat.)
etc	et cetera
ex	Out of from (Lat.)
$FeSO_4.7H_2O$	Ferrous sulphate
Fig.	Figure
HCl	Hydrochloric acid
Hrs	Hours
H_3BO_3	Boric acid
IBA	Indole Butyric acid
i.e	That is
KH_2PO_4	Dihydrogen potassium phosphate
KI	Potassium iodide

KNO_3	Potassium nitrate
Lab	Laboratory
mg/L	Milligram per litre
$MgSO_4.7H_2O$	Magnesium sulphate
MHA	Mueller Hinton Agar
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
$MnSO_4.H_20$	Manganese sulphate
$MnSO_4.4H_2O$	Manganese sulphate tetrahydrate
MS	Murashige and Skoog
MTCC	Microbial type culture collection
NAA	Naphthalene acetic acid
Na_2EDTA	Disodium ethylene diamine tetraacetic acid
$NaH_2PO_4.H_2O$	Dihydrogen Sodium phosphate
NaOH	Sodium hydroxide
NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulphate
$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate
nm	Nano metre
OD	Optical density

PDA	Potato Dextrose Agar
pH	Potential hydrogen
psi	Pounds per square inch
UV	Ultra violet
viz	Videlicet (Lat.-videre licet)
w/v	Weight per volume
X	Multiplication sign
$ZnSO_4.7H_2O$	Zinc sulphate
1N	1 Normal
2,4- D	2,4- Dichlorophenoxy acetic acid
^oC	Degree celsius
%	percentage
µg	microgram
µl	Micro litre
µM	Micromolar
µg/ml	Microgram per millilitre
±	Plus minus sign
≤	Less than or equal to

LIST OF TABLES

LIST OF PLATES

CONTENTS

INTRODUCTION

Brucea mollis Wall. *ex* Kurz. belonging to the family Simaroubaceae under the order Sapindales is an important medicinal plant of Northeast India. In addition to India, it occurs in Bhutan, China, Bangladesh, Cambodia, Myanmar, Laos, Indonesia, Malaysia, Sri Lanka, Nepal, Vietnam, Philippines and Thailand (Hajra *et al*., 1997; Pullaiah, 2006). In India, it is distributed in Sikkim, Arunachal Pradesh, Nagaland, Assam, Manipur, Meghalaya and West Bengal (Hajra *et al.,* 1997; Gupta *et al.,* 2004; Bharati and Singh, 2009).

The family Simaroubaceae to which *Brucea mollis* belongs has been placed, in the order Geraniales of the series Disciflorae (Bentham and Hooker, 1862-1883). However, in APG III System of classification (2009), the family has been placed under the order Sapindales of the clade Rosids of the Eudicots. It includes 30 genera and *ca* 200 species, of which 5 genera and 11 species are reported from India (Hajra *et al.*,1997). The habit of the members of the family is either dioecious or monoecious tree or shrub, usually with bitter substances. The family is distinguished by the presence of quassinoids, a chemical constituent responsible for antimalarial activity which was first isolated from *Quassia amara,* a species of this family (Alves *et al.,* 2014). Besides it has numerous other chemical constituents like alkaloids, coumarins, triterpenes, anthraquinones, flavonoids, steroids and metabolites. The members of the family possess numerous biological properties like antimalarial, anticancer, antigastric-ulcer effect, antitumor, antiplasmodial, antiasthmatic, antiviral, anti inflammatory, antituberculosis, anti-

amoebic, antileukemic, antiallergenic, insecticide, pesticide and amebicide (Liu *et al.*, 2009; Alves *et al.*, 2014; Ehata *et al.*,2012).

The members of the genus *Brucea* are distributed in several parts of tropical eastern hemisphere (Liu *et al.*, 2009) and comprises of 10 species (Bharati and Singh,2012) *viz.*, *Brucea antidysenterica* J.F.Mill., *Brucea erythraeae* Chiov., *Brucea guineensis* G.Don, *Brucea bruceadelpha* (Noot.) Kosterm., *Brucea javanica* (L.) Merr., *Brucea macrocarpa* Stannard, *Brucea mollis* Wall. ex Kurz, *Brucea trichotoma* Spreng, *Brucea tenuifolia* Engl. and *Brucea tonkinensis* (Lecomte) Gagnep. Members of *Brucea* possess a number of phytochemical compounds which have been used to cure diseases like malaria, colonic diseases, parasitosis, gastrointestinal cancer, dysentery (Bharati and Singh, 2012; Chen *et al.*, 2013c; Gillin *et al.*, 1982; Ehata *et. al*; 2012). In India it is represented by only two members *viz*., *B. mollis* and *B. javanica.*

B. mollis is well known in traditional medicine and extensively used for curing malaria in Assam. Moreover, there are reports about the use of this plant in stomach disorders, cardiovascular problems, anticancer and diuretic activities (Bhutani, 2008; Pullaiah, 2006; Singh *et al*., 2003; Borthakur, 1976). Inspite of its uses in traditional healthcare practices, the population of *B. mollis* has been diminishing in alarming rate due to several anthropogenic activities such as habitat fragmentation and over-exploitation and for which it has already been listed in endangered category by Conservation Assessment and Management Plan (CAMP), Foundation for Revitalization of Local Health Tradition (FRLHT), Bangalore (India) in Arunachal Pradesh and Assam (Kakati and Borthakur, 2016). Also it has been reported to be rare in Meghalaya (Chauhan, 1983; Haridasan and Rao , 1985).

The habit of the plant varies from perennial shrub or small tree. Stem is branched and with lenticels. Leaves alternate, pinnate. Leaflets opposite, lowest pair of leaflets smallest, gradually larger upwards, oblong-lanceolate or ovate, unequal at base, acuminate or acute at apex, entire along the margins, rarely undulate and membranous, pubescent on both surfaces. Flowers minute, in axillary pubescent panicle of cymes, *ca* 25 cm long. Sepals 4, *ca* 1 mm long, imbricate; petals 4, linear, minute, imbricate, green, white, creamy or red. Stamens 4, *ca* 1 mm long. Disk 4-lobed, ovary 4- lobed, glabrescent; styles linear and deflexed. Nuts 1-2, or 3-4, ovoid and pointed at apex, orange-red in color when ripe. The flowering and fruiting period starts from November and ends in May (Hajra *et al.* 1997).

Brucea mollis contains a number of phytochemical compounds like quassinoids, triterpenoids, alkaloids which are responsible for biological activities like antimalarial, anticancer, antitumor, antileukemic, antiplasmodial, pesticide, antibacterial, trypanocide, cytotoxic, etc. (Bharati and Singh, 2012). The whole plant possesses an immense medicinal property. It has been reported that the powdered dried seeds mixed with water is taken thrice in a day for the treatment to cure malaria and stomach complaints (Bhutani, 2008; Pullaiah, 2006; Singh *et al.*, 2003; Borthakur, 1976). Its fruits and root decoction are also used by the local people of Karbi Anglong District of Assam to cure malaria.

Rationale of the present study

India is endowed with abundant plant resources with wide variety of medicinal plants. Some of these resources have been employed in traditional systems of medicine, folk remedies and in pharmaceutical industries for use in modern medicine. As a result

many of the medicinal plants have been collected from wild habitats resulting in depletion of their population. In addition, other anthropogenic factors, natural calamities like flood, landslides and climate change also directly or indirectly contributed to the risk of their survival. As a part of conservation strategies very often conventional techniques of propagation through seed is useful, but it not always successful for some of these plants. Low seed set, low seed germination and limited availability of seeds are some of the factors associated with some of these plants (Kakati and Borthakur, 2017). In *B. mollis*, it has been reported that the pollination as well as seed dispersal mechanism is ineffective (Kakati and Borthakur, 2017). As a result the plant becomes rare in its natural habitat. This is aggravated by overexploitation of plants from the wild and resulted in decline of its population. Therefore, there is a need to develop an efficient protocol for its mass propagation as a part of conservation initiatives. Further, no work has been carried out on the antimicrobial activity of *B. mollis.*

Objectives:

1. To develop the protocol for micropropagation of the plant
2. To study the antimicrobial activity of the plant.

REVIEW OF LITERATURE

2.1. Habit and Distribution

The members of Simaroubaceae are trees or shrubs characterized by the bitter taste and are distributed in tropical and subtropical regions of both the hemispheres, particularly in tropical America, Mexico to Argentina, tropical West Africa and Southeast Asia. The family comprises of 200 species under 30 genera and of which 11 species under 5 genera occur in India. One of the important genera of the family Simaroubaceae is *Brucea* J.F. Miller. Bharati and Singh (2012) reported 10 species under the genus which are distributed in the tropics. However, Santapau and Henry (1973) and Hajra *et al.* (1997) reported 6 species under the genus that are distributed in Asia and North Australia and out of which only 2 species *viz.*, *B. javanica* (L.) Merr. and *B. mollis* Wallich ex Kurz. occur in India.

B. mollis is distributed in India, Bhutan, Bangladesh, China, Cambodia, Indonesia, Myanmar, Malaysia, Philippines, Sri Lanka, Thailand and Vietnam (Hajra *et al.*,1997). However, Pullaiah (2006) reported the species from Nepal, China, Bhutan, Cambodia, Laos, Malaysia, Myanmar, Philippine, Thailand and Vietnam. In India, the species is found in parts of West Bengal, Meghalaya, Arunachal Pradesh, Sikkim, Assam, Manipur and Nagaland (Hajra *et al.*, 1997). In Assam, it is restricted only to Karbi Anglong district (Kakati and Borthakur, 2017).

2.2. Economic importance

B. mollis is a potent medicinal plant used traditionally by several ethnic communities of Northeast India. The plant is well-known for its potent antimalarial activity (Prakash *et al*., 2013). Besides, Liu *et al*. (2009); Bharati and Singh (2012); Chen *et al*. (2013a,b) reported anticancer, diuretic, cardiovascular, antiplasmodial, amebicidal, antigiardial, antiviral, antileukemic, pesticidal activities of the plant. Dhawan *et al*. (1977) and Rastogi and Dhawan (1990) reported the presence of diuretic activity and cardiovascular effects of the ethanolic extract (50%) of the whole plant respectively. It is also reported to be used in Chinese traditional medicines for the treatment of certain types of cancer and malaria (Liu *et al.,* 2009; Bharati and Singh, 2012). Borthakur (1976) reported the use of fresh fruit or root decoction of *B. mollis* to cure fever. One or two fresh fruit is taken orally as per severity of the ailment. Kakati and Borthakur (2017) reported that about 4-5 cm of root is taken and the bark is removed, then washed, crushed and boiled in a glass of water for half an hour to prepare a decoction which is taken with one or two tablespoon twice or thrice daily to treat malaria. For malaria and stomach problem, 50 g of powder of dried seeds mixed in 50 ml water is taken thrice a day (Bhutani, 2008; Pullaiah, 2006; Singh *et al.,* 2003; Borthakur, 1976).

2.3. Phytochemical constituents

Studies on the phytochemical constituents of the family Simaroubaceae have been carried out by a number of workers and several compounds have been isolated and identified. Alves *et al.*(2014) reported the presence of quassinoids, alkaloids, triterpenes, steroids, coumarins, anthraquinones, flavanoids and metabolites which have

been isolated from the members of the family Simaroubaceae and of these quassinoids are the most dominant and abundant group in the family. Quassinoids are derivatives of a chemical compound named "quassin" which are the degraded triterpenoids isolated from the bark of *Quassia amara,* a member of Simaroubaceae family. Lavhale and Mishra (2007) described quassinoids as the bitter constituents possessing many biological activities such as antimalarial, anticancer, antifeedant, insecticidal, herbicidal and antiparasitic. Polonsky (1985) isolated many bitter constituents from the species of the genus *Brucea*. Ouyang *et al.* (1995) isolated quassinoids like Brusatol, Brucein B, Brucein D, Yadanziolide A, Bruceoside B, Soulameanone from the root wood and root bark of *B. mollis* var. *tonkinensis.* They also isolated β-carboline alkaloids and by using spectral and chemical evidences illuminated the structures of 1-(2′- hydroxyethyl) β-carboline, 1- hydroxymethyl- β-carboline, 1-ethyl- β-carboline and β-carboline-1-propionic acid from the root wood and root bark of *B. mollis* var. *tonkinensis*. Some other compounds isolated by them include Canthin-6-one- alkaloids like Bruceolline C, Bruceolline G, 11- hydroxycanthin-6-one, 1 hydroxy -11-methoxycanthin-6-one, 11-hydroxyl-1-methoxycanthin-6-one .

Ouyang *et al*. (1994) isolated and determined the structures of three new alkaloids *viz*., 11-O-β D-glucopyranosyl (1->6)-beta-D-glucopyranosylcanthin-6-one, 5-O-β-D-glucopyranosyl-(1->6)-β-D-glucopyranosylcanthin-6-one and 11-hydroxycanthin-6-one-N-oxide from the rootwood of *B. mollis*. Yadanziolides T-V, Yadanziolide B and Brucein D from ethanol extract of stem of *Brucea mollis* have been isolated by Chen *et al*. (2011). They also isolated and reported the indole alkaloids like Bruceollines H-N, Bruceolline E and Bruceolline F from the species. In an another study, Chen *et al.*(2014) reported compounds like Bruceolline O, 1-(1-beta-glucopyranosyl)-1H-

indole-3-carbaldeyde, canthin-6-one, 11-hydroxycanthin-6-one, 9-methoxycanthin-6-one, 4-methoxycanthin-6-one, infractin and beta- carboline-1-propionic acid from the stems of *B. mollis.* Of these, Bruceolline O was a new indole alkaloid and 1-(1-beta-glucopyranosyl)-1H-indole-3-carbaldehyde, 9-methoxy canthin-6-one, 4-methoxycanthin-6-one and infractin were isolated for the first time from the species. Chen *et al.* (2013b) also isolated triterpenoids like brumollisols A-C; piscidinol A; (23R, 24S)-23, 24, 25-trihydroxytirucall-7-ene-3, 6-dione; 21α-methylmelianodiol; 24-epipiscidinol A and 21β-methylmelianodiol from ethanol extract of the stem of *Brucea mollis* and reported that 24-epipiscidinol A have cytotoxic activity against human gastric cancer (BGC 823) and human lung epithelia cancer (A549) with IC_{50} values 1.16 and 3.01μM respectively.

2.4. Medicinal properties

Medicinal plants which constitute a main source of natural organic compounds are extensively used in different healthcare practices. The medicinal effects of plants are basically due to the presence of secondary metabolites synthesized in specific regions or parts of the plant (Joseph and Raj, 2010). Secondary metabolites are responsible for various defence mechanisms of plants but do not play a direct role in growth and development of plants. They protect the plant from predators, harmful microbial pathogens, abiotic stresses and also play an important role for communication of the plants with other organisms (Mazid *et al.,* 2011). It is reported that the leaves are the suitable storage site for desired compounds and fruits also contain a considerable amount of pharmaceutical active ingredients, and to obtain the desired compounds these parts of the plants are very often used as the sources of these compounds. Other parts of

plants that have therapeutic compounds are roots, aerial parts, flowers, seeds, stem barks, etc. (Chan *et al.*, 2012).

Medicinal properties of members of *Brucea* have been well documented by a number of workers (Gillin *et al.*,1982; O'Neill *et al.*, 1987; Liu *et al.*, 2009; Bharati and Singh, 2012; Prakash *et al.*,2013; Chen *et al.*,2013a,b,c and Sornwatana *et al.*,2013). Kupchan *et al*. (1973) reported anti-inflammatory effect of *B. antidysenterica* while Gillin *et al.*(1982) reported its usefulness in the treatment of dysentery in Ethiopia. They also investigated the compound Bruceantin (an amoebicide) present in the plant, that killed *Entamoeba histolytica in vitro*. There are records of the use of this species in traditional health practices in a number of countries for treating malaria, helminthic infections, fever, dysentery, etc. (Grace and Fowler, 2008). Tian and Xie (2011) reported antitumor effect of *B. javanica*. In an investigation on Chinese medicine, Chen *et al*. (2013c) reported its efficacy in the treatment of prostrate, lung and gastrointestinal cancer and also found to have antimalarial, anti-inflammatory and antiviral activities. *B. javanica* has long been used in folk medicine of Thailand to treat dysentery and diarrhoea (Sornwatana *et al*., 2013). Its anti-dysenteric effect is believed to be due to the active quassinoids present in the kernel extract (Sato *et al*., 1980; Wright *et al*., 1993).

2.5. Biological activities of *B. mollis*

2.5.1. Antimalarial activity

Traditionally, *B. mollis* has been used to cure malaria in many parts of India and China. Quassinoids, the characteristic compound of the family Simaroubaceae act as one of the chief antimalarial compounds (Alves *et al.*, 2014).

Prakash *et al.* (2013) carried out antiplasmodial activities *in vitro* and *in vivo* from the root extracts of *B.mollis* against chloroquine sensitive and chloroquine resistant strains of *Plasmodium falciparum* and found to be significant in chloroquine sensitive strain with 5.1 µg/ml IC_{50} value compared to chloroquine resistant strain with IC_{50} value 334 µg/ml in methanolic chloroform extract.

2.5.2. Anticancer activity

Tung *et al.* (2013) carried out studies on cytotoxic activity against 4 cancer cell lines *viz.*, human carcinoma of the mouth, human lung adenocarcinoma, human prostrate adenocarcinoma and human promyelocytic leukemia from the ten compounds isolated from the methanolic extract of leaves, stems and roots and was found to have cytotoxic activity.

2.5.3. Antitumor, antileukemic, antiplasmodial and pesticide activity

The quassinoids such as Bruceine D and Brusatol which were isolated from the root wood and root bark of *B. mollis* var *tonkinensis* were reported to have antititumor activity (Ouyang *et al*., 1995). Bharati and Singh in 2012 reported that Brucein D, Brusatol, 11-hydroxy-canthin-6-one and canthin-6-one isolated from the root bark and root wood have antileukemic activity while Bruceine B, D and Brusatol have antiplasmodial activity and Bruceine B, Bruceine D, Brusatol, Yadanziolide A, 1-ethyl-β-carboline and canthin-6-one have pesticidal effect.

2.5.4. Antibacterial

1-ethyl-β-carboline and canthin-6-one isolated from *B.mollis* reported to possess antibacterial activity by Bharati and Singh (2012).

2.6. Micropropagation

Plant cell and tissue culture is a useful technique for micropropagation where *in-vitro* culture of meristematic tissues of any part of a plant under aseptic conditions are used to regenerate a new plant under controlled nutritional and environmental condition. It produces the desired plant material in abundant quantities and provides opportunities for the production of large scale propagation of plants from the selected elite plant material in a short period of time (Debnath *et al.,* 2006; Kumar *et al*., 2013).

Tissue culture is becoming quite popular from the last two decades especially for species propagated through vegetative means in general and those considered as endangered and threatened species in particular. Unlike the conventional method of propagation, it is the only method of achieving rapid and large scale production of disease free plants. Tissue culture is also well employed for conservation of medicinal plants. For commercial plant propagation, it is one of the important techniques which contribute in the field of agriculture, horticulture, production of disease free plants, in industry and in germplasm conservation. The clonal propagation using *in-vitro* culture techniques was first tried in orchid genus *Cymbidium* using shoot tip explants (Morel, 1960).

The *in-vitro* method of propagation is based on the totipotency of plant cells. The steps involved in the explants during *in vitro* culture are (a) dedifferentiation of the tissues or organs through the activation of physiological mechanism by the endogenous and exogenous hormones to cell division, (b) active cell division at the cut end portion or localized meristematic activity in the specific regions of the explants which results in the formation of shoot meristem or root meristems or both, (c) redifferentiation of the cells which can be categorized as unipolar or bipolar. Unipolar redifferentiation results

in the regeneration of new shoot or root meristem (organogenesis) whereas the bipolar redifferentiation results in the formation of both, *i.e.* somatic embryogenesis (Jha and Ghosh, 2016).

2.6.1. Factors necessary for plant tissue culture

2.6.1.1. Explant type and its establishment

The part of the plant which is taken for tissue culture is known as the explant. The explants can be any part of the leaf, stem, roots, apical portion of the stem, axillary bud, etc. A successful plant tissue culture technique depends on the removal of endogenous or exogenous microorganisms present in the explants (Constantine, 1986; Buckley and Reed, 1994). The factors needed for the selection of the explants are the age of the explants, season in which the explants are obtained, size of the explants, quality of the explants and the ultimate goal of the tissue culture and genotype. Smith (2013) illustrated that the *in vitro* response may vary depending upon the endogenous level of phytohormones and different hormonal balances present in the plant species.

Different sterilization processes are utilized by the different protocols in the explants. For successful tissue culture procedures, the maintenance of aseptic condition is very much essential (Ndakidemi *et al.,* 2013). Different sterilizing agents are used during surface sterilization of the explants which are toxic to plant tissues. So, proper concentration of the sterilants, time of exposure of the explants to the sterilants and the order of using the sterilants are to be standardized for better establishments of the explants. Mihaljevic *et al*. (2013) suggested the use of different sterilization agents like sodium hypochlorite, calcium hypochlorite, sodium dichloro isocyanurate, mercuric chloride, silver nitrate, hydrogen peroxide during surface sterilization process.

2.6.1.2. Plant growth regulators

Plant growth regulators are the plant hormones and their respective synthetic analogues which are important components in culture media required for determining certain developmental pathways of a plant cell. The plant growth regulators include auxins, cytokinins, gibberallic acid, abscisic acid. Auxins and cytokinins are very essential which are used to regulate growth and development of morphological characteristics in plant tissue culture. According to Razdan (2014), the ratio of plant growth regulators required for rooting or shooting varies with the tissue which is related to the quantum of hormones synthesized endogenously within the cells of the explants. Charriere *et al*. (1999) reported that the optimum concentration of the plant growth regulators varies with respect to the species. Auxins are widely used and form an integral part of the basal nutrient media. They promote cell division and cell elongation, formation of meristems as a result of which either undifferentiated cells or organized tissues developed. Cytokinins are the most complex plant growth regulators available in natural and synthetic forms. They promote cell division, shoot proliferation, axillary branching, adventitious shoot formation and embryogenesis. The key role of auxin and cytokinin was highly appreciated for the induction of callus, organogenesis and embryogenesis by Skoog and Miller (1957). They emphasized the importance of high amount of cytokinin and low amount of auxin for promotion of shoot formation and low cytokinin and high auxin for induction of root. An intermediate concentration of auxin and cytokinin promote callus formation (Jha and Ghosh, 2016).

2.6.1.3. Basal media

According to Razdan (2014), the principal components of plant tissue culture are media comprising of inorganic nutrients (macro and micro nutrients), carbon source, organic nutrients, plant growth regulators and a gelling agent. The culture medium used in plant tissue culture contains mineral nutrients which are the crucial factors for the growth, development and maintenance of the tissue in culture. The formulation of the medium is another important factor for the successful establishment of the cultures. Jha and Ghosh (2016) suggested that the suitable nutritional requirement should be determined by trial and error for a particular tissue or plant species. A number of workers formulated different media (White, 1943; Hildebrandt *et al*., 1946; Burkholder and Nickall, 1949; Heller, 1953; Nitsch and Nitsch, 1956; Murashige and Skoog, 1962; Linsmaier and Skoog, 1965 and Gamborg *et al*.1968). However, media formulation by Murashige and Skoog (1962) has been extensively used.

Several studies were carried out to work out the effectiveness of different media using plants like *Aristolochia indica* (Shah *et al*., 2013), *Saraca asoca* (Shirin *et al*., 2015), *Cunila galioides* (Fracaro and Echeverrigaray, 2001), etc. There are a number of literatures reporting comparative effectiveness of different media on *in vitro* shoot multiplication (Mehta *et al*., 2000; Lu, 2005; Jain *et al*., 2009).

2.6.1.4. pH of the culture medium

Plant cells and tissues require an optimum pH for growth and development of the cultures. Maintenance of pH in the culture medium is very important as it influences the uptake of nutrients and plant growth regulators by regulating the solubility in the culture media (George, 1993). It regulates a wide range of biochemical reactions occurring in

the plant tissues (Bhatia and Ashwath, 2005). pH of tissue culture media are generally adjusted between 5-5.8. pH of media below 5 does not give satisfactory gelling and above 6 gives too hard medium (Jha and Ghosh, 2016).

2.7. Steps necessary for micropropagation

2.7.1. Callus culture

According to Reinert and Bajaj (1976), callus culture is one of the important technique to develop clonal propagation, genetic manipulation and plant regeneration in both monocotyledonous and dicotyledonous plants. It is a mass of undifferentiated cells produced when the explants are cultured on the appropriate medium supplemented with desired ratio of auxin and cytokinin. Various studies have been investigated to enhance callus induction from different explants and subsequent plantlet regeneration through callus formation (Sen *et al.,* 2014; Ahmad *et al.,* 2010; Elangomathavan *et al*., 2017; Biswas *et al.*, 2007). A protocol was established for *in vitro* callus induction and plantlet regeneration from leaf derived callus of *Ruta graveolans* by Ahmad *et al*. (2010) and maximum callus induction frequency (70.6±2.33%) was observed at 10 µM 2,4,5-T (2,4,5-trichloro phenoxy acetic acid) in MS media. Ali and Mirza (2006) studied the effect of explant type and hormone concentration on callus induction of *Citrus jambhiri* and was found that the stem explants showed highest response for callus induction (92%) at 1.5 mg/L 2,4-D. Biswas *et al.* (2007) established a protocol for *in vitro* propagation of *Abru sprecatorius* from nodal explants through inducing callus. It was found that maximum percentage of callus induction (80%) took place at BAP (5 mg/L) in combination with NAA (0.5 mg/L) which developed yellowish green color callus. Sen *et al.* (2014) studied callus induction and plantlet propagation from leaf, internode

and root explants of *Achyranthes aspera* and emphasized the effectiveness of 2,4-D along with BAP, IBA and NAA which developed profuse callusing from all the three explants. Another study on callus induction from different explants *viz.* leaf, petiole and internode explants of *Orthosiphon stamineus* was conducted by Elangomathavan *et al.*(2017) where 100% callus induction was obtained from petiole and internode explants at BAP(0.5 mg/L) with NAA(4 mg/L). They also emphasized that 2,4-D (5 mg/L) concentration induced high efficiency callus (100%) at petiole explants compared with other explants.

There are reports on callus induction of the species belonging to Simaroubaceae. Rout and Das (1994) studied somatic embryogenesis in *Simarouba glauca* from immature cotyledon explants and obtained highest frequency of somatic embryos from callus at 11.1µM benzyladenine and 13.42 µM α naphthalene acetic acid. Another study on callus induction of *S. glauca* using shoot tips and axillary buds was made by Dudhare *et al*. (2014) at different concentrations of 2,4-D and considered 2,4-D (5 mg/L) to be the best for callus induction with maximum fresh and dry weight of callus. Kakuturu *et al.* (2014) studied callus induction in *S. glauca* from leaf explants in MS media and emphasized the importance of using 2,4-D with NAA for better callusing response. Also it was reported that the medium supplemented with NAA (2.5 mg/L) was found to be effective for initiation of friable embryogenic callus. In *Eurycoma longifolia*, an attempt was made by Hussein *et al*. (2005a) to establish embryogenic callus from different explants such as leaf, zygotic embryo, stem, taproot, cotyledon and petiole in MS media supplemented with different concentrations of auxins where only cotyledon explants were able to form embryogenic callus at 2,4-D 1 mg/L concentration.

2.7.2. Organogenesis

Several reports on direct and indirect organogenesis of Simaroubaceae family have been established on *Simarouba glauca*, *Ailanthus malabarica*, *Eurycoma longifolia*. D'Silva and D'Souza (1992) obtained direct regeneration of *Ailanthus malabarica* DC. from the nodes of mature trees and cotyledonary node of seedlings. Natesha and Vijayakumar (2004) reported MS medium to be the best medium for establishment of cultures and shoot growth of *Ailanthus triphysa*. Hussein *et al*. (2005a) examined plantlet regeneration through somatic embryogenesis in *Eurycoma longifolia* in six different basal media *viz.* MS, WPM, NM, DKW, B_5 and White media supplemented with various concentrations of BAP, kinetin and zeatin and revealed lower concentration of kinetin (1-4 mg/L) as an ideal cytokinin for regeneration of shoots from somatic embryos. Gatti (2008) reported the effect of different concentrations of BAP and IBA on shoot formation and shoot length in different strength of MS media and obtained best results in full strength MS media at BAP 1.32 or 2.64 µM concentration. Hassan *et al*. (2012) studied the effect of BAP and Kinetin on shoot multiplication in *Eurycoma longifolia* and emphasized the effectiveness of BAP in shoot multiplication in contrast with Kinetin. Shukla and Padmaja (2013) developed an efficient regeneration protocol of *Simarouba glauca* from the shoot tips and nodal explants which exhibited highest regeneration frequency (90%) and shoot number (7.00±1.00 shoots/explants) in nodal explants in MS medium when supplemented with 6-benzylaminopurine (BAP) 4.43 µM and α-naphthalene acetic acid (NAA) 5.36 µM. The micropropagation protocol established by Lavanya *et al.* (2016) from different explants of *S. glauca* exhibited the maximum number of shoots (55.3) with 100% response from cotyledonary node explants on MS medium fortified with 6-Benzyl

adenine (3 mg/L), Kinetin (0.5 mg/L), α-Naphthalene acetic acid (0.5 mg/L) and glutamine (10 mg/L).Study of *in vitro* regeneration of *S. glauca* was undertaken by Dudhare *et al.*(2014) with the objective to observe the possibility of developing faster and reliable *in-vitro* regeneration using shoot tips and axillary buds as explants where the callus failed to regenerate although successful establishment of shoot tip explants occurred. Rathore *et al.* (2007) established a protocol for micropropagation of *Citrus limon* using nodal shoot segments through direct regeneration where 3-4 shoots initiated per axillary meristem at 9 µM BAP in MS media. Studies on indirect organogenesis of different medicinal plants have been carried out on *Ruta graveolens* L. by Ahmad *et al*. (2010), on *Achyranthes aspera* L. by Sen *et al.*(2014), on *Ephedra gerardiana* by Sharma *et al.*(2012) and on *Caralluma pauciflora* Wight by Kiranmai *et al*. (2015) etc. Sen *et al*. (2014) studied indirect shoot organogenesis from leaf and internode derived callus of *Achyranthes aspera* using BAP alone or in combination with NAA and Kinetin. It was reported that BAP (2 mg/L) along with NAA (0.5 mg/L) exhibited maximum frequency of shoot regeneration and recorded maximum shoot number at BAP (4 mg/L) + Kinetin (0.5 mg/L). Ahmad *et al*. (2010) established a protocol for multiple shoot bud induction from leaf derived callus of *Ruta graveolans* in different concentrations and combinations of BAP, Kinetin, IBA, IAA and NAA in MS media and obtained highest shoot multiplication (92.3%) at BAP (7.5 µM) with NAA (1 µM). An efficient protocol of somatic embryogenesis and indirect organogenesis of *Caralluma pauciflora* was established by Kiranmai *et al.* (2015) where best response of callus regeneration (65%) was obtained at 2ip (2 mg/L) + Zeatin (0.5 mg/L). Mroginski *et al*. (2003) studied *in vitro* plantlet regeneration from *Toona ciliate* of family

Meliaceae and reported that the best response of shoot regeneration was obtained in ¼ strength MS media supplemented with IBA 0.1 mg/L and BAP 0.5 mg/L.

2.7.3. Root regeneration

Rooting of shoots obtained *in vitro* is a prerequisite for successful establishment in soil. Natesha and Vijayakumar (2004) reported that the rooting of *in vitro* derived microshoots of *Ailanthus triphysa* was successfully accomplished only at IBA 0.4 mg/L and IAA 4 mg/L concentration with 6.3% rooting in half strength MS media. Hussein *et al.* (2005b) reported that, root formation of shootlets in *Eurycoma longifolia* occurred only on MS media at 0.4 and 0.5 mg/L IBA with 90% rooting at 0.5 mg/L IBA. Another study was conducted by Hassan *et al.* (2012) of *E. longifolia* which reported that the highest number of roots per shoot (derived from nodal explants) was observed at IBA 10 mg/L in half strength MS media. Gatti (2008) studied the effect of IBA on rooting percentage, root number and root length in *Ailanthus altissima* and highlighted about its strong influence on rooting percentage. Shukla and Padmaja (2013) recorded better rooting response in WPM than MS media with 90% root regeneration frequency at half strength media. Dudhare *et al.* (2014) observed profuse rooting in *in-vitro* derived shootlets of *Simarouba glauca* when cultured in MS media supplemented with IAA 3 mg/L, with 80% rooting in least number of days 22.8±0.52 for root induction. Lavanya *et al.* (2016) reported to obtain 95% rooting at NAA 3 mg/L in *Simarouba glauca* DC. when cultured on MS medium containing NAA, IBA and IAA either singly or in combination with Kinetin.

2.7.4. Hardening or acclimatization

Success of micropropagation depends on the ability of transferring *in vitro* derived plantlets from culture to the soil at large scale with high survival rate (Chandra *et al*, 2010). Research has been focused to control the environmental attributes for increasing growth and reducing mortality in plantlets during acclimatization stage (Mathur *et al*., 2008).

The *in vitro* plantlets are grown under low light intensity (1,200–3,000 lux) and low temperature (25 ± 2°C) and therefore, direct transfer to broad spectrum of sunlight (4,000 - 12,000 lux) and temperature (26-36°C) might cause leaf charring and wilting of plantlets. Hence, the plant requires natural conditions for acclimatization (Lavanya *et al.,* 2009). Hussein *et al.* (2005a) reported 80% survival during acclimatization of *Eurycoma longifolia*. To identify the best potting media for acclimatization in *E. longifolia* by using sealed glass chamber, Yahya *et al.* (2015) used different potting media like jiffy 7, baked soil, sand, mixture of cocoa peat + sand, sand + top soil and lastly cocoa peat + baked soil and found that jiffy is the best potting media where 100% survival was obtained and sealed glass chamber as a good technique for acclimatization. Shekhawat *et al.* (2015) successfully acclimatized *Morinda coreia* with 100% survival where flowering and fruiting was observed on transplantation after six months.

2.8. Antimicrobial activity of plant

An antimicrobial is an agent that kills or hinders the growth of microorganisms. Antimicrobials of plant origin have enormous potential to cure various ailments (Kuete *et al.,* 2008; Vats *et al.,* 2009). Studies on antimicrobial properties of plants can be traced back to the works of Osborn in 1943 who reported the presence of antimicrobial

substances in green plants. Since then screening of new plants for drug discoveries continued in great pace. Antibiotics are important weapons to fight bacterial infections and to provide healthy life to people since time immemorial.

Medicinal plants have been used in healthcare systems since time immemorial and their knowledge has been passed from generation to generation (Adedapo *et al*., 2005).There are wide range of bioactive molecules produced by the plants which make them a rich source of medicines (Nair *et al.*, 2005). Several plants containing active constituents such as volatile oils, polyphenols and alkaloids are utilized either as popular folk medicines or in the form of finished products collectively known as phytomedicines (Al-Bakri and Afifi, 2007). It is proved that different parts of the plants like stem, root, flower, barks, leaves, etc. possess antimicrobial property (Muhammad and Muhammad, 2005). Iwu (1993) stated that resistance exhibited by pathogenic microorganisms has led to the screening of several medicinal plants for their potent anti microbial activity.

A number of studies on antimicrobial activity of the plants belonging to Simaroubaceae have been undertaken. Antimicrobial activity of leaf extract of *Ailanthus excelsa* Roxb. were studied by Manikandan *et al.*(2015) using different solvents like chloroform, ethanol, methanol and water against clinical isolates of bacterial and fungal species and was found to have a potent inhibitory effect against pathogenic bacterial and fungal species . Similarly, studies on seed extract of *Brucea javanica* against antifungal susceptibility patterns and growth inhibitory effect against *Candida* species were carried out and found as a potential antifungal agent in oral health products (Nordin *et al.*, 2013). Viswanad *et al.* (2011) studied on antimicrobial activity of *Samadera indica* from leaf extracts and revealed significant activity against *Bacillus subtilis*,

Staphylococcus aeruginosa, *Pseudomonas aeruginosa*, *E.coli*, *Proteus vulgaris* and *Candida albicans* but was found resistant against *Aspergillus niger* and *A. fumigatus.*

2.9. Microorganisms

2.9.1. *Bacillus subtilis*

Numerous works on antimicrobial activity of crude plant extracts against *B. subtilis* have been reported by Viswanad *et al.*(2011); Abhiramasundari *et al*. (2011); Mohammed (2013); Jouda *et al.* (2016); etc. Viswanad *et al*. (2011) carried out a study on antimicrobial activity of methanolic extract of *Samadera indica* against *Bacillus subtilis* in different concentrations of plant extract (250, 500 and 1000 µg/ml) which revealed significant activity against *B. subtilis* with maximum zone inhibition (20.33±0.58 mm) at 1000 µg/ml. Abhiramasundari *et al*. (2011) evaluated antimicrobial activity on *Cocculus hirsutus* from leaf and stem extracts of different solvents and found pronounced antimicrobial activity against *B. subtilis*. Another study was conducted by Mohammed (2013) for antimicrobial activity of pepper, black seed, olive and mustard against *B. subtilis* and found positive effect against the organism. Manikandan *et al*. (2015) studied antimicrobial effect on *Ailanthus excelsa* against *B. subtilis* which showed significant zone of inhibition in different solvent extracts of ethanol, methanol, chloroform and water.

2.9.2. *Staphylococcus aureus*

Number of literatures have been reported which worked on antimicrobial activity of plant extracts against *S. aureus* by Viswanad *et al.*(2011); Jouda *et al.*(2016); Manikandan *et al.* (2015); Abhiramasundari *et al*. (2011); Bhalodia and Shukla (2011);

Nagarajan *et al*. (2009) etc. Viswanad *et al*. (2011) studied antimicrobial activity of methanolic extract of *Samadera indica* against *S. aureus* in different concentrations of plant extract (250, 500 and 1000 µg/ml) and found significant effect of antimicrobial activity with maximum zone inhibition (17.33±0.58 mm) at 1000 µg/ml. Bhalodia and Shukla (2011) investigated antimicrobial potential of leaves of *Cassia fistula* against *S. aureus* from hydroalcoholic extracts and found out that the extracts were active on the organism. An antimicrobial activity of *Ailanthus excels*a was studied against *S. aureus* by Manikandan *et al*. (2015) using different solvents like chloroform, ethanol, methanol and water which found better antimicrobial effect in methanol extracts compared to the other extracts. Jouda *et al*. (2016) studied antimicrobial effect of some medicinal plant extracts against *S. aureus* in aqueous and alcoholic extracts and found that ethanolic extracts showed better antimicrobial effect than methanolic and aqueous extract.

2.9.3. *Pseudomonas aeruginosa*

There are numerous reports on antimicrobial activity of plant extracts against *P. aeruginosa* by Tanti *et al*. (2010); Bhalodia and Shukla (2011); Viswanad *et al*.(2011); Manikandan *et al*.(2015) etc. An assessment of antimicrobial activity on *Dendrocnide sinuata* was carried out by Tanti *et al*. (2010) against *P. aeruginosa* from methanol and hot aqueous extract of leaves which found activity against the organism. Bhalodia and Shukla (2011) found effective against *P. aeruginosa* on antimicrobial study of *Cassia fistula* leaf extracts. Manikandan *et al*. (2015) found antimicrobial effect from leaves and stems of *Ailanthes excelsa* against *P. aeruginosa* in different solvent extracts.

2.9.4. *Serratia marcescens*

Bonjar (2004) worked on antimicrobial activity of 195 plant species out of which only 3 species showed inhibition against *S. marcescens.* Nwankwo *et al.* (2017) evaluated for antimicrobial activity of prodigiosin produce by *S. marcescens* against some pathogenic microorganisms which exhibited marked inhibitory effect against the pathogenic microorganisms.

2.9.5. *Aspergillus fumigatus*

A number of workers have reported about the antimicrobial activity of plant extracts against *A. fumigatus*. Manikandan *et al*. (2015) reported the antifungal activity of *Ailanthus excels* against *A. fumigatus* using ethanol, methanol chloroform and water extract of which water extract showed maximum antifungal activity with 14 mm zone inhibition. Joshi *et al*. (2016) studied antimicrobial activity of *Bryophyllum calycinum* against *A. fumigatus* from leaf and stem in different solvents and found better effect from ethanol solvent of stem extracts.

2.9.6. *Aspergillus niger*

There are numerous reports on antimicrobial activity of plant extracts against *A. niger.* Vats *et al.* (2009) studied antimicrobial activity of *Nyctanthes arbortristis* from stem bark extracts of petroleum ether, chloroform and ethanol against *A. niger* where only chloroform extract showed zone inhibition. Duraipandiyan and Ignacimuthu (2007) studied antifungal activity from flowers of *Cassia fistula* in different solvent extracts against *A. niger* where MIC was found to be more than 1 mg/ml in all the extracts. Viswanad *et al*. (2011) studied antifungal activity from leaves of *Samadera indica* of

methanol extract where *A. niger* was found to be resistant to the extract. Manikandan *et al*. (2015) reported the antifungal activity of *Ailanthus excelsa* against *A. niger* using ethanol, methanol chloroform and water extract of which water extract showed maximum antifungal activity with 16 mm zone inhibition.

2.9.7. Significance of antimicrobial susceptibility test

The antimicrobial susceptibility test is essential in order to determine the susceptibility of the microorganisms against the antimicrobial agents or to determine the resistance of microbial strains used against the antimicrobial compounds of plant extracts. In pharmacology, it is very much essential to identify the nobel compounds of the plant extract used against different microbes (Das *et al.,* 2010). It is useful for anticipating therapeutic outcome, epidemiology and drug discovery (Balouiri *et al.*, 2016). The most commonly used antimicrobial susceptibility methods are the disk diffusion method and broth micro dilution method. Agar disk diffusion method which was developed in 1940 is used in many clinical microbiological laboratories for testing antimicrobial susceptibility. Both these methods are used to determine the minimum inhibitory concentration (MIC) of the antimicrobial agent that completely inhibits the growth of the microorganism in micro dilution wells. It is used to quantitatively measure the *in vitro* antimicrobial activity against the bacterial and fungal organism. MIC is usually expressed in µg/ml or mg/L. There are many guidelines to measure for dilution antimicrobial susceptibility testing and the most recognized standards are provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI).

MATERIALS AND METHODS

3.1. Habit and Distribution

Brucea mollis is a bitter deciduous shrub or small tree growing on gentle hill slopes along the roadsides in forested areas of Karbi Anglong District of Assam situated in between 25º 33' to 26º 35' N latitude and 92º10' to 92º50' E longitude. In Karbi Anglong, *Brucea mollis* is occurring in Lahorijan Reserve Forest and Diphu Recreation Park. The fruits of the species were collected from Diphu Recreation Park of the district. The seeds were germinated in the seedbed and seedlings were planted and maintained in the experimental garden of the Department of Botany, Gauhati University, Assam for the present study (Plate 1).

3.2. Study Material

3.2.1. Explants

Fresh and healthy leaves, nodes and internodes of *Brucea mollis* maintained in the experimental garden of the Department of Botany, Gauhati University were used as explants for micropropagation.

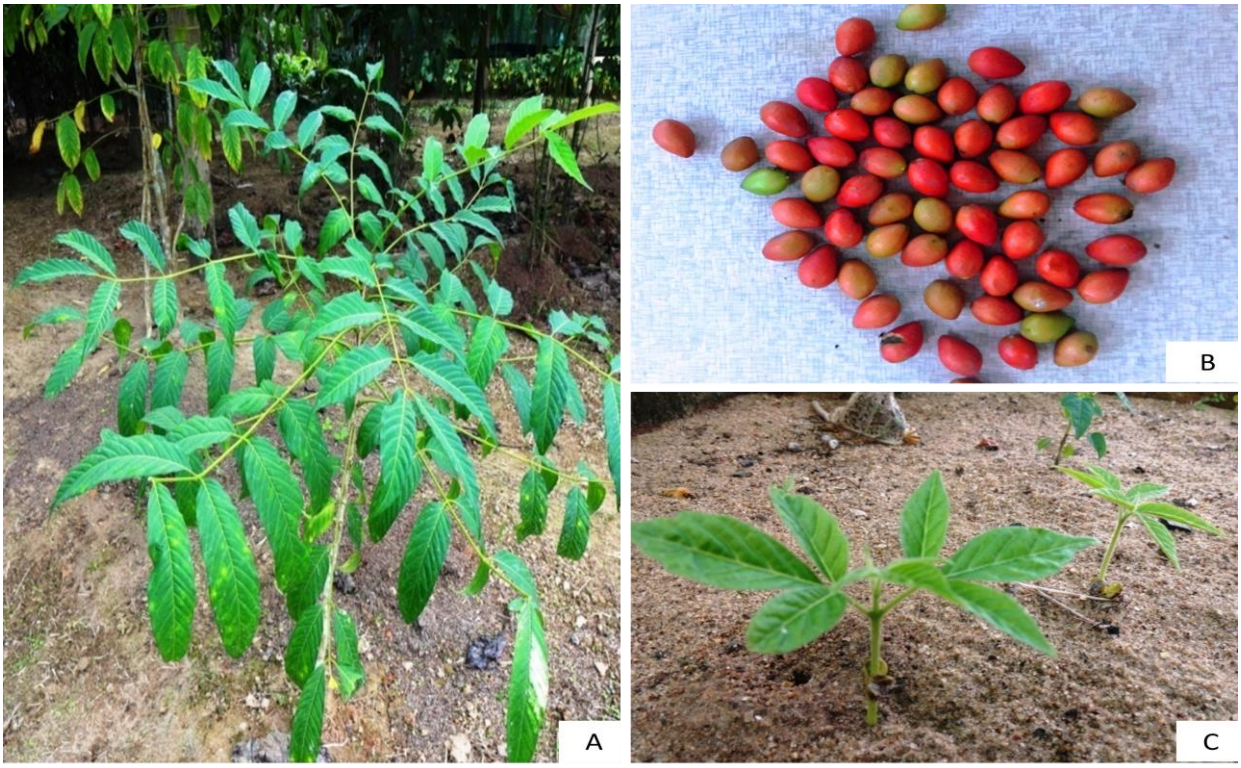

Plate 1: *Brucea mollis* A- Habit of the plant; B- Ripe fruits; C- Seedlings

3.2.2. Microbial strains

For antibacterial activity, two Gram positive strains *viz., Bacillus subtilis* MTCC441, *Staphylococcus aureus* MTCC3160 and two Gram negative strains *viz., Pseudomonas aeruginosa* MTCC424and *Serratia marcescens* MTCC2645 and for antifungal activity *Aspergillus fumigatus* MTCC2550 and *Aspergillus niger* MTCC282 were obtained from Microbial Type Culture Collection (MTCC), IMTECH (India).Bacterial strains were maintained in MHA (Muller Hinton agar medium) and fungal strains were maintained in PDA (Potato Dextrose agar medium) at 4°C for further experiments.

3.2.3. Chemical compounds

Murashige and Skoog's (MS) medium (1962) and Gamborg's B_5 medium (1968) were used to test the response of the selected explants in different combinations and concentrations of growth regulators for micropropagation. The MS media (macronutrients, micronutrients, iron, vitamins), growth regulators, agar and B_5 basal salt mixtures were purchased from Hi media.

For antimicrobial assay, Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) media were also procured from Hi Media.

3.3. METHODS

3.3.1. Preparation of the explants and surface sterilization

The explants *viz.*, leaves, nodes and internodes were collected from the experimental garden of Gauhati University. The collected explants were young and

healthy and about 10-15 cm in length. The fresh leaves and stems (containing both node and internode) were excised from the source plant and proceded for surface sterilization. The surface sterilization method was done following the protocol as suggested by Dudhare *et al.*(2014) with slight modification. The explants were washed under running tap water for 5 min in order to remove the dust particles. They were then treated with 0.1% $HgCl_2$ for 1-10 min and washed with sterile distilled water for 3-4 times under laminar air flow chamber. Finally the explants were soaked in sterilized blotting paper and cut with sterilized blade.

3.3.2. Establishment of cultures

3.3.2.1. Basal media

MS (Murashige and Skoog, 1962) and B_5 (Gamborg *et al.,* 1968) were used as basal media for *in-vitro* cultures of the explants for callus induction, shoot regeneration and root induction.

3.3.2.2. Preparation of stock solution

The stock solution of major salts (200X), minor salts, iron and vitamin (20X) were prepared separately by following the standard protocols for MS and B_5 media. They were prepared by dissolving the required quantities in distilled water and stored in the refrigerator. The growth regulators were prepared for 1mM by dissolving the plant growth regulators in ethanol or 1N HCl/NaOH for 100 ml stock solutions and stored in the refrigerator for long term use.

3.3.2.3. Growth regulators

Auxins and cytokinins were used as growth regulators for callus induction and shoot regeneration in different concentrations and combinations in both MS and B_5 media. For root induction only auxins were used in both MS and B_5 media.

Auxins

NAA (Naphthalene acetic acid), 2, 4 - D (2, 4- dichlorophenoxyacetic acid), IBA (Indole butyric acid) were used for the present study. The stock solutions of NAA and 2, 4 - D were used for 1 mM in 100 ml distilled water. NAA and 2, 4 - D were used for callus induction in different concentrations and combinations with cytokinin. For shoot regeneration, NAA and IBA were used with cytokinin in different concentrations and combinations.

Cytokinins

BAP (Benzyl aminopurine) and Kinetin were used in the present study. The stock solution of BAP and Kinetin were also prepared in 1 mM solution in 100 ml distilled water. BAP was used for callus induction at 2 mg/L in combination with auxins at different concentrations. BAP and Kinetin were used in different concentrations either singly or in combination with auxins for shoot regeneration.

3.3.2.4. Preparation of working media

The working media was prepared by adding stock solution in required quantity and dissolved in distilled water. Sucrose 3% for MS media or 2% for B_5 media and myo inositol 100mg/L were added freshly as required. They were dissolved properly with the help of magnetic stirrer. The plant growth regulators were added in required concentrations and combinations before making up the media to the required volume.

pH was maintained at 5.8 by using 1N NaOH/ 1N HCl. Finally for solidification of media 0.8% w/v agar (Hi Media Lab. India) was added to the media and mixed properly (Table 1).

Table1. Media composition for MS and B_5 media

Constituents	Media (mg/l)	
	MS	B_5
KNO_3	1900	2500
NH_4NO_3	1650	-
$NaH_2PO_4.H_2O$	-	150
KH_2PO_4	170	-
$(NH_4)_2SO_4$	-	134
$MgSO_4.7H_2O$	370	250
$CaCl_2.2H_20$	440	150
KI	0.83	0.75
H_3BO_3	6.20	3.00
$MnSO_4.4H_2O$	22.3	-
$MnSO_4.H_20$	-	10
$ZnSO_4.7H_2O$	8.60	2.00
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_20$	0.025	0.025
$CaCl_2.2H_2O$	440	150
$FeS0_4.7H_2O$	27.80	27.80
Na_2EDTA	37.30	37.30
Myoinositol	100	100
Nicotinic acid	0.50	1.00
Pyridoxine HCl	0.50	1.00
Thiamine HCl	0.10	10
Glycine	2.00	-
Sucrose	30000	20000

3.3.2.5. Sterilization

The glassware, forceps, scalpel, surgical blade and tissue papers were wrapped with aluminium foil, packed nicely in polybags and sterilized in autoclave at 121ºC for 20 min and 15 psi pressure. The media was also sterilized in a conical flask in the same procedure.

The laminar air flow was cleaned properly by swiping with cotton dipped in 70% alcohol before pouring the media in the test tubes or petriplates. UV light was exposed for 20 min to sterilize the interior of the laminar flow cabinet before usage to prevent contamination. The forceps and scalpels were again sterilized by dipping in alcohol and flamed in spirit lamp before handling the explants.

3.3.2.6. Inoculation and incubation

The explants were surface sterilized under laminar air flow chamber before inoculation. The leaf explants were cut in 1 cm^2, node and internode in 0.5-1 cm with sterilized blade and inoculated in the test tubes plugged with non-absorbent cotton. In case of petriplates, they were sealed tightly with parafilm tape.

The cultures were incubated for 16 hrs light, 8 hrs dark period at temperature 25ºC and 3000 lux light intensity with a relative humidity 55-60%.

3.3.2.7. Induction of callus

Callus was induced from leaf, node and internode explants in both MS and B_5 media at different combination and concentrations of BAP (2.0 mg/L), NAA(0.3, 0.5,

1.0and 2.0 mg/L) and 2, 4- D (0.5,1.0,2.0, and 3.0mg/L). The explants were cultured in test tubes (55 ml) and subcultured in 12-15 days interval in the same culture media containing plant growth regulators. The data pertaining to percentage of callus induction, number of days taken for callus induction, texture, color, were recorded after six weeks of culture. After 3-4 repeated subculturing the callus were excised and transferred to different treatments for shoot regeneration. The formula followed for calculating percentage of callus induction was the one suggested by Ray *et al.*(2011).

$$\text{Callus induction}(\%) = \frac{\text{No.of explants induced callus}}{\text{Total no.of explants in the culture}} \times 100$$

3.3.2.8. Shoot regeneration (Shoot initiation)

The callus derived from the explants were cultured separately in both MS and B_5 medium containing BAP (1.0, 2.0, 3.0, 4.0 mg/L) + Kinetin (0.5 mg/L) , BAP (2.0 mg/L) + NAA (0.5 mg/L) + Kinetin (0.5, 1.0, 2.0, 3.0 mg/L) and BAP (2.0 mg/L) + NAA(0.3 mg/L) + IBA(0.5,1.0,2.0 and 3.0mg/L) in 4 different concentrations. The parameters like percentage of shoot regeneration, number of days taken for shoot regeneration, number of shoots regenerated, number of leaves and shoot length(cm)were noted after 30 days. The formula suggested by Ray *et al.* (2011) was used to calculate the percentage of shoot regeneration.

$$\text{Shoot regeneration } \% = \frac{\text{No.of shoots initiated from callus}}{\text{Total no.of callus in the culture}} \times 100$$

3.3.2.9. *In-vitro* rooting

In vitro derived shoots were subjected to rooting in half strength MS and B_5media containing IBA and NAA at 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L concentration respectively. The recorded parameters were percentage of root initiation, number of days taken for root initiation, number of roots regenerated and root length(cm).The formula for root initiation percentage was calculated using the following formula (Sarker *et al.,* 2015):

$$\text{Root initiation\%} = \frac{\text{No.of roots initiated from the shootlets}}{\text{Total no.of shootlets in the culture}} \times 100$$

3.3.2.10. Hardening and acclimatization

The process of acclimatization was done following the method of Shekhawat *et al.* (2015) with slight modification. *Invitro* rooted plantlets were taken out from the culture test tubes and washed with autoclaved distilled water to remove the agar sticked into the roots. The plantlets were then planted in sterile soilrite in plastic cups and covered with transparent plastic bag in order to maintain humidity. Plastic bags were perforated in every 2-3 days in order to lower down the humidity. The plantlets were also sprayed with autoclaved distilled water manually in alternate days by carefully removing the plastic bags. The covered plastic bags were removed after 3 weeks. When the plantlets start appearing new leaves, they were transferred to polybags containing soilrite: garden soil: farmyard manure in the ratio of 1:1:1. They were kept in the culture room for 1 week. Finally, the hardened plantlets were shifted to greenhouse and carefully maintained for 3 weeks and then transferred to outside in open air.

3.3.2.11. Data collection and statistical analysis

The experiments were repeated three times with 5 replicates per treatment. Data were recorded as mean ± standard deviation. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The data pertaining to percentage of callus induction, shoot regeneration, root initiation; number of days taken for callus induction, shoot regeneration, root initiation; number of shoots, number of roots initiation, number of leaves; shoot length and root length were analysed separately by one way ANOVA (Analysis of variance) and the mean values were separated by using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ (Gomez and Gomez, 1976).

3.3.3. Antimicrobial activity

3.3.3.1. Preparation of the plant extract

For antimicrobial activities crude plant extracts were prepared from fresh leaves as well as callus obtained from leaves through micropropagation. The leaf samples were air-dried at room temperature for about one month. The dried samples were then coarsely powdered in a mechanical grinder. On the other hand, callus extracts were prepared following the method suggested by Johnson *et al.* (2011) with slight modification. These were dried at 40-45°C in hot air oven for 7 days till a constant weight was observed. The dried callus thus obtained was powdered in a mechanical grinder.

3.3.3.2. Solvent extraction

The solvent extraction was done using soxhlet method. 10 gm each of coarsely powdered leaf samples and the callus samples were extracted separately in 100 ml of ethanol, methanol and water using soxhlet apparatus for 24 hrs. The extracts were then filtered with Whatmann filter paper. The filtrate was evaporated in hot air oven at 45ºC. The crude extract thus obtained was lyophilized. The dried extract were then stored at 4°C for further use.

3.3.3.3. Preparation of the standard concentration of the plant extract

Stock solutions of the leaf and callus extracts were prepared by dissolving the dried extract in 10% DMSO (Dimethyl sulfoxide) at the concentration of 200 mg/ml (Parekh and Chanda, 2006; Jouda *et al.,* 2016). From the stock solution, different volumes of the extracts were prepared to get the final amount of 20, 10, 5, 2.5 and 1.25 mg/ml concentration. Sterile filter paper discs were loaded with different concentrations of the extracts and allowed to dry at room temperature under aseptic condition.

3.3.3.4. Preparation of the bacterial suspension

The bacterial slants were prepared on Mueller Hinton Agar (MHA) medium in test tubes and stored at 4°C. Active cultures were prepared by transferring a loopful of cultures in Mueller Hinton broth medium incubated at 37°C for 24 hrs.

3.3.3.5. Preparation of the fungal suspension

The fungal cultures were prepared in Potato Dextrose Agar (PDA) media and incubated at 27°Cfor 48 hrs and stored at 4°C. Active cultures were prepared by transferring a loopful of fungi in 100 ml Potato Dextrose broth (PDB) in 250 ml conical flask and incubated at 27°C for 48-72 hrs.

3.3.3.6. Determination of antibacterial activity

The antibacterial activity of plant extracts were screened by disc diffusion method of Kirby-Bauer sensitivity test (Bauer *et al.,* 1966; Murray *et al*., 1995). The MHA plates were spread uniformly with 100 µl of bacterial cultures (10^8 CFU/ml) of all the bacterial strains. They were allowed to dry for 10 min. Then, the discs (0.6 cm) were loaded with 20 µl of 20, 10, 5, 2.5 and 1.25 mg/ml extract respectively. The loaded discs were allowed to remain for diffusion for 30 min at room temperature. Ceftazidime disc (30 µg, Hi Media) was used as positive control. The plates were incubated at 37°C for 24-48 hrs. Zone inhibition formed around the discs were measured in millimeters and recorded. The experiment was repeated twice with three replicas per experiment.

3.3.3.7. Determination of antifungal activity

For study of antifungal activity of plant extracts (leaf and callus extracts of ethanol, methanol and water) against two fungal strains, agar disc diffusion method was used (Bauer *et al.,* 1966; Nordin *et al.,* 2013). Amphotericin B was taken as positive control. The Potato Dextrose Agar plates were inoculated with a loopful of fungal

culture (10^5spore/ml) by cross streaking at right angle over the agar plates. Each disc were loaded with 20 µl of solvent extract containing 20, 10, 5, 2.5 and 1.25 mg/ml respectively, so that the extract can diffuse to the medium. One disc was loaded with Amphotericin B (20 µg/ml stock) which served as positive control. The plates were incubated at 27°C for 24-72 hrs. The zone of inhibition created around the discs were measured and recorded in millimeter.

3.3.3.8. Determination of MIC for antibacterial activity

MIC is defined as the lowest concentration of the compound to inhibit the growth of microorganisms. The MIC was determined in 96 well microtitre plates following the broth microdilution method based on Clinical Laboratory Standard Institute M07-A8 (CLSI, 2009). Two fold serial dilution of the plant extracts (both leaf and callus extracts prepared in ethanol and methanol separately) were prepared. From the stock solution (2.5 mg/ml) of previously studied zone inhibition test, six different dilutions were prepared as 1.25, 0.625, 0.3125, 0.156, 0.078and 0.039 mg/ml respectively. 50 µl of MH broth was dispensed into 96 well plates vertically from WA (first well) to WH (eighth well). Then, 50 µl of 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.039 mg/ml extracts were poured in each well from WA to WF. In the seventh well (WG), Ceftazidime was used in place of the plant extract as positive control and the eighth well (WH) was used as negative control which consists of MH broth and extract. 50 µl of bacterial suspension (1×10^5 CFU/ml) was inoculated in each of the wells (from Well A to Well G) (Plate 12).The microdilution plates were incubated at 37º C for 24 hrs. After

incubation the bacterial growth was observed by taking absorbance at 405 nm (Taye *et al.,* 2011).The presence of turbidity was considered when the difference of OD value (after incubation-before incubation) of the tested extracts was more than the control (broth+extract) (Taye *et al.,* 2011).

3.3.3.9. Determination of MIC for antifungal activity

The MIC for antifungal activity was determined in the same procedure as that of antibacterial activity (Nordin *et al*., 2013). 50 µl of PDB was poured vertically in all the wells from WA to WH. Then, 50 µl of 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.039 mg/ml extracts (both leaf and callus extracts prepared in ethanol and methanol solvent separately) were poured in each well from WA to WF. In the seventh well (WG), Amphotericin B was used as positive control.50 µl of fungal suspension (10^5 spore /ml) was added in all the wells. The eighth well (WH) was taken as negative control which consists of PDB and plant extract (Plate 13).The microtitre plate was incubated at 30ºC for 24hrs. The growth inhibition of the fungus in microdilution wells was observed by taking the absorbance at 405 nm and the presence of turbidity was observed by taking the OD value as described by Taye *et al.,* 2011.

RESULTS

4.1. *In-vitro* propagation of *Brucea mollis*

Leaf, node and internode of *B. mollis* were used as explants for *in-vitro* propagation using different combinations and concentrations of plant growth regulators on two different culture media *viz.*,MS and B_5 through callus induction. For callus induction effect of three different sets of combinations of growth regulators *viz.*, BAP, NAA and 2,4-D at different concentrations were studied. The calluses so obtained were cultured for shoot regeneration at different sets of combinations and concentrations of growth regulators *viz.*, BAP, Kinetin, NAA and IBA. The *in vitro* derived shootlets were transferred to half strength MS and B_5 media at different concentrations of IBA and NAA for rooting. The explants did not exhibit any response to callus induction, shoot regeneration and root induction on media free of growth regulators which were used as control.

4.1.1. Sterilization of explants

Leaf, node and internode explants were surface sterilized at 0.1% $HgCl_2$ for 1-10 min. In leaf explants, there was 100% contamination when treated for 1 min, 2 min and 3 min duration and it was found to be ineffective when treated for 7 min, 8 min, 9 min and 10 min duration because of death of the explants. However, 4 min, 5min and 6 min duration were found to be effective with less contamination. In node and internode

explants, it was found that treatment for 1 min, 2 min, 3 min and 4 min duration was ineffective causing more than 50%contamination while on increasing the time duration to 5, 6 and 7 min duration, contamination gradually reduced. However, the duration of 8, 9 and 10 min treatment with $HgCl_2$ caused death of the explants. Although the treatment of node and internode explants in between 5-7 min duration was found to be suitable with less contamination and death of the explants yet treatment for 5min duration with 0.1% $HgCl_2$ was found to be the best for all the explants *i.e.* leaf, node and internode (as shown in the Table 2). Therefore, further experiments were carried out in 0.1 % $HgCl_2$ for 5 min duration exposure.

Table 2: Effect of sterilant on surface sterilization of leaf, node and internode explants

Sterilant used	Duration (min)	Contamination (%)			Death of the culture (%)		
		Leaf	Node	Internode	Leaf	Node	Internode
$HgCl_2$ 0.1%	1	100.00±0.00	100.00±0.00	100.00±0.00	0	0	0
	2	100.00±0.00	100.00±0.00	100.00±0.00	0	0	0
	3	100.00±0.00	76.66±5.77	73.33±5.77	0	0	0
	4	26.66±5.77	53.33±5.77	53.33±5.77	0	0	0
	5	16.66±5.77	16.66±5.77	20.00±10.00	0	0	0
	6	16.66±5.77	16.66±5.77	16.66±11.54	0	10.00±0.00	0
	7	16.66±5.77	13.33±5.77	13.33±5.77	46.66±5.77	0	0
	8	16.66±5.77	10.00±0.00	10.00±0.00	60.00±0.00	20.00±0.00	50.00±0.00
	9	10.00±0.00	10.00±0.00	0	63.33±5.77	20.00±0.00	63.33±5.77
	10	10.00±0.00	10.00±0.00	0	80.00±0.00	20.00±0.00	63.33±5.77

Values are mean ±SD of 10 replicas per treatment repeated three times per experiment.

4.1.2. Callus induction

4.1.2.1. Effect of BAP, NAA and 2, 4-D on callus induction from leaf explants on MS media

The effect of callus induction on leaf explants were studied in the combination of BAP + NAA, BAP + 2,4-D and 2,4-D singly. Among the concentrations of growth regulators used, both BAP (2 mg/L) with NAA (0.3 and 0.5 mg/L) and BAP (2 mg/L) with 2,4-D (0.5, 1 mg/L) concentrations were found to produce 100% callus induction. Regarding the time taken for callus induction, no significant differences were observed in different concentrations of BAP + NAA combination as it took 14-15 days in all the concentrations. While, in case of BAP + 2,4-D concentrations, the minimum number of days (10.80±0.80) taken for callus induction was shown at BAP (2 mg/L) + 2,4-D (0.5 mg/L) concentration. In 2,4-D alone, the percentage of callus induction decreased with increase in concentration but significantly low as compared to BAP + 2, 4-D and BAP + NAA combinations. Contrary to this, the time taken to initiate callus was less in 2,4-D (0.5 mg/L and 1mg/L) than in BAP + 2,4- D and BAP + NAA combinations (Table 3). The overall growth of the callus was found slower at 2,4-D as compared with other growth regulators used. Profuse growth could be observed only in BAP + NAA combination. The colors of the callus were creamish in BAP + NAA and BAP + 2,4- D but in 2,4-D color appeared as whitish. The textures were friable in all the concentrations and combinations of plant growth regulators (Table 6).

Table 3. Effect of BAP, NAA and 2, 4- D on callus induction from leaf explants on MS media

Concentration of plant growth regulators in MS media(mg/L)			Percentage of callus induction (%)	No. of days taken for callus induction
BAP	NAA	2,4- D		
2	0.3	-	100.00±0.00^{a}	15.53±0.50^{d}
2	0.5	-	100.00±0.00^{a}	15.33±0.30^{d}
2	1	-	80.00±20.00^{b}	14.06±2.15^{d}
2	2	-	73.33±11.54^{bc}	15.20±0.80^{d}
2	-	0.5	100.00±0.00^{a}	10.80±0.80^{bc}
2	-	1	100.00±0.00^{a}	12.00±0.52^{c}
2	-	2	86.66±11.54^{ab}	15.00±0.60^{d}
2	-	3	86.66±11.54^{ab}	15.53±0.41^{d}
-	-	0.5	73.33±11.54^{bc}	7.06±0.61^{a}
-	-	1	60.00±0.00^{cd}	9.73±1.28^{b}
-	-	2	53.33±11.54^{d}	10.66±1.66^{bc}
-	-	3	46.66±11.54^{d}	14.40±1.20^{d}

Values are mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.2.2. Effect of BAP, NAA and 2,4-D on callus induction from nodal explants on MS media

The effect of BAP + NAA, BAP + 2,4-D and only with 2,4-D on callus induction was studied on the nodal explants in the similar way as studied for the leaf explants. In nodal explants, the percentage of callus induction was found to be maximum *i.e.*, 93.33±11.54% at BAP (2 mg/L) + 2,4-D (1 mg/L) concentration. In the combination of BAP + NAA, the percentage of callus induction decreased with increase in concentration and was maximum (60.00±11.54) % at BAP (2 mg/L) + NAA (0.3 mg/L). At 2,4-D concentration, the percentage of callus induction was maximum (86.66±11.54 %) at 1 mg/L. Among all the growth regulator used, the minimum time duration for callus induction 8.73±1.40 days was recorded at 2,4-D (0.5 mg/L) concentration followed by 10.86±0.30 days at 2,4- D (1 mg/L). In general, it took 8-16 days by the nodal explants to initiate callus (Table 4). The color of the callus was light greenish in case of BAP + NAA and BAP +2, 4- D combination, while it was creamish to light brownish in 2,4-D concentrations. The textures were compact in case of all the combinations of growth regulators used in the study (Table 6).

Table 4. Effect of BAP, NAA and 2,4-D on callus induction from nodal explants on MS media

Concentration of plant growth regulators on MS media(mg/L)			Percentage of callus induction (%)	No. of days taken for callus induction
BAP	NAA	2,4- D		
2	0.3	-	60.00±11.54^{cd}	13.06±0.23^{cd}
2	0.5	-	53.33±11.54^{cde}	11.46±0.57^{b}
2	1	-	53.33±11.54^{cde}	12.73±0.70^{c}
2	2	-	46.66±11.54^{de}	13.26±0.94^{cd}
2	-	0.5	73.33±11.54^{abc}	13.53±0.92^{cde}
2	-	1	93.33±11.54^{a}	13.06±0.75^{cd}
2	-	2	53.33±11.54^{cde}	14.80±0.60^{ef}
2	-	3	33.33±11.54^{e}	14.26±0.80^{de}
-	-	0.5	66.66±11.54^{bcd}	8.73±1.40^{a}
-	-	1	86.66±11.54^{ab}	10.86±0.30^{b}
-	-	2	53.33±11.54^{cde}	12.80±0.40^{c}
-	-	3	53.33±11.54^{cde}	15.80±0.52^{f}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.2.3. Effect of BAP, NAA and 2,4-D on callus induction from internodal explants on MS media

The effect of BAP + NAA, BAP + 2, 4-D and only with 2,4-D on callus induction from internode explants was also studied at the same combinations and concentrations as that of leaf and nodal explants on MS media. Among all the plant growth regulators used in the study, the percentage of callus induction in internode explants was maximum of 100.00±0.00 % at BAP (2 mg/L) + NAA (0.3, 0.5 mg/L) as was observed in the leaf explants. In case of BAP + 2,4-D,maximum callus induction *i.e.*, 93.33±11.54 % was recorded at BAP (2 mg/L) + 2,4-D (0.5 mg/L) which subsequently decreased with increase in concentration of 2,4- D. In case of 2,4-D concentration, the maximum of 93.33±11.54% was recorded at 2,4-D (1.0 mg/L) concentration. There was significance in terms of time taken for callus induction both at BAP + 2,4-D and 2,4-D concentrations. The least time duration (10.66±0.46 days) was recorded in the combination of BAP (2 mg/L) + 2,4-D (0.5 mg/L) for callus initiation (Table 5). The color of the callus were light green in the combination of BAP with NAA and BAP with 2,4-D concentrations, while in case of 2,4-D concentrations, the color was creamish. The texture was compact in all the plant growth regulators used (Table 6).

Table 5. Effect of BAP, NAA and 2, 4-D on callus induction from internodal explants on MS media

Concentration of plant growth regulators in MS media(mg/L)			Percentage of callus induction (%)	No. of days taken for callus induction
BAP	NAA	2,4- D		
2	0.3	-	100.00 ± 0.00^{a}	12.93 ± 1.36^{d}
2	0.5	-	100.00 ± 0.00^{a}	11.06 ± 0.64^{ab}
2	1	-	80.00 ± 20.00^{abc}	12.06 ± 0.83^{bcd}
2	2	-	86.66 ± 11.54^{abc}	11.73 ± 0.75^{abcd}
2	-	0.5	93.33 ± 11.54^{ab}	10.66 ± 0.46^{a}
2	-	1	73.33 ± 11.54^{bcd}	11.13 ± 0.41^{ab}
2	-	2	73.33 ± 11.54^{bcd}	12.73 ± 0.46^{d}
2	-	3	53.33 ± 11.54^{d}	12.46 ± 0.50^{cd}
-	-	0.5	73.33 ± 11.54^{bcd}	14.60 ± 0.40^{e}
-	-	1	93.33 ± 11.54^{ab}	11.40 ± 0.52^{abc}
-	-	2	66.66 ± 11.54^{cd}	12.40 ± 0.40^{cd}
-	-	3	66.66 ± 11.54^{cd}	16.86 ± 0.70^{f}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p\leq0.05$ according to Duncan's multiple range test (DMRT).

Table 6: Effect of BAP, NAA and 2, 4-D on color and texture of callus on MS media

Plant growth regulator (MS media)			Texture			Color		
BAP	NAA	2,4- D	Leaf	Node	Internode	Leaf	Node	Internode
2	0.3	-	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	0.5	-	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	1	-	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	2	-	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	-	0.5	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	-	1	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	-	2	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
2	-	3	Friable	Compact	Compact	Creamish	Light greenish	Light greenish
-	-	0.5	Friable	Compact	Compact	Whitish	Light brownish	Light brownish
-	-	1	Friable	Compact	Compact	Whitish	Creamish	Creamish
-	-	2	Friable	Compact	Compact	Whitish	Creamish	Creamish
-	-	3	Friable	Compact	Compact	Whitish	Creamish	Creamish

4.1.2.4. Effect of BAP, NAA and 2,4-D on callus induction from leaf explants on B_5 media

The effect of BAP + NAA, BAP + 2,4-D and only with 2,4-D on callus induction from leaf explants were also studied in the similar way on B_5 media. The explants exhibited positive response on callus induction in all the concentrations of growth regulators used. There was no significant difference in case of percentage of callus induction in BAP + NAA concentrations. Among all the growth regulators used, the highest percentage (93.33±11.54%) of callus induction was recorded in BAP (2 mg/L) + 2,4-D (0.5 mg/L). In case of BAP + NAA concentrations, the maximum percentage (86.66±11.54%) of callus induction was recorded at BAP (2 mg/L) + NAA (0.3, 0.5 mg/L). Similarly, in case of 2,4-D concentrations, the maximum (86.66±11.54%) percentage of callus induction was recorded at 0.5 mg/L concentration which subsequently decreased with increase in concentration. In general, it took 7-14 days to initiate callus in the leaf explants on B_5 media with different combinations and concentrations of growth regulators (Table 7). The least time *i.e.,* 7.66±1.20 days were required at 2,4-D (0.5 mg/L). There were significances in time taken for callus induction in all the sets of growth regulators used in the study. The color of the callus was creamish and texture friable in all the combinations and concentrations of growth regulators used in the study (Table 10).

Table 7. Effect of BAP, NAA and 2,4-D on callus induction from leaf explants on B_5 media

Explant	Concentration of plant growth regulators on B_5 media(mg/L)			Percentage of callus induction (%)	No. of days taken for callus induction
	BAP	NAA	2,4 -D		
Leaf	2	0.3	-	86.66 ± 11.54^{ab}	10.80 ± 0.91^{c}
	2	0.5	-	86.66 ± 11.54^{ab}	10.66 ± 1.81^{c}
	2	1	-	83.33 ± 15.27^{ab}	8.53 ± 0.94^{ab}
	2	2	-	73.33 ± 11.54^{ab}	8.06 ± 0.41^{a}
	2	-	0.5	93.33 ± 11.54^{a}	10.13 ± 0.30^{bc}
	2	-	1	86.66 ± 11.54^{ab}	11.20 ± 0.34^{c}
	2	-	2	66.66 ± 11.54^{bc}	13.93 ± 0.46^{d}
	2	-	3	66.66 ± 11.54^{bc}	14.73 ± 1.33^{d}
	-	-	0.5	86.66 ± 11.54^{ab}	7.66 ± 1.20^{a}
	-	-	1	73.33 ± 11.54^{ab}	10.26 ± 1.41^{bc}
	-	-	2	66.66 ± 11.54^{bc}	13.53 ± 1.30^{d}
	-	-	3	46.66 ± 11.54^{c}	14.73 ± 1.36^{d}

Values are mean ± standard deviation of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p\leq0.05$ according to Duncan's multiple range test (DMRT).

4.1.2.5. Effect of BAP, NAA and 2, 4-D on callus induction from nodal explants on B_5 media

Similar studies on the effect of BAP + NAA, BAP + 2, 4-D and only with 2,4-D of nodal explants on B_5 media were carried out (Table 8). Among all the growth regulators used in the study, the percentage of callus induction was maximum of 86.66±11.54% at BAP (2 mg/L) + NAA (0.5 mg/L) and BAP (2 mg/L) + 2,4-D (0.5 mg/L). The time required for callus induction was minimum (7.16±0.57 days) at BAP (2 mg/L) + NAA (2 mg/L) followed by 8.06±0.30 days at BAP (2 mg/L) + NAA (1 mg/L). In case of BAP + 2,4-D concentration, the minimum 8.73±0.75 days was required to initiate callus at BAP (2 mg/L) + 2,4-D (2 mg/L).While at 2,4-D concentrations, the percentage of callus induction was maximum (80.00±20.00%) at 2,4-D (1 mg/L) concentration but the minimum days required to initiate callus was 8.86±0.90 days at 2,4-D (0.5 mg/L). The color and texture of the callus were light yellowish and compact in case of BAP + NAA and BAP + 2,4-D concentrations whereas in 2,4-D concentrations, the color were light brownish to creamish and compact texture (Table 10).

Table 8. Effect of BAP, NAA and 2,4-D on callus induction from nodal explants on B_5 media

Concentration of plant growth regulators on B_5 media(mg/L)			Percentage of callus induction (%)	No. of days taken for callus induction
BAP	NAA	2,4-D		
2	0.3	-	80.00±20.00^{a}	11.80±1.24^{d}
2	0.5	-	86.66±11.54^{a}	10.06±1.00^{c}
2	1	-	80.00±20.00^{a}	8.06±0.30^{b}
2	2	-	80.00±20.00^{a}	7.16±0.57^{a}
2	-	0.5	86.66±11.54^{a}	11.66±0.83^{d}
2	-	1	80.00±20.00^{a}	11.80±1.00^{d}
2	-	2	66.66±11.54^{ab}	8.73±0.75^{bc}
2	-	3	66.66±11.54^{ab}	9.33±0.94^{bc}
-	-	0.5	73.33±11.54^{ab}	8.86±0.90^{bc}
-	-	1	80.00±20.00^{a}	13.26±0.70^{e}
-	-	2	66.66±11.54^{ab}	14.33±0.46^{e}
-	-	3	46.66±11.54^{b}	14.53±0.70^{e}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.2.6. Effect of BAP, NAA and 2,4-D on callus induction from internodal explants on B_5 media

The effect of BAP+NAA, BAP + 2,4-D and only with 2,4-D were studied in the similar way in the internodal explants as studied on MS media (Table 9). In BAP + NAA concentrations, the percentage of callus induction was found to be significantly higher (93.33±11.54%) at BAP (2 mg/L) + NAA (0.3 mg/L) which decreased with increase in concentration of NAA. But the minimum time required was 8.33±0.57 days for callus induction at BAP (2 mg/L) + NAA (2 mg/L) followed by 8.40±0.52 days at BAP (2 mg/L) + 2,4-D (3 mg/L) concentration. In BAP + 2,4-D, the highest percentage of 80.00±20.00% callus induction was recorded at BAP (2 mg/L) + 2,4-D (0.5 mg/L) but the time required for callus induction was 8.40±0.52days at BAP (2 mg/L) + 2,4-D (3 mg/L). While in case of 2,4-D concentrations, the percentage of callus induction was found to be maximum 73.33±11.54% at 0.5 mg/L which also required minimum *i.e.* 12.66±1.10 days for callus induction. The color and texture of callus varied according to plant growth regulators used which were light yellowish to light greenish in BAP + NAA, light greenish in BAP + 2,4-D and light brownish to creamish in color in 2,4-D (Table 10).

Table 9. Effect of BAP, NAA and 2,4-D on callus induction from internodal explants on B_5 media

Explant	Plant growth regulators on B_5 media(mg/L)			Percentage of callus induction (%)	No. of days taken for callus induction
	BAP	NAA	2,4- D		
Internode	2	0.3	-	93.33±11.54^{a}	12.00±0.87^{cde}
	2	0.5	-	86.66±11.54^{ab}	10.66±0.46^{b}
	2	1	-	73.33±11.54^{abc}	10.06±0.57^{b}
	2	2	-	73.33±11.54^{abc}	8.33±0.57^{a}
	2	-	0.5	80.00±20.00^{ab}	11.93±0.23^{cd}
	2	-	1	73.33±11.54^{a}	11.20±0.34^{bc}
	2	-	2	66.66±11.54^{bcd}	10.26±0.30^{b}
	2	-	3	46.66±11.54^{d}	8.40±0.52^{a}
	-	-	0.5	73.33±11.54^{abc}	12.66±1.10^{de}
	-	-	1	66.66±11.54^{bcd}	13.26±0.57^{e}
	-	-	2	53.33±11.54^{cd}	13.20±1.05^{de}
	-	-	3	46.66±11.54^{d}	15.26±1.10^{f}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

Table 10: Effect of BAP, NAA and 2,4-D on color and texture of callus on B_5 media

Concentration of plant growth regulators in B5 media (mg/L)			Texture			Color		
BAP	NAA	2,4- D	Leaf	Node	Internode	Leaf	Node	Internode
2	0.3	-	Friable	Compact	Compact	Creamish	Light yellowish	Light yellowish
2	0.5	-	Friable	Compact	Compact	Creamish	Light yellowish	Light yellowish
2	1	-	Friable	Compact	Compact	Creamish	Light yellowish	Light yellowish
2	2	-	Friable	Compact	Compact	Creamish	Light yellowish	Light greenish
2	-	0.5	Friable	Compact	Compact	Creamish	Light yellowish	Light greenish
2	-	1	Friable	Compact	Compact	Creamish	Light yellowish	Light greenish
2	-	2	Friable	Compact	Compact	Creamish	Light yellowish	Light greenish
2	-	3	Friable	Compact	Compact	Creamish	Light yellowish	Light greenish
-	-	0.5	Friable	Compact	Compact	Creamish	Creamish	Creamish
-	-	1	Friable	Compact	Compact	Creamish	Creamish	Creamish
-	-	2	Friable	Compact	Compact	Creamish	Creamish	Creamish
-	-	3	Friable	Compact	Compact	Creamish	Creamish	Creamish

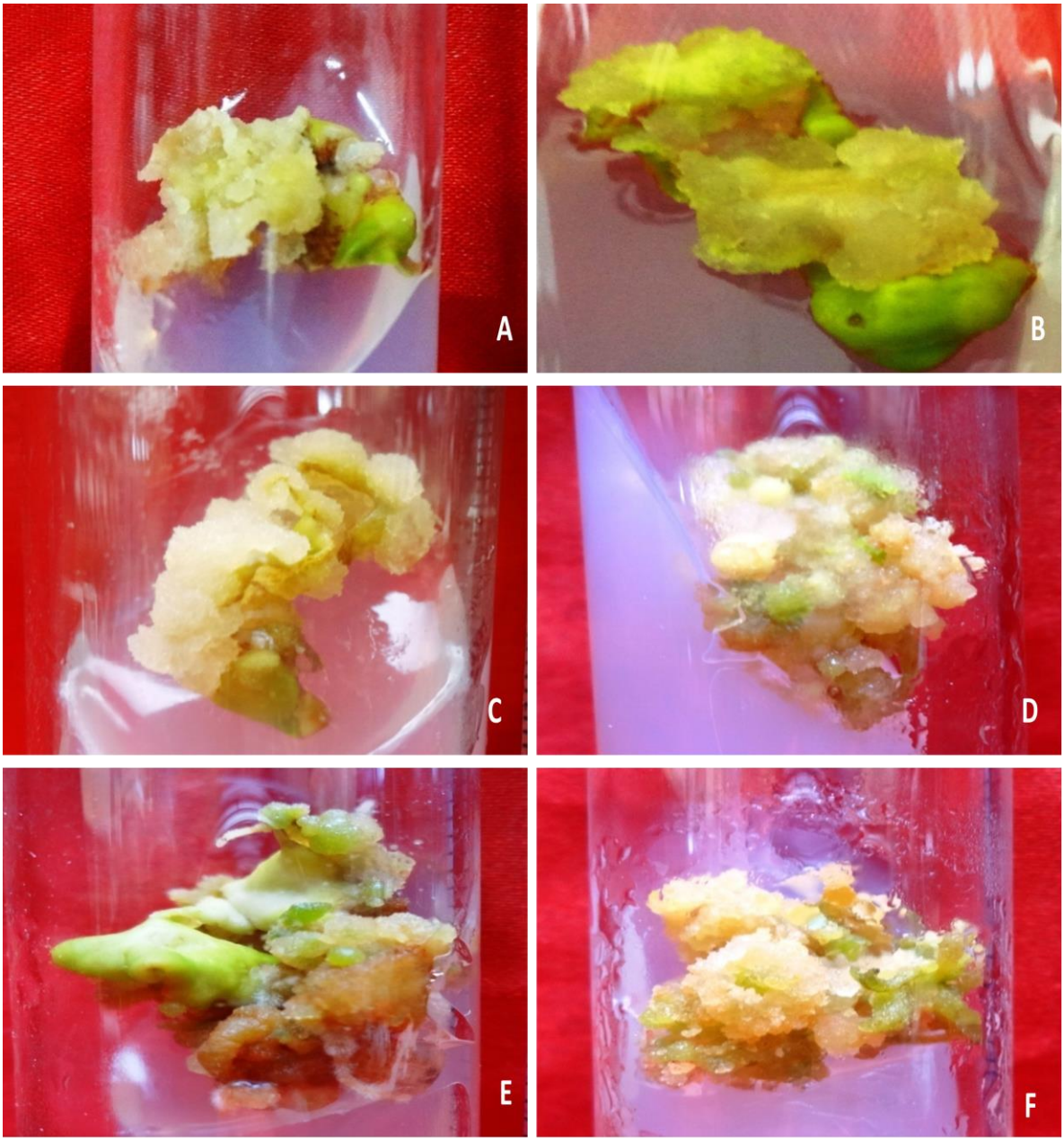

Plate 2: Callus obtained from the leaf explants of *B. mollis* (**A**)**:** Friable callus at BAP (2 mg/L) + NAA (0.5 mg/L) on MS media; (**B**)**:** Friable callus at BAP (2 mg/L) + 2,4-D (0.5 mg/L) on MS media; **(C):** Friable and cream color callus at 2,4-D (1 mg/L) on MS media; **(D):** Callus at BAP (2mg/L) + NAA (1 mg/L) on B_5 media; **(E):** Callus at BAP (2 mg/L) + 2,4-D (2 mg/L) on B_5 media; **(F):** Callus changing its color after 30 days on B_5 media.

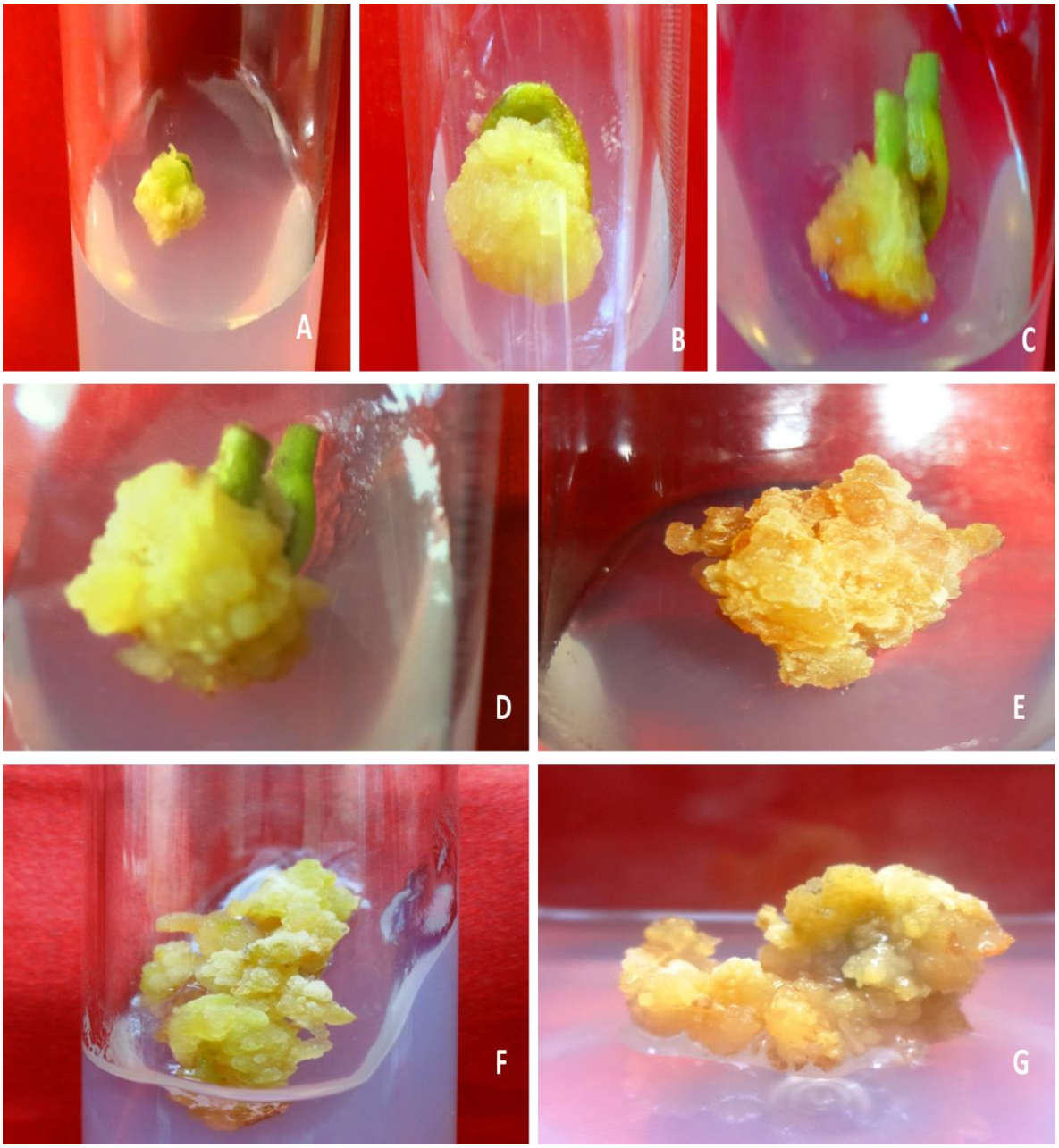

Plate 3: Callus obtained from the nodal explants of *B. mollis* **(A):** Compact callus at BAP (2 mg/L) + NAA (0.3 mg/L) concentration on MS media; **(B):** Compact callus at 2,4-D (1 mg/L) concentration; **(C):** Callus initiation from the cut end portion of the nodal explants on B_5 media; **(D):** Callus growing over the entire explants; **(E):** Callus turning brown in color after 40 days at 2,4-D; **(F):** Callus changing its color after subculture for regeneration on MS media; **(G):** Callus in 60 days.

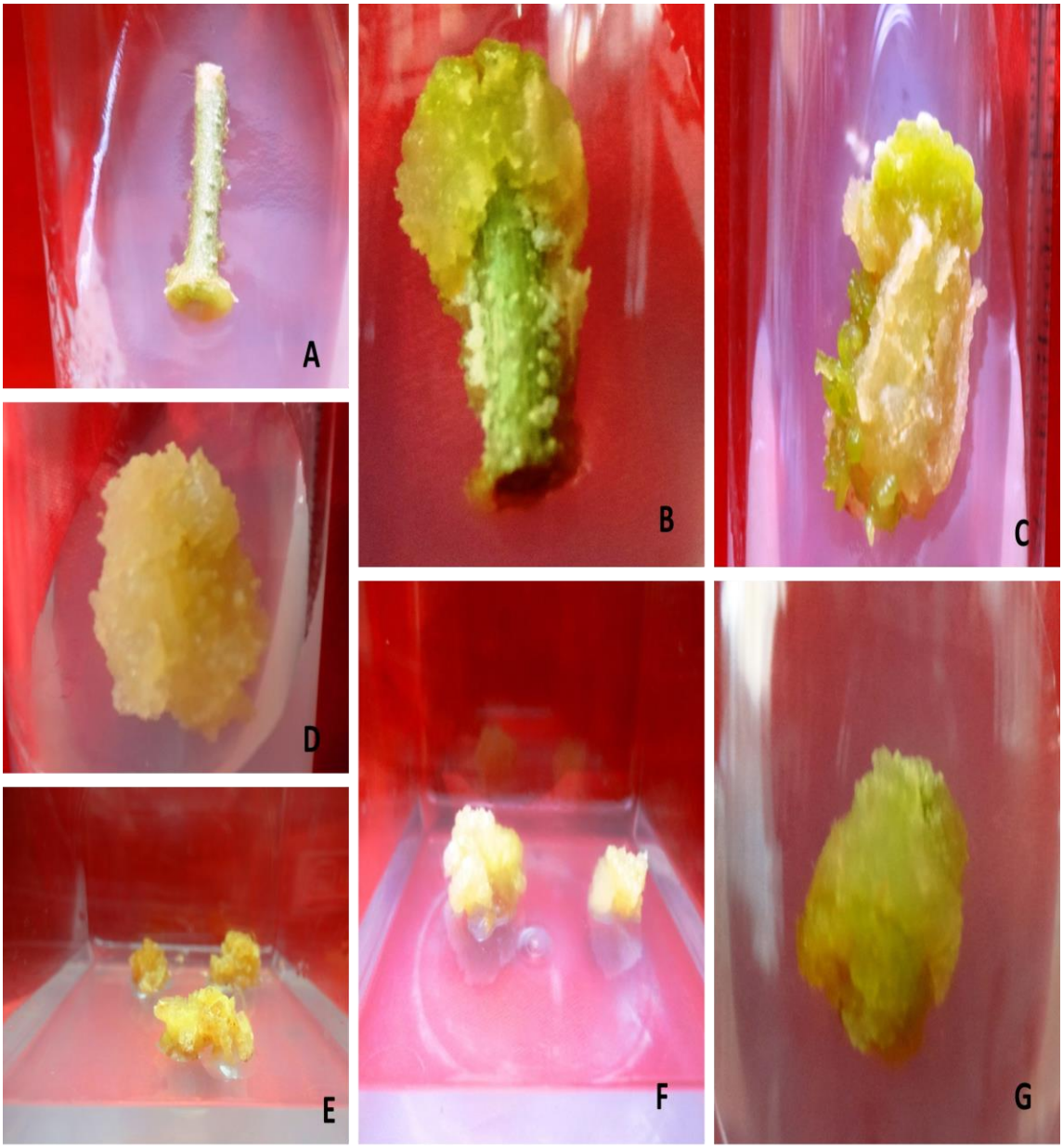

Plate 4: Callus obtained from the internodal explants of *B. mollis* **(A):** Callus initiation from the cut end portion of the explants ; **(B)**: light greenish color callus at BAP (2 mg/L) + NAA (0.5 mg/L); **(C):**Callus growing all over the entire explants; **(D)** Cream color callus at 2,4-D (0.5 mg/L) on MS media; **(E):** Compact callus at 2,4- D 1 mg/L concentration; **(F) :**Compact and cream color callus at 2,4-D **(**2 mg/L)on B_5 media; **(G)**: Light greenish callus at BAP (2 mg/L) + 2,4-D (3 mg/L) on MS media.

4.1.3. Shoot proliferation through indirect regeneration

Callus from leaf, node and internode were subcultured for shoot regeneration on both MS and B_5 media supplemented with BAP, Kinetin, NAA and IBA in different sets of combinations and concentrations. The callus when subculture for regeneration in BAP + Kinetin combination, its color and texture changed into green and hard compact callus. Shoots proliferated from callus directly without embryo formation. Similarly, in case of BAP + NAA + IBA and BAP + NAA + Kinetin combinations, the color and texture of callus changed into green and soft friable callus, when shoots proliferated. The data pertaining to percentage of shoot regeneration, number of days taken to initiate shoots, shoot number, number of leaves and length of shoots were recorded.

4.1.3.1. Effect of growth regulators on shoot proliferation from leaf callus on MS media

Effects of BAP, NAA, IBA and Kinetin on shoot regeneration on MS media were studied on calli obtained from leaf explants in three different sets of combinations *viz.*, BAP + NAA + Kinetin, BAP + Kinetin and BAP + NAA + IBA. The calli showed regeneration of shoots after 2-3 subcultures. In case of BAP + Kinetin and BAP + NAA + IBA, 100% shoot regeneration was recorded from the calli of leaf explants on MS medium and there were no significant differences in percentage of shoots regeneration among the different concentrations. At BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L) concentration the number of shoots regenerated was maximum (7.86±0.50) and the time required was also minimum (17.80±1.03 days) (Table 11). However, no significant difference was recorded in leaf number in all the concentrations of plant growth regulators used. As compared to other combinations, maximum shoot length (4.69±0.27 cm) was recorded at combination of BAP (4 mg/L) + Kinetin (0.5 mg/L).

Table 11. Effect of BAP, NAA, Kinetin and IBA on shoot proliferation from leaf callus on MS media.

Concentration of plant growth regulators in MS media(mg/L)				Percentage of shoot regeneration (%)	No. of days taken for shoot regeneration	No. of shoots regenerated	No. of leaves	Shoot length (cm)
BAP	NAA	Kinetin	IBA					
2	0.5	0.5	-	100.00±0.00^{a}	23.46±1.20^{cd}	4.86±0.83^{bcd}	2.86±1.02^{a}	2.00±0.72^{cd}
2	0.5	1	-	86.66±11.54^{a}	22.13±0.64^{bc}	4.40±1.21^{bcd}	2.53±1.10^{a}	1.52±0.17^{d}
2	0.5	2	-	73.33±11.54^{a}	20.53±1.28^{b}	4.60±0.52^{bcd}	3.00±1.00^{a}	1.91±0.95^{cd}
2	0.5	3	-	73.33±11.54^{a}	17.80±1.03^{a}	7.86±0.50^{a}	3.33±1.10^{a}	2.08±1.00^{cd}
1	-	0.5	-	100.00±0.00^{a}	34.73±0.98^{f}	3.86±0.30^{d}	3.06±0.50^{a}	1.91±0.21^{cd}
2	-	0.5	-	100.00±0.00^{a}	35.80±0.87^{f}	4.53±0.50^{bcd}	3.53±0.30^{a}	2.58±0.16^{bc}
3	-	0.5	-	100.00±0.00^{a}	36.33±1.20^{fg}	4.66±0.61^{bcd}	3.73±0.11^{a}	3.12±0.23^{b}
4	-	0.5	-	100.00±0.00^{a}	37.46±1.10^{g}	5.46±1.41^{b}	3.46±0.41^{a}	4.69±0.27^{a}
2	0.3	-	0.5	100.00±0.00^{a}	21.46±0.41^{b}	5.26±0.41^{bc}	3.33±0.30^{a}	1.95±0.11^{cd}
2	0.3	-	1	100.00±0.00^{a}	23.86±0.11^{d}	4.40±0.60^{bcd}	3.46±0.30^{a}	1.64±0.04^{d}
2	0.3	-	2	100.00±0.00^{a}	25.80±0.40^{e}	3.93±0.23^{cd}	2.73±0.41^{a}	1.80±0.17^{cd}
2	0.3	-	3	100.00±0.00^{a}	27.00±0.87^{e}	4.33±0.30^{bcd}	3.33±0.30^{a}	2.03±0.11^{cd}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.3.2. Effect of growth regulators on shoot proliferation from nodal callus on MS media

Effects of BAP, NAA, IBA and Kinetin on shoot regeneration were also studied on calli obtained from nodal explants in three different sets of combinations (Table 12). Similar to the calli obtained from leaf explants, there was 100% shoot regeneration from calli obtained from nodal explants at all combinations of BAP + Kinetin and BAP + NAA + IBA concentrations. Among all the combinations and concentrations of plant growth regulators used in the study, the minimum time duration *i.e.*, 23.26±0.98 days was exhibited by the nodal callus to regenerate shoots at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (0.5 mg/L). The maximum number of shoots (5.53±0.30) initiated at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (0.5 mg/L). There were no such significant differences exhibited in number of leaves developed at different concentrations of BAP + NAA + Kinetin and BAP + NAA + IBA combinations. The maximum number of leaves (6.33±2.92) was recorded at BAP (2 mg/L) + Kinetin (0.5 mg/L) and maximum shoot length (3.48±0.81 cm) at BAP (4 mg/L) + Kinetin (0.5 mg/L).

Table12. Effect of BAP, NAA, Kinetin and IBA on shoot proliferation from nodal callus on MS media

Concentration of plant growth regulators in MS media(mg/L)				Percentage of shoot regeneration (%)	No. of days taken for shoot regeneration	No of shoots regenerated	No of leaves	Shoot length (cm)
BAP	NAA	Kinetin	IBA					
2	0.5	0.5	-	66.66±11.54^{c}	24.00±1.00^{ab}	4.06±0.23^{c}	2.53±0.23^{b}	1.40±0.04^{ef}
2	0.5	1	-	73.33±11.54^{c}	23.46±0.23^{ab}	4.53±0.11^{bc}	3.60±0.20^{b}	1.71±0.01^{de}
2	0.5	2	-	86.66±11.54^{b}	24.06±0.23^{ab}	4.60±0.34^{bc}	3.06±0.23^{b}	1.94±0.12^{cd}
2	0.5	3	-	93.33±11.54^{ab}	25.46±0.23^{cd}	5.00±0.60^{ab}	3.20±0.20^{b}	2.23±0.14^{c}
1	-	0.5	-	100.00±0.00^{a}	35.00±0.20^{g}	2.93±0.30^{e}	4.13±2.50^{b}	1.02±0.08^{f}
2	-	0.5	-	100.00±0.00^{a}	34.26±0.90^{g}	3.40±0.20^{de}	6.33±2.92^{a}	1.23±0.14^{f}
3	-	0.5	-	100.00±0.00^{a}	32.73±0.90^{f}	4.00±0.34^{cd}	4.53±0.23^{ab}	2.70±0.26^{b}
4	-	0.5	-	100.00±0.00^{a}	29.13±0.70^{e}	5.06±0.30^{ab}	3.86±0.23^{b}	3.48±0.81^{a}
2	0.3	-	0.5	100.00±0.00^{a}	23.26±0.98^{a}	5.53±0.30^{a}	3.53±0.11^{b}	2.19±0.06^{cd}
2	0.3	-	1	100.00±0.00^{a}	25.40±0.72^{cd}	4.86±0.23^{ab}	3.33±0.50^{b}	2.28±0.04^{bc}
2	0.3	-	2	100.00±0.00^{a}	24.66±0.61^{bc}	4.86±0.41^{ab}	3.13±0.11^{b}	2.15±0.05^{cd}
2	0.3	-	3	100.00±0.00^{a}	26.26±0.57^{d}	4.66±0.57^{bc}	3.13±0.30^{b}	1.84±0.11^{cde}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.3.3. Effect of growth regulators on shoot proliferation from internodal callus on MS media

Effects of BAP, NAA, IBA and Kinetin on shoot regeneration from calli developed from internode explants in three different sets of combinations exhibited similar results as those of calli obtained from leaf and nodal explants in terms of percentage of shoot regeneration (Table 13).There were no significant differences recorded in percentage of shoot regeneration in different concentrations of BAP + Kinetin and BAP + NAA + IBA combinations. Of all the concentrations of BAP + NAA + Kinetin combination, the percentage of shoot regeneration was maximum with 93.33±11.54% at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L) but the minimum time required for shoot regeneration *i.e.,* 21.20±0.23 days was recorded at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L).The maximum number of shoots (7.60±0.40 and 7.53±0.75) was initiated at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L) and BAP (4 mg/L) + Kinetin (0.5 mg/L) respectively. Similarly, maximum number of leaves (6.40±1.40) and maximum length of shoot (6.36±0.80 cm) were recorded at BAP (4 mg/L) + Kinetin (0.5 mg/L).

Table13. Effect of BAP, NAA, Kinetin and IBA on shoot proliferation from internodal callus on MS media

Concentration of plant growth regulators in MS media (mg/L)				Percentage of shoot regeneration (%)	No. of days taken for shoot regeneration	No of shoots regenerated	No of leaves	Shoot length (cm)
BAP	NAA	Kinetin	IBA					
2	0.5	0.5	-	66.66±11.54^{c}	21.46±0.23^{a}	4.86±0.83^{bc}	3.00±0.52^{d}	1.70±0.29^{c}
2	0.5	1	-	73.33±11.54^{bc}	23.40±0.69^{b}	4.80±1.40^{bc}	3.26±0.30^{d}	1.95±0.07^{c}
2	0.5	2	-	80.00±0.00^{b}	22.73±0.46^{b}	5.06±0.92^{bc}	3.73±0.30^{d}	1.98±0.15^{c}
2	0.5	3	-	93.33±11.54^{a}	23.80±0.34^{b}	5.86±0.23^{b}	3.86±0.11^{d}	2.06±0.24^{c}
1	-	0.5	-	100.00±0.00^{a}	34.60±0.60^{f}	3.86±0.50^{c}	4.00±0.00^{cd}	2.55±0.26^{c}
2	-	0.5	-	100.00±0.00^{a}	33.93±1.00^{ef}	5.46±0.30^{b}	4.93±0.75^{bc}	3.98±1.08^{b}
3	-	0.5	-	100.00±0.00^{a}	33.40±0.52^{e}	5.26±0.30^{b}	5.13±0.41^{b}	4.38±0.88^{b}
4	-	0.5	-	100.00±0.00^{a}	29.60±0.34^{d}	7.53±0.75^{a}	6.40±1.40^{a}	6.36±0.80^{a}
2	0.3	-	0.5	100.00±0.00^{a}	23.00±0.52^{b}	5.73±0.90^{b}	3.20±0.40^{d}	2.02±0.17^{c}
2	0.3	-	1	100.00±0.00^{a}	21.20±0.23^{a}	7.60±0.40^{a}	3.33±0.30^{d}	1.98±0.07^{c}
2	0.3	-	2	100.00±0.00^{a}	23.80±0.34^{b}	5.53±0.23^{b}	3.13±0.50^{d}	1.74±0.11^{c}
2	0.3	-	3	100.00±0.00^{a}	27.00±0.87^{c}	5.13±0.30^{bc}	3.13±0.70^{d}	1.70±0.12^{c}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.3.4. Effect of growth regulators on shoot proliferation from leaf callus on B_5 media

Effects of BAP, NAA, IBA and Kinetin on shoot regeneration on B_5 media were also studied on calli obtained from leaf explants in three different sets of combinations of BAP + NAA + Kinetin, BAP + Kinetin and BAP + NAA + IBA (Table 14). The percentage of shoot regeneration was recorded to be maximum (93.33±11.54%) both at BAP (4mg/L) + Kinetin (0.5 mg/L) and BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L) while the percentage of shoot regeneration was found to be decreased with increase in concentration of Kinetin in BAP + NAA + Kinetin and IBA in BAP + NAA + IBA respectively. In BAP + Kinetin, the percentage of shoot regeneration increased with increase in concentration of BAP. It was recorded that the minimum time (24 days) required for shoot regeneration was at BAP (2mg/L) + NAA (0.3 mg/L) + IBA (0.5 mg/L) and BAP (2mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L).The numbers of leaves in the regenerated shoots were in between 2-5 and the maximum shoots *i.e.*, 5.53±1.10 initiated at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (0.5 mg/L). There is no significant difference in number of leaves developed at BAP + NAA + Kinetin and BAP + Kinetin combinations. The length of shoot was in between 1-2 cm and recorded to be maximum (2.32±0.68 cm) at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L).

Table14. Effect of BAP, NAA, Kinetin and IBA on shoot proliferation from leaf callus on B_5 media.

Concentration of plant growth regulators in B_5 media(mg/L)				Percentage of shoot regeneration (%)	No. of days taken for shoot regeneration	No of shoots regenerated	No of leaves	Shoot length (cm)
BAP	NAA	Kinetin	IBA					
2	0.5	0.5	-	80.00±20.00^{ab}	33.20±1.05^{f}	2.13±0.90^{c}	2.40±1.00^{b}	1.46±0.22^{ab}
2	0.5	1	-	60.00±20.00^{b}	27.53±1.41^{bcd}	3.86±1.00^{abc}	3.00±1.00^{b}	1.71±0.15^{ab}
2	0.5	2	-	60.00±0.00^{b}	25.13±1.10^{ab}	3.26±1.02^{c}	2.66±0.64^{b}	1.88±0.43^{ab}
2	0.5	3	-	60.00±0.00^{b}	24.80±1.05^{ab}	5.53±1.10^{a}	3.13±1.00^{b}	2.32±0.68^{a}
1	-	0.5	-	73.33±11.54^{ab}	31.73±1.10^{ef}	2.40±1.00^{c}	3.06±0.64^{b}	2.10±2.07^{ab}
2	-	0.5	-	66.66±11.54^{ab}	29.00±0.91^{cd}	3.33±1.10^{bc}	3.60±1.00^{b}	0.93±0.18^{c}
3	-	0.5	-	80.00±20.00^{ab}	27.20±1.00^{bcd}	3.80±1.63^{abc}	3.73±1.10^{b}	1.18±0.19^{ab}
4	-	0.5	-	93.33±11.54^{a}	26.80±1.24^{abcd}	4.66±0.92^{ab}	3.73±0.57^{b}	1.61±0.21^{ab}
2	0.3	-	0.5	86.66±11.54^{ab}	24.06±1.44^{a}	2.93±1.02^{bc}	3.66±0.57^{b}	0.80±0.22^{c}
2	0.3	-	1	93.33±11.54^{a}	26.26±1.70^{abc}	3.66±0.57^{abc}	5.33±0.57^{a}	1.02±0.10^{ab}
2	0.3	-	2	73.33±11.54^{ab}	27.26±2.30^{bcd}	3.53±0.98^{abc}	2.93±1.10^{b}	1.25±0.44^{ab}
2	0.3	-	3	66.66±11.54^{ab}	29.40±2.49^{de}	2.26±1.17^{c}	3.06±0.11^{b}	0.81±0.17^{c}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.3.5. Effect of growth regulators on shoot proliferation from nodal callus on B_5 media

The callus obtained from the nodal explants was similarly studied for shoot regeneration at BAP, NAA, IBA and Kinetin in three different sets of combinations. The percentage of shoot regeneration was maximum *i.e.,* 100% at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (0.5 mg/L) which decreased with increasing concentration of Kinetin (Table 15). In all the combinations and concentrations the time required for shoot regeneration was recorded to be in between 24-33 days. However, minimum time for shoot regeneration was recorded as 24.26±0.94 days at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (2 mg/L). In general, the number of shoots regenerated varies in between 2-4 and better performance was exhibited at BAP (4 mg/L) + Kinetin (0.5 mg/L) with 4.80±0.87 shoots. There were no significant differences recorded in the number of leaves developed and in length of shoots in all the combinations and concentrations. However, at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L), the shoot length was recorded to be maximum *i.e.,* 1.86±0.43 cm. There was no significant effect recorded in length of shoots at BAP + NAA + IBA and BAP + NAA + Kinetin combinations.

Table15. Effect of BAP, NAA, Kinetin and IBA on shoot proliferation from nodal callus on B_5 media

Concentration of plant growth regulators in B_5 media(mg/L)				Percentage of shoot regeneration (%)	No. of days taken for shoot regeneration	No. of shoots regenerated	No. of leaves	Shoot length (cm)
BAP	NAA	Kinetin	IBA					
2	0.5	0.5	-	100.00 ± 0.00^{a}	26.20 ± 1.00^{ab}	3.86 ± 2.04^{ab}	3.73 ± 1.40^{a}	1.29 ± 0.28^{bc}
2	0.5	1	-	73.33 ± 11.54^{bcd}	25.13 ± 1.02^{ab}	4.66 ± 1.15^{a}	3.33 ± 1.10^{a}	1.23 ± 0.09^{bcde}
2	0.5	2	-	60.00 ± 0.00^{cdef}	24.26 ± 0.94^{a}	3.06 ± 1.15^{ab}	3.26 ± 1.15^{a}	1.62 ± 0.14^{ab}
2	0.5	3	-	53.33 ± 11.54^{def}	25.53 ± 1.62^{ab}	2.26 ± 1.15^{b}	3.66 ± 1.15^{a}	1.86 ± 0.43^{a}
1	-	0.5	-	80.00 ± 20.00^{abc}	29.46 ± 0.90^{c}	2.26 ± 0.83^{b}	2.80 ± 0.20^{a}	0.79 ± 0.04^{de}
2	-	0.5	-	86.66 ± 11.54^{ab}	27.06 ± 0.30^{b}	2.53 ± 0.80^{b}	3.20 ± 0.40^{a}	0.87 ± 0.03^{cde}
3	-	0.5	-	86.66 ± 11.54^{ab}	26.53 ± 0.30^{b}	3.40 ± 0.40^{ab}	3.66 ± 0.23^{a}	1.28 ± 0.28^{bc}
4	-	0.5	-	86.66 ± 11.54^{ab}	26.13 ± 0.50^{ab}	4.80 ± 0.87^{a}	3.66 ± 0.46^{a}	1.63 ± 0.24^{ab}
2	0.3	-	0.5	86.66 ± 11.54^{ab}	29.13 ± 1.10^{c}	2.73 ± 1.33^{ab}	3.13 ± 1.90^{a}	1.24 ± 0.36^{bcd}
2	0.3	-	1	66.66 ± 11.54^{bcde}	31.60 ± 1.83^{d}	2.40 ± 0.52^{b}	4.40 ± 2.35^{a}	1.06 ± 0.21^{cde}
2	0.3	-	2	46.66 ± 11.54^{ef}	32.73 ± 1.40^{d}	2.86 ± 0.80^{ab}	4.06 ± 1.74^{a}	1.03 ± 0.18^{cde}
2	0.3	-	3	40.00 ± 20.00^{f}	33.40 ± 1.40^{d}	2.32 ± 1.01^{b}	3.20 ± 0.20^{a}	0.78 ± 0.18^{e}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p\leq0.05$ according to Duncan's multiple range test (DMRT).

4.1.3.6. Effect of growth regulators on shoot proliferation from internodal callus on B_5 media

In internodal callus, significant effect was observed on shoot proliferation in terms of percentage of shoot regeneration among all the concentrations of growth regulators and maximum of 93.33±11.54% was observed at BAP (4 mg/L) + Kinetin (0.5 mg/L) combination (Table 16). However, the minimum time recorded as 20.46±1.84 days to regenerate shoots at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (0.5 mg/L).There was no significant difference on time duration for shoot regeneration in BAP + NAA + IBA and BAP + NAA + Kinetin combinations. Of all the combinations and concentrations, the maximum number of shoots (4.26±0.46) initiated was recorded at BAP (4 mg/L) + Kinetin (0.5mg/L). The number of leaves developed ranged from 1-4 in different combinations and concentrations and the shoot length was recorded to be maximum at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L) with 1.42±0.17 cm and BAP (4 mg/L) + Kinetin (0.5 mg/L) with 1.38±0.24 cm.

Table16. Effect of BAP, NAA, Kinetin and IBA on shoot proliferation from internodal callus on B_5 media

Concentration of plant growth regulators in B5 media(mg/L)				Percentage of shoot regeneration (%)	No. of days taken for shoot regeneration	No of shoots regenerated	No of leaves	Shoot length (cm)
BAP	NAA	Kinetin	IBA					
2	0.5	0.5	-	33.33±11.54^{e}	20.46±1.84^{e}	2.00±0.72^{b}	1.73±0.30^{f}	0.82±0.11^{e}
2	0.5	1	-	73.33±11.54^{abc}	24.00±3.55^{bc}	2.20±0.52^{b}	2.13±0.46^{def}	0.88±0.03^{cde}
2	0.5	2	-	46.66±11.54^{ab}	24.20±3.30^{bc}	2.20±0.91^{b}	2.20±0.60^{def}	1.22±0.15^{ab}
2	0.5	3	-	26.66±11.54^{e}	22.80±2.02^{ab}	3.36±1.26^{ab}	1.86±0.50^{ef}	1.42±0.17^{a}
1	-	0.5	-	60.00±0.00^{cd}	32.80±1.31^{e}	3.00±0.52^{ab}	3.53±0.50^{bc}	0.96±0.12^{bcde}
2	-	0.5	-	73.33±11.54^{abc}	29.93±0.11^{de}	2.80±0.72^{ab}	4.00±0.52^{ab}	1.12±0.35^{abcd}
3	-	0.5	-	86.66±11.54^{ab}	27.33±1.41^{cd}	3.33±0.30^{ab}	4.66±0.57^{a}	1.13±0.06^{abcd}
4	-	0.5	-	93.33±11.54^{a}	25.46±0.46^{bc}	4.26±0.46^{a}	4.26±0.80^{ab}	1.38±0.24^{a}
2	0.3	-	0.5	66.66±11.54^{bcd}	24.93±1.10^{bc}	3.40±1.00^{ab}	3.46±0.30^{bc}	1.16±0.07^{abc}
2	0.3	-	1	86.66±11.54^{ab}	25.13±1.10^{bc}	3.20±1.00^{ab}	2.93±0.41^{cd}	0.96±0.10^{bcde}
2	0.3	-	2	60.00±11.54^{cd}	25.93±1.10^{bc}	2.73±0.90^{ab}	3.00±0.52^{cd}	0.98±0.14^{bcde}
2	0.3	-	3	46.66±11.54^{de}	25.33±0.80^{bc}	2.33±0.90^{b}	2.80±0.52^{cde}	0.75±0.05^{e}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

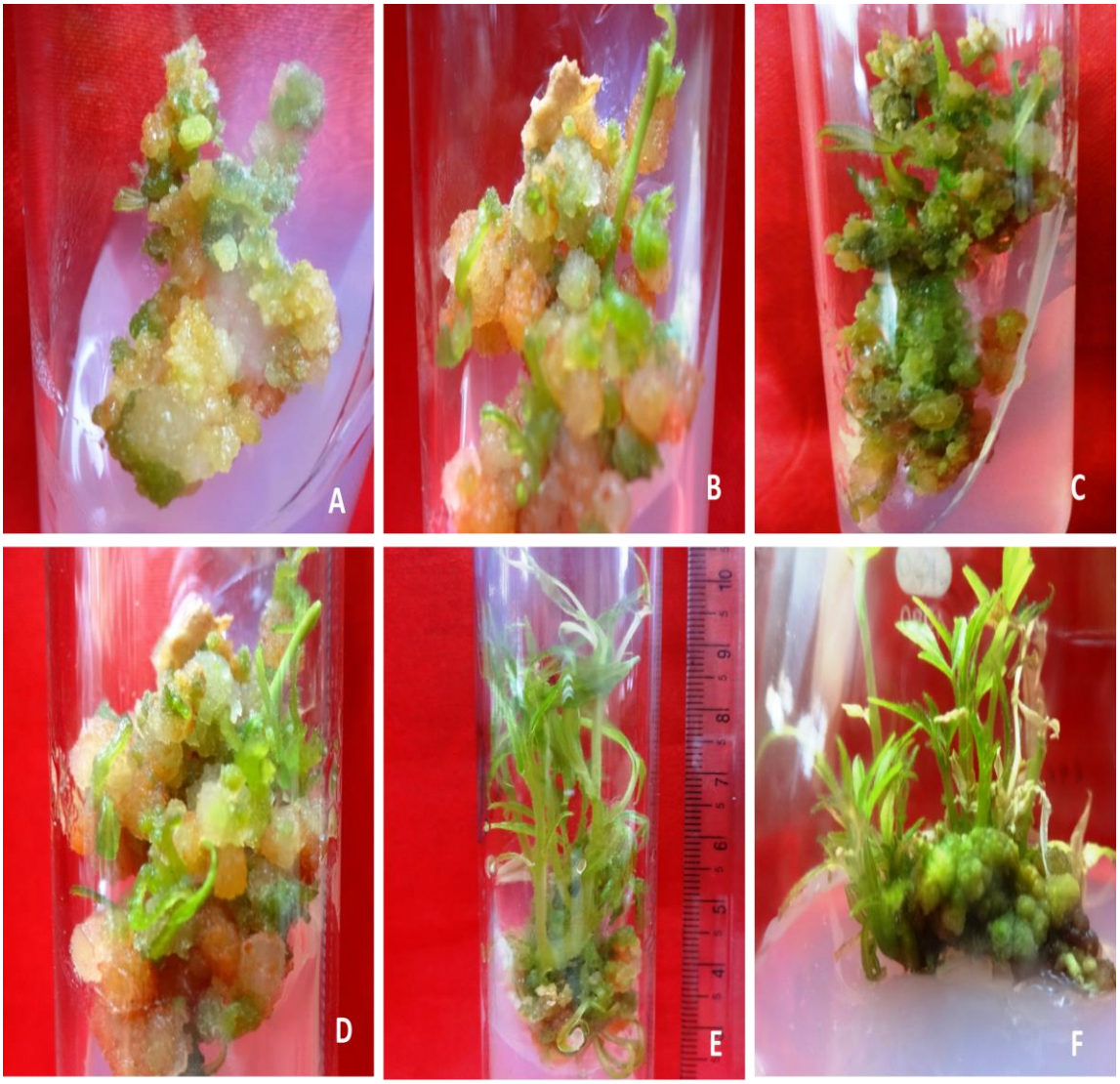

Plate 5: Shoot proliferation from leaf, node and internode callus on MS and B_5 media **(A):** Shoot regeneration after subculture at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (0.5 mg/L) from nodal callus on MS media; **(B):** shoot regeneration from leaf callus at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L) concentration on MS media; **(C):** callus changing its color into green during regeneration; **(D):** shoots proliferation on B_5 media; **(E):** shoot elongation after 3-4 subcultures on MS media at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (0.5 mg/L); **(F):** Multiple shoot initiation from internodal callus at BAP (4 mg/L) + Kinetin (0.5 mg/L) on MS media.

4.1.4. Root initiation

Effect of IBA and NAA on both full strength and half strength MS and B_5 media were studied for root initiation and it was recorded that irrespective of media the time taken for initiation of roots was much faster on half-strength media than the full strength media. Therefore, further subcultures to study the root initiation were undertaken on half strength media. It was also recorded that roots did not initiate on media free of growth regulator.

4.1.4.1. Effect of IBA and NAA on root initiation on MS media

Effects of IBA and NAA on half strength MS media exhibited no significant differences in percentage of root initiation (Table 17). All the concentrations of both IBA and NAA exhibited 100 % root initiation except in 4mg/L IBA and 4 mg/L NAA where the percentage were 80 and 66 respectively. Although the time taken for initiation of roots ranges from 21-44 days in different concentrations of IBA and NAA yet there were differences in their effect in different concentrations of both the growth regulators. The maximum number of roots (26.46±1.62) with maximum root length (1.82±0.20 cm) was recorded in minimum time duration of 21.80±0.20 days at IBA 3 mg/L but in NAA concentrations, the better effect was recorded at NAA (3 mg/L) concentration with maximum number of roots (19.80±1.40) with maximum root length (1.68±0.20 cm).

Table17. Effect of IBA and NAA on root initiation at half strength MS media

Concentration of plant growth regulators in half strength MS media(mg/L)		Percentage of root induction/initiation	No. of days taken for root initiation	No. of roots initiated	Root length (cm)
IBA	NAA				
0.5	-	100.00±0.00^{a}	44.40±1.00^{e}	5.06±0.80^{f}	0.87±0.13^{d}
1	-	100.00±0.00^{a}	26.13±1.20^{b}	12.46±0.83^{e}	1.56±0.22^{a}
2	-	100.00±0.00^{a}	26.60±1.21^{b}	16.40±1.44^{d}	1.66±0.13^{a}
3	-	100.00±0.00^{a}	21.80±0.20^{a}	26.46±1.62^{a}	1.82±0.20^{a}
4	-	80.00±0.00^{b}	23.33±0.57^{a}	23.60±2.25^{b}	1.30±0.12^{bc}
-	0.5	100.00±0.00^{a}	42.20±2.22^{e}	5.46±0.90^{f}	0.84±0.03^{d}
-	1	100.00±0.00^{a}	35.73±3.33^{d}	11.46±0.30^{e}	1.54±0.12^{ab}
-	2	100.00±0.00^{a}	29.86±1.52^{c}	13.40±1.05^{e}	1.64±0.05^{a}
-	3	100.00±0.00^{a}	23.20±0.40^{a}	19.80±1.40^{c}	1.68±0.20^{a}
-	4	66.66±23.09^{c}	28.00±0.87^{bc}	11.53±2.10^{e}	1.08±0.13^{cd}

Values are mean ± standard deviation of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.1.4.2. Effect of IBA and NAA on root initiation on B_5 media

Effect of IBA and NAA on half strength B_5 media on root initiation exhibited better response in IBA than in NAA (Table 18). There were no significant differences among the concentrations of IBA and NAA in terms of percentage of root initiation. However, the maximum of 86.66±11.54 % root initiation was recorded in both IBA and NAA at 3 mg/L concentration. Out of both the combinations of IBA and NAA, IBA exhibited better response. IBA at 3mg/L produced maximum number of roots (10.40±1.77) in minimum 22.33±3.07 days with root length of 1.41±0.30 cm, which was found to be best among all the concentrations of both IBA and NAA.

Table18. Effect of IBA and NAA on root initiation at half strength B_5 media

Concentration of plant growth regulators in half strength B_5 media(mg/L)		Percentage of root induction/initiation	No. of days taken for root initiation	No. of roots initiated	Root length (cm)
IBA	NAA				
0.5	-	73.33±11.54^{a}	46.26±0.80^{d}	3.40±0.20^{d}	0.52±0.20^{c}
1	-	73.33±11.54^{a}	28.20±0.52^{bc}	6.40±2.10^{b}	0.66±0.03^{bc}
2	-	73.33±11.54^{a}	30.13±2.71^{c}	3.60±0.40^{d}	0.88±0.20^{b}
3	-	86.66±11.54^{a}	22.33±3.07^{a}	10.40±1.77^{a}	1.41±0.30^{a}
4	-	80.00±0.00^{a}	23.60±1.56^{a}	5.53±0.75^{bcd}	0.73±0.18^{bc}
-	0.5	73.33±11.54^{a}	47.40±1.03^{d}	3.66±0.50^{d}	0.68±0.09^{bc}
-	1	73.33±11.54^{a}	29.86±0.41^{c}	6.00±1.11^{bc}	0.68±0.11^{bc}
-	2	80.00±20.00^{a}	31.53±0.41^{c}	4.06±0.30^{cd}	0.80±0.15^{bc}
-	3	86.66±11.54^{a}	24.26±3.72^{a}	9.73±1.61^{a}	1.34±0.20^{a}
-	4	73.33±11.54^{a}	25.80±2.25^{ab}	4.93±0.50^{bcd}	0.72±0.04^{bc}

Values are Mean ± SD of 5 replicas from 3 repeated experiments. Means with common superscript within each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

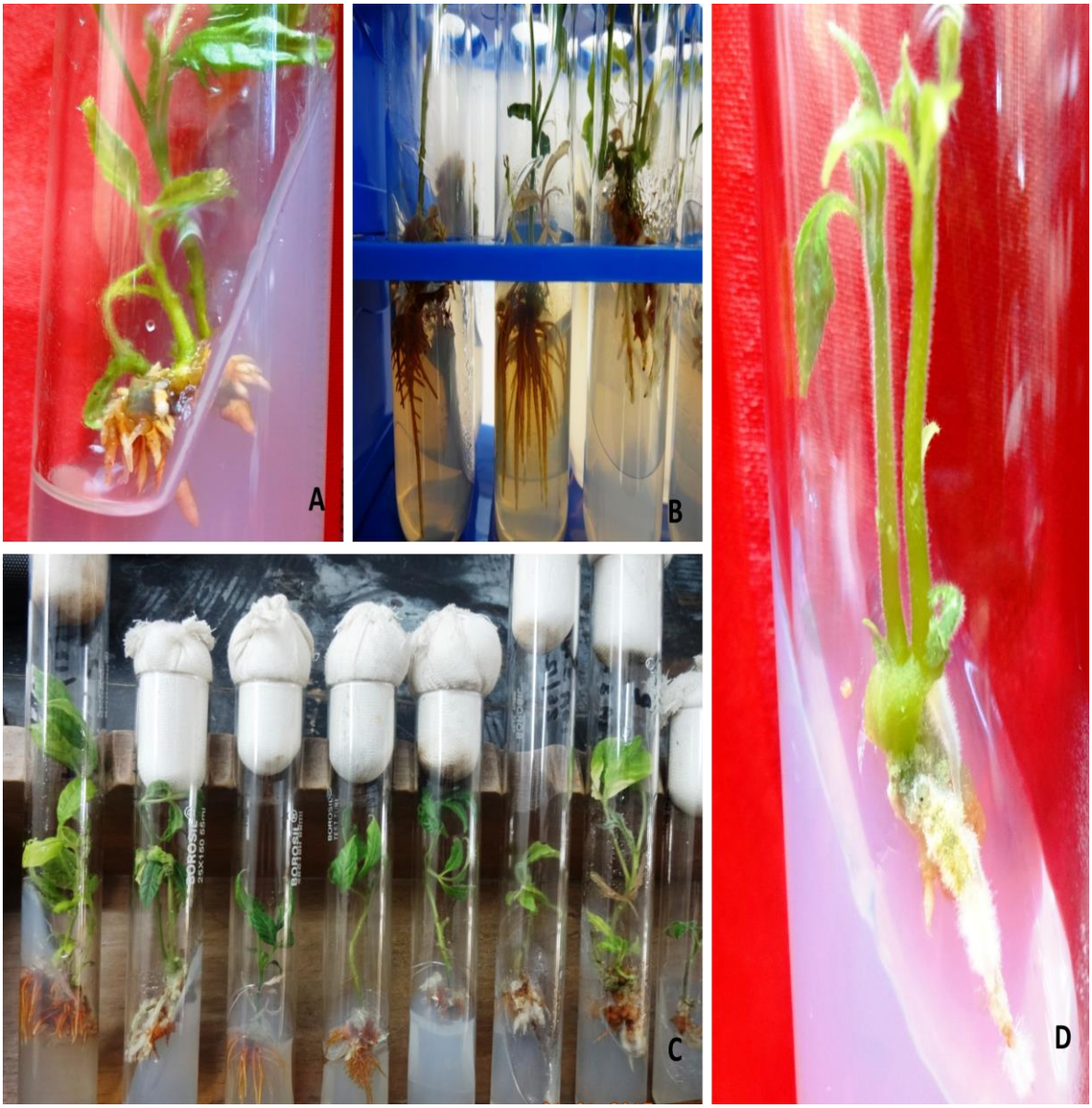

Plate 6: Root initiation on MS and B_5 media **(A):** Root initiation after transferring the regenerated shoots at IBA (0.5 mg/L) concentration within 30 days; (**B**)**:** Elongated roots at IBA (3 mg/L) concentration; **(C):** 100% root initiation on MS media; **(D):** Root initiation at IBA (0.5 mg/L) on B_5 media.

4.1.5. Acclimatization

The healthy plantlets with well developed roots were transferred from both MS and B_5 media to soilrite. The soilrite were autoclaved in a beaker before transferring the plantlets. The rooted plantlets were washed in sterilized distilled water by carefully removing the agar sticked to the roots. They were then planted in small plastic cups containing soilrite and covered with transparent plastic bag in order to maintain relative humidity in the culture room. The plantlets were sprinkled with water in every alternate days and the covered plastic bags were gradually perforated to lower down the atmospheric humidity. New leaves appeared within 10-15 days. After 21 days, the plantlets were transferred to polybags containing soilrite : garden soil : farmyard manure (1:1:1) and kept in the culture room for another 4-5 days. It took 25-30 days for hardening in the culture room. After proper acclimatization, they were transferred from culture room to greenhouse and carefully maintained. There was no detectable phenotypic aberration observed in the growth of the plantlets. Within a week, the shootlets grew with appearance of new green leaves. About 60% plantlets survived during acclimatization.

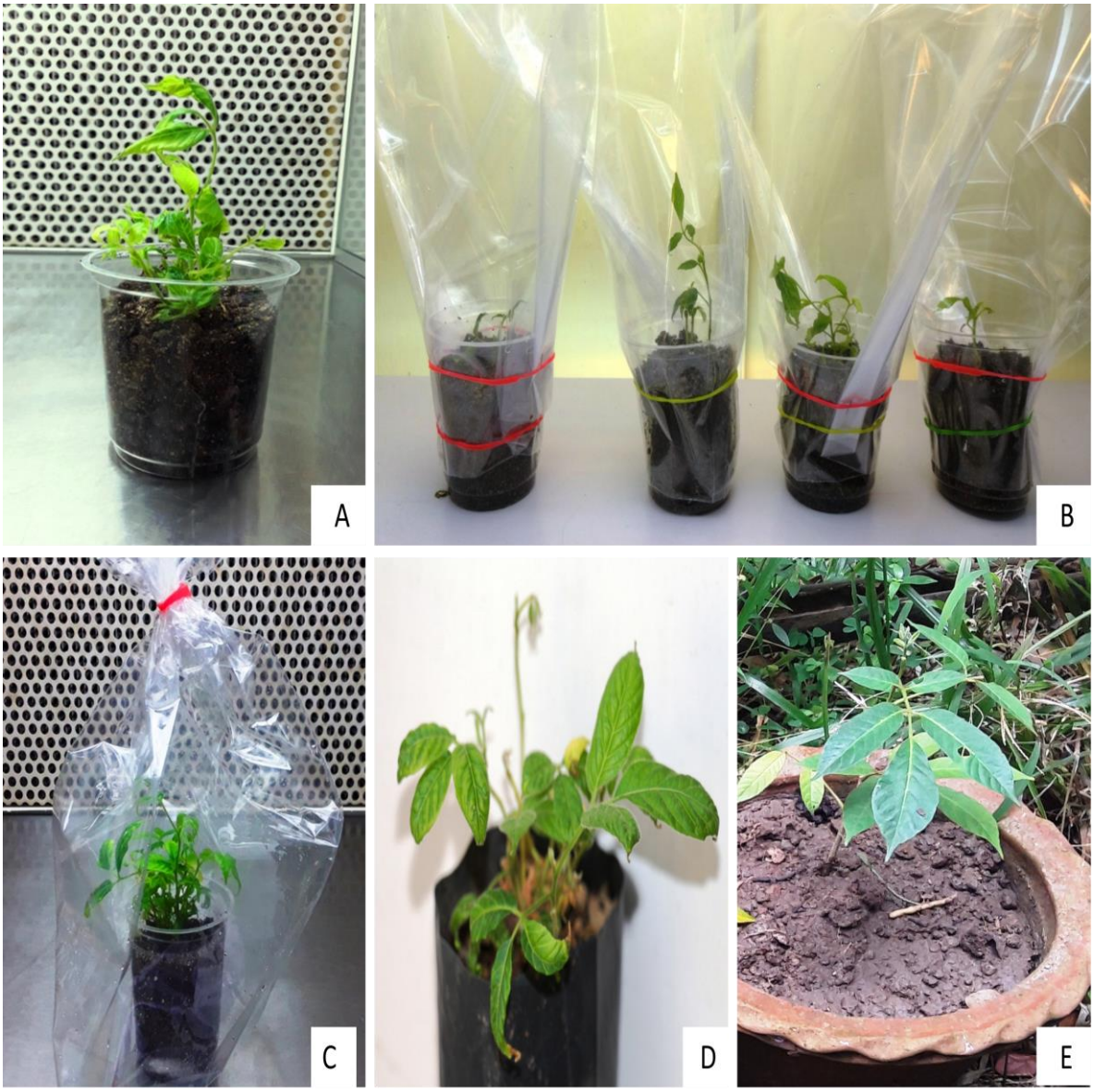

Plate 7: Process of Acclimatization **(A):** Transferring the rooted plantlets into soilrite; **(B):**Plantlets were covered with transparent polybags in order to maintain humidity; **(C):** Plantlets during acclimatization; **(D):**Acclimatized plantlet; **(E):** Plantlet under natural environment after transferring to the soil.

4.2. Antimicrobial activity

The antimicrobial activity test was determined by disc diffusion method. The antimicrobial activity of water, methanol and ethanol extracts of leaves and callus derived

from leaves explants were studied against four bacterial strains *viz.*, *Bacillus subtilis* MTCC441, *Staphylococcus aureus* MTCC3160, *Pseudomonas aeruginosa* MTCC424, *Serratia marcescens* MTCC2645 and two fungal strains *viz.*, *Aspergillus fumigatus* MTCC2550 and *Aspergillus niger* MTCC282. The extracts were prepared in five different concentrations *i.e.*, 1.25, 2.5, 5, 10 and 20 mg/L. No antimicrobial activity was exhibited by the water extracts of both leaves and callus derived from the leaves against all the bacterial and fungal strains. The methanol and ethanol extracts exhibited antimicrobial activity against all the bacterial and fungal strains. Ceftazidime and Amphotericin B were used as positive controls for bacteria and fungi respectively. Moreover, DMSO was taken as negative control which did not show any inhibition.

4.2.1. Effect of crude extracts against *Bacillus subtilis*

The effect of 1.25, 2.5, 5.0, 10.0 and 20.0 mg/ml concentration of the crude extracts of leaves and callus derived from the leaves prepared in ethanol and methanol against *Bacillus subtilis* was studied for antimicrobial activity. All the crude extracts of leaves and callus derived from the leaves showed effect of antimicrobial activity in all the concentrations of the extracts. With increasing concentration of the extracts, the zone inhibition was found to be increasing in all the concentrations of ethanol and methanol extracts. However, ethanol extracts of callus exhibited better antimicrobial effect than the other extracts. In case of ethanol and methanol extracts of leaves, the maximum zone inhibition was recorded at 20 mg/ml concentration with12.66±0.66 mm and 12.00±0.00 mm inhibition respectively. Whereas, in case of ethanol and methanol extracts of callus, the maximum zone inhibition was recorded as 17.33±0.33 mm and 12.00±0.57 mm at 20 mg/ml respectively. The zone inhibition of positive control Ceftazidime (30 µg) was

12.33±0.88 mm. The callus extracts of both ethanol and methanol showed better antimicrobial activity than the leaf extracts against *B. subtilis* (Table 19).

4.2.2. Effect of crude extracts against *Staphylococcus aureus*

The antimicrobial activity of ethanol and methanol extracts of leaves and callus derived from leaves was studied against *Staphylococcus aureus* and all the concentrations of the extracts exhibited antimicrobial activity. The callus extracts showed better effect than the leaves extracts against *S. aureus*. Among all the concentrations of the extracts, the maximum zone inhibition was recorded in ethanol extracts of callus with 14.66±0.33 mm inhibition at 20 and 10 mg/ml concentration as compared with the positive control Ceftazidime (30 µg) which showed 15.00±0.57 mm inhibition. In case of methanol extracts of callus, the maximum zone inhibition was recorded with 13.00±0.57mm inhibition at 20 mg/ml concentration. On the other hand, in case of ethanol and methanol extracts of leaf, the maximum zone inhibition was recorded at 20 mg/ml concentration with 12.66±0.88mm and 12.33±0.66 mm inhibition respectively. It was observed that with the increasing concentration of the crude extracts, antimicrobial activity was also increased (Table 19).

4.2.3. Effect of crude extracts against *Pseudomonas aeruginosa*

The antimicrobial activity of leaves and callus derived from leaves against gram negative *Pseudomonas aeruginosa* was studied and all the concentrations of the extracts (leaves and callus extracts prepared in ethanol and methanol solvent) exhibited antimicrobial activity. The highest effect was observed in ethanol extracts ofcallus at 20 mg/ml and 10 mg/ml concentration with 12.00±1.15 mm and 12.00±0.57 mm inhibition

followed by 11.66±1.20 mm at 20 mg/ml methanol extracts of callus. In ethanol extracts of leaves, the maximum antimicrobial activity was observed at 20 mg/ml concentration with 11.33±0.66 mm inhibition whereas in methanol extracts of leaves, the highest activity was observed at 20 mg/ml concentration with 11.00±0.57 mm inhibition. Among all the extracts, callus extracts exhibited better activity as compared with leaves extracts. The zone of inhibition of the positive control was 17.00±0.57 mm (Table 19).

4.2.4. Effect of crude extracts against *Serratia marcescens*

The gram negative *Serratia marcescens* showed more sensitivity in antimicrobial activity than *Pseudomonas aeruginosa*. Among the plant extracts *i.e.,* leaves and callus derived from the leaves used for antimicrobial activity, extracts of callus was found to be much effective than the leaf extracts. The highest zone inhibition of the extracts was observed at 20 mg/ml with 13.33±0.66 mm and13.00±0.57 mm in callus ethanol and methanol extract respectively. The zone inhibition of the positive control was 14.66±0.66 mm. In ethanol extract of leaves, the maximum zone of inhibition with 12.33±0.66mmwas observed at 20 mg/ml concentration whereas in methanol extract of leaves, the maximum zone of inhibition was recorded as 12.33±0.33 mm at 20 mg/ml (Table 19).

4.2.5. Effect of crude extracts against *Aspergillus fumigatus*

The effect of crude extracts of leaves and callus derived from the leaves against *Aspergillus fumigatus* on antimicrobial activity were studied and showed positive response in all the concentrations of the extracts. In case of methanol extract of leaves, itexhibited better effect with 17.66±0.33 mm inhibition at 20 mg/ml concentration as

compared to ethanol extract which showed 14.66±0.33 mm inhibition at 20 mg/ml concentration. In case of callus extract in ethanol showed better antimicrobial activity than methanol extract. The maximum zone of inhibition with 17.33±0.33 mm inhibition was recorded at 20 mg/ml concentration in the ethanol extract as compared with 17.00±0.57 mm inhibition in the same concentration of methanol extract. The positive control showed 21.00±0.57 mm inhibition. It was also observed that with increasing concentration of the extracts, the zone of inhibition was increasing (Table 19).

4.2.6. Effect of crude extract against *Aspergillus niger*

The effect of crude extracts of both leaves and callus derived from the leaf explants against *Aspergillus niger* was studied in the same manner and was found to have antimicrobial activity**.** With increasing concentration of the extracts, the effect was found to be increasing. Of ethanol and methanol extracts of leaves, it was observed that ethanol extracts with18.33±0.33 mm inhibition at 20 mg/ml concentration exhibited better result as compared with 16.00±0.57 mm inhibition in methanol extract at the same concentration. In case of callus extracts, the maximum zone of inhibition was recorded as 16.33±0.66 mm at 20 mg/ml concentration in ethanol extracts as compared to 13.66±0.33 mm inhibition in methanol extract at the same concentration. The positive control Amphotericin B showed 15.66±0.33 mm inhibition (Table 19).

Table 19: Antimicrobial activity of callus and leaf extract of *B. mollis*

Extract	Concentration (mg/mL)	Zone of inhibition (mm)					
		B.subtilis (MTCC 441)	*S.aureus*(MTCC 3160)	*P.aeruginosa* (MTCC 424)	*S.marcescens* (MTCC 2645)	*A. fumigatus* (MTCC2550)	*A. niger* (MTCC 282)
Leaf ethanol	1.25	11.33±0.66	10.66±0.33	10.00±1.15	11.00±0.57	12.66±0.33	12.83±0.16
	2.5	12.00±0.57	11.66±0.88	10.66±0.88	11.33±0.66	13.00±0.57	16.33±0.33
	5.0	12.00±0.57	12.00±1.54	10.66±0.66	11.66±0.33	13.66±0.33	16.33±0.88
	10.0	12.33±0.88	12.33±0.66	10.66±0.88	11.66±0.88	14.00±0.57	17.33±0.33
	20.0	12.66±0.66	12.66±0.88	11.33±0.66	12.33±0.66	14.66±0.33	18.33±0.33
Leaf methanol	1.25	10.33±0.33	10.33±0.33	9.66±0.33	11.00±0.57	12.00±0.57	14.00±0.57
	2.5	11.00±1.00	10.66±0.33	9.66±0.88	11.33±0.33	15.66±0.66	14.33±0.33
	5.0	11.33±0.66	11.00±0.57	10.00±0.57	11.66±0.33	16.00±0.57	15.33±0.88
	10.0	11.66±0.33	11.66±0.33	10.33±0.66	11.66±0.88	16.66±0.33	15.66±0.33
	20.0	12.00±0.00	12.33±0.66	11.00±0.57	12.33±0.33	17.66±0.33	16.00±0.57
Callus ethanol	1.25	13.33±0.88	13.33±0.33	10.66±0.33	12.00±0.57	14.33±0.66	13.66±0.33
	2.5	14.33±0.66	13.66±0.33	11.00±0.57	12.33±0.88	15.66±0.33	13.00±1.00
	5.0	15.00±0.57	14.00±0.57	10.33±0.33	12.33±0.33	16.00±0.57	15.33±0.33
	10.0	15.66±0.33	14.66±0.33	12.00±0.57	12.33±0.66	16.33±0.33	16.00±0.57
	20.0	17.33±0.33	14.66±0.33	12.00±1.15	13.33±0.66	17.33±0.33	16..33±0.66
Callus methanol	1.25	10.66±0.33	11.33±0.33	9.66±0.33	11.33±0.88	10.33±0.33	11.66±0.33
	2.5	11.33±0.33	11.33±0.66	11.00±0.57	12.33±0.33	10.66±0.88	13.00±0.57
	5.0	11.66±0.33	11.66±0.33	11.33±0.66	12.33±0.88	12.00±0.57	13.00±0.28
	10.0	11.00±0.57	12.33±0.33	11.33±0.88	12.66±0.33	15.00±1.00	13.33±0.33
	20.0	12.00±0.57	13.00±0.57	11.66±1.20	13.00±0.57	17.00±0.57	13.66±0.33
Ceftazidime	30 µg	12.33±0.88	15.00±0.57	17.00±0.57	14.66±0.66	-	-
Amphotericin B	20 µg	-	-	-	-	21.00±0.57	15.66±0.33

Values are Mean± SD of three replicates repeated thrice per experiment. mm: millimeter; µg:microgram;mg/ml: milligram per milliliter.

4.2.7. Minimum inhibitory concentration (MIC) of plant extracts

Minimum inhibitory concentration of the ethanol and methanol extracts of leaves and callus derived from the leaf explants was determined using broth microdilution method which revealed that all the ethanol and methanol extracts of leaves and callus showed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Aspergillus fumigatus* and *Aspergillus niger* ranging from0.156 to 1.25 mg/ml. Depending upon the species the tested extracts showed different level of antimicrobial activity (Table 20).

4.2.7.1. MIC of *Bacillus subtilis*

The MIC of the ethanol and methanol extracts of leaves and callus extracts against *Bacillus subtilis* ranged from 0.156 to 0.625 mg/ml. The ethanol extracts showed better inhibitory than the methanol extract. In case of leaf extract, the MIC for ethanol extract was found to be 0.3125 mg/ml and for methanol extract, it was found to be 0.625 mg/ml. Whereas, in case of callus extracts, the MIC for ethanol was the lowest *i.e.*,0.156 mg/ml and for methanol extract, it was 0.625 mg/ml. In general, it was observed that the callus ethanol extracts showed the lowest MIC against *B. subtilis* among all the extracts.

4.2.7.2. MIC of *Staphylococcus aureus*

The MIC of the ethanol and methanol extracts of both leaves and callus against *Staphylococcus aureus* was found to be in the range between 0.156 to 0.625 mg/ml. Of all the extracts, the lowest MIC was recorded at 0.156 mg/ml in callus ethanol extracts which was followed by callus methanol extracts at 0.3125 mg/ml. Whereas, in case of leaf extracts, the MIC for both ethanol and methanol extracts was found to be 0.625

mg/ml. In general, it can come to the conclusion that callus ethanol extract was more potent compared to the other extracts against *S. aureus*.

4.2.7.3. MIC of *Pseudomonas aeruginosa*

The MIC of the ethanol and methanol extracts of both leaves and callus against *Pseudomonas aeruginosa* showed better effect in callus extracts which showed 0.625 mg/ml MIC in both ethanol and methanol extracts. Whereas, in case of leaf extracts, it showed similar MIC at 1.25 mg/ml in both ethanol and methanol extracts.

4.2.7.4.MIC of *Serratia marcescens*

The MIC of the ethanol and methanol extracts of leaves and leaf derived callus against *Serratia marcescens* was found to be in the range between 0.156 to 0.625 mg/ml. The lowest MIC was found to be 0.156 mg/ml in callus methanol extract which was followed by 0.3125 mg/ml in callus ethanol extract. In case of leaf extracts, the MIC for both ethanol and methanol extracts was found to be 0.625 mg/ml.

4.2.7.5. MIC of *Aspergillus fumigatus*

The MIC of the ethanol and methanol extracts of leaves and leaf derived callus against *Aspergillus fumigatu s*was found to be in the range between 0.156 to 1.25 mg/ml. Among all the extracts, the lowest MIC was found in callus ethanol extracts at 0.156 mg/ml. Whereas, in case of callus methanol extracts, the MIC was found at 1.25 mg/ml. The MIC for leaf ethanol extracts was found to be 0.625 mg/ml and in leaf methanol extract, it was found to be 1.25 mg/ml.

4.2.7.6. MIC of *Aspergillus niger*

The MIC of the ethanol and methanol extracts of leaves and leaf derived callus against *Aspergillus niger* was found to be in the range between 0.156 to 0.625 mg/ml. Among all the extracts, the leaf methanol extract showed the lowest MIC against *A. niger* at 0.156 mg/ml which was followed by 0.3125 mg/ml in both ethanol extracts of leaf and callus. In callus methanol extract, the MIC was found to be 0.625 mg/ml.

Table 20: Minimum inhibitory concentration of the plant extracts

Microorganisms	Minimum inhibitory concentration (mg/mL)			
	Leaf		Callus	
	Ethanol extract	Methanol extract	Ethanol extract	Methanol extract
B. subtilis	0.3125	0.625	0.156*	0.625
S. aureus	0.625	0.625	0.156*	0.3125
P. aeruginosa	1.25	1.25	0.625	0.625
S. marcescens	0.625	0.625	0.3125	0.156*
A. fumigatus	0.625	1.25	0.156*	1.25
A. niger	0.3125	0.156*	0.3125	0.625

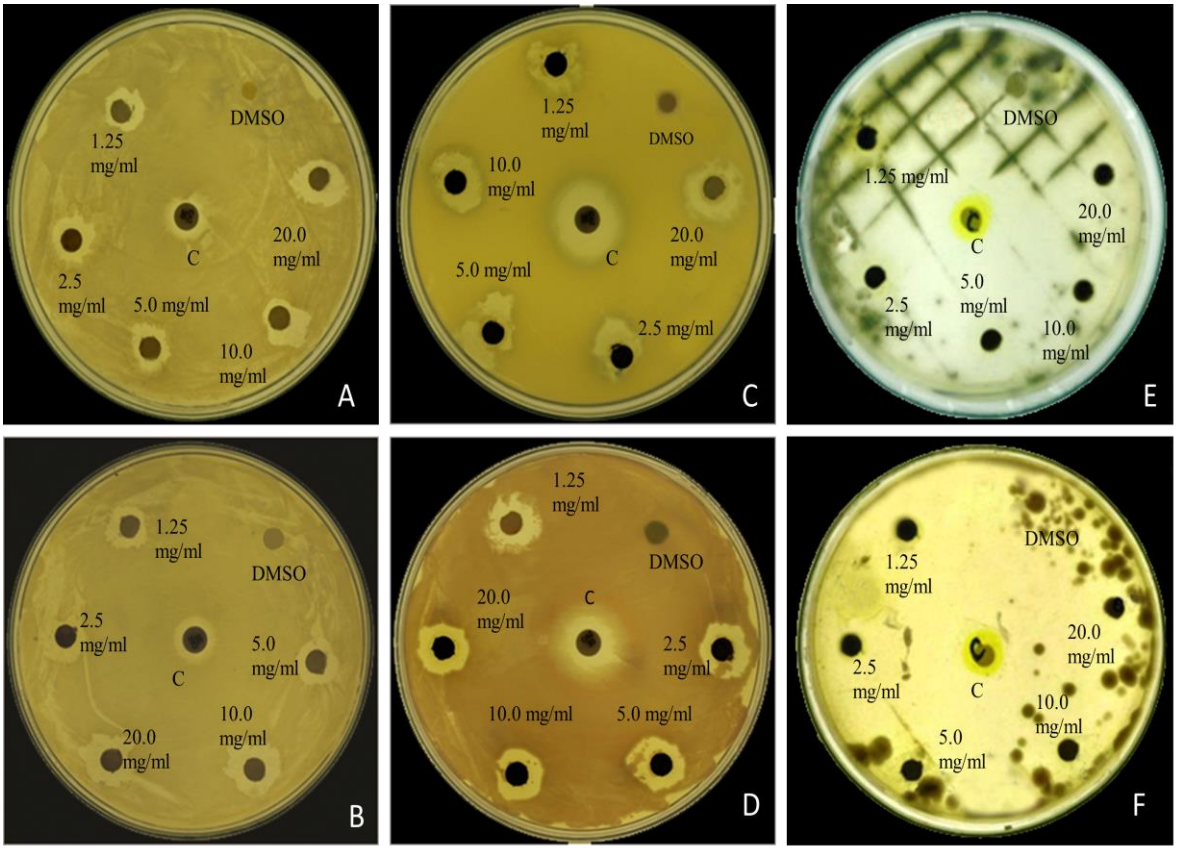

Plate 8: Antimicrobial activity of leaf ethanol extract against **(A)**: *Bacillus subtilis*; **(B):** *Staphylococcus aureus*; **(C):** *Pseudomonas aeruginosa*; **(D):** *Serratia marcescens*; **(E):** *Aspergillus fumigatus*; **(F):** *Aspergillus niger*

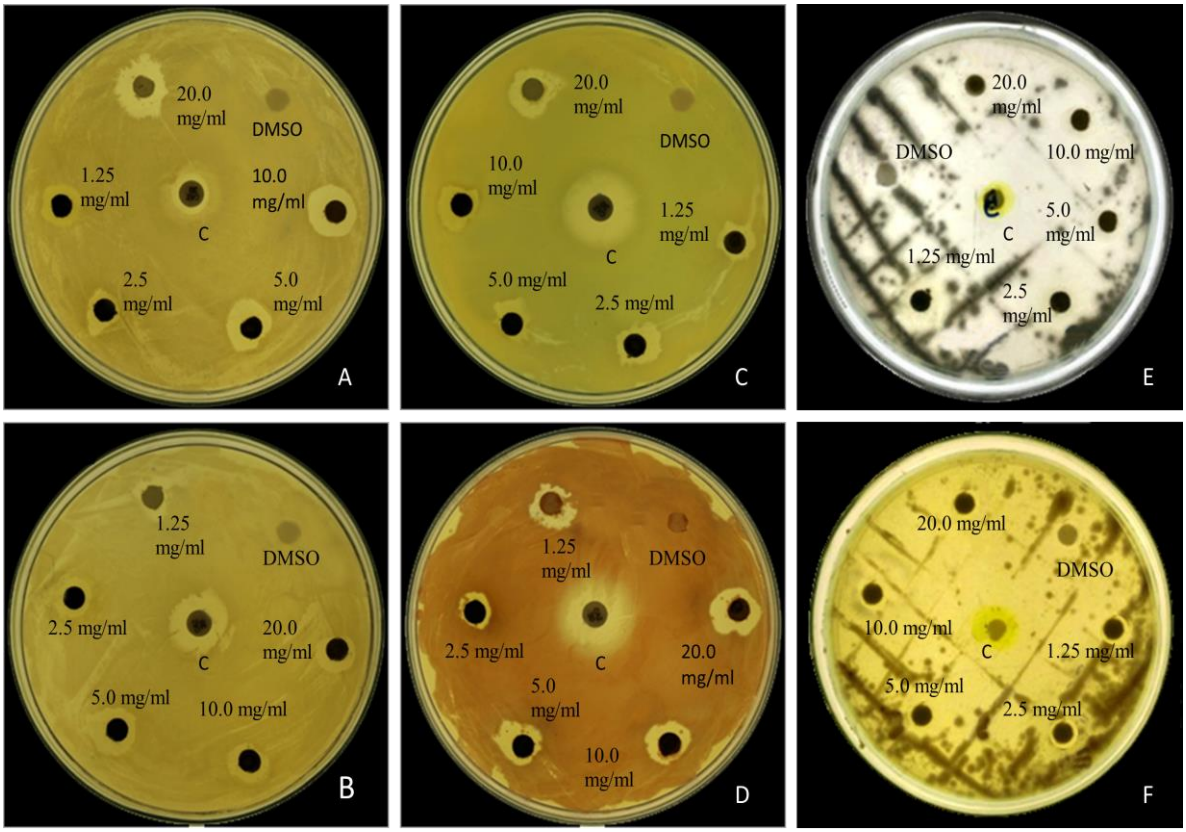

Plate 9: Antimicrobial activity of leaf methanol extract against **(A):** *Bacillus subtilis*; **(B):** *Staphylococcus aureus*; **(C):** *Pseudomonas aeruginosa*; **(D):** *Serratia marcescens*; **(E):** *Aspergillus fumigatus*; **(F):** *Aspergillus niger*

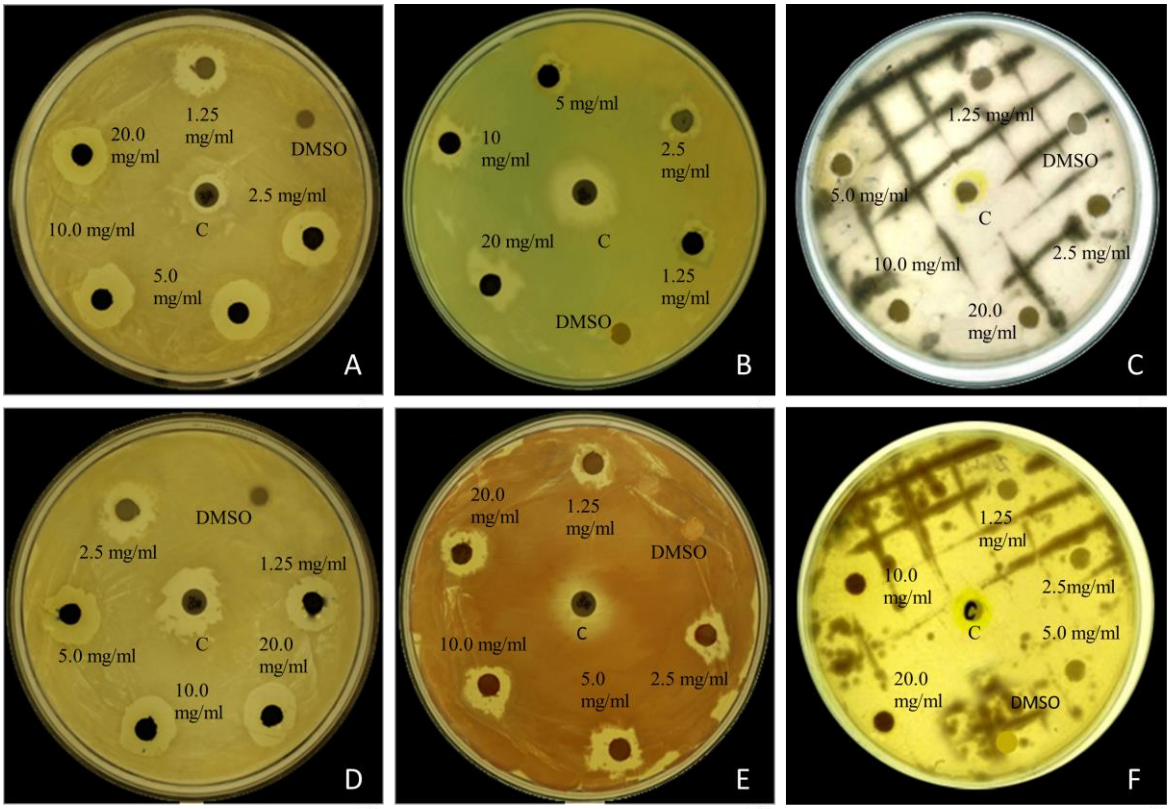

Plate 10: Antimicrobial activity of callus ethanol extract against **(A):** *Bacillus subtilis*; **(B):** *Staphylococcus aureus*; **(C):** *Pseudomonas aeruginosa*; **(D):** *Serratia marcescens*; **(E):** *Aspergillus fumigatus*; **(F):** *Aspergillus niger*

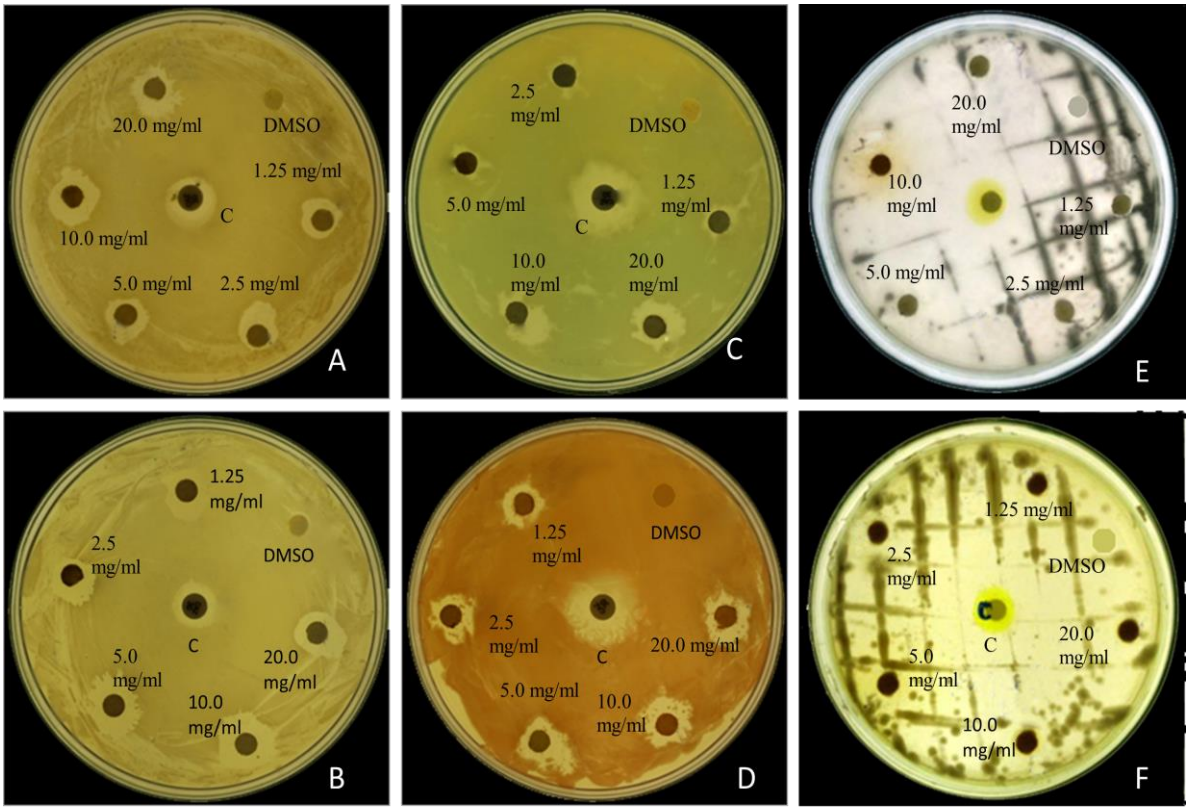

Plate 11: Antimicrobial activity of callus methanol extract against (A): *Bacillus subtilis*;(B): *Staphylococcus aureus*; (C): *Pseudomonas aeruginosa*; **(D):** *Serratia marcescens*; **(E):** *Aspergillus fumigatus*; **(F):** *Aspergillus niger*

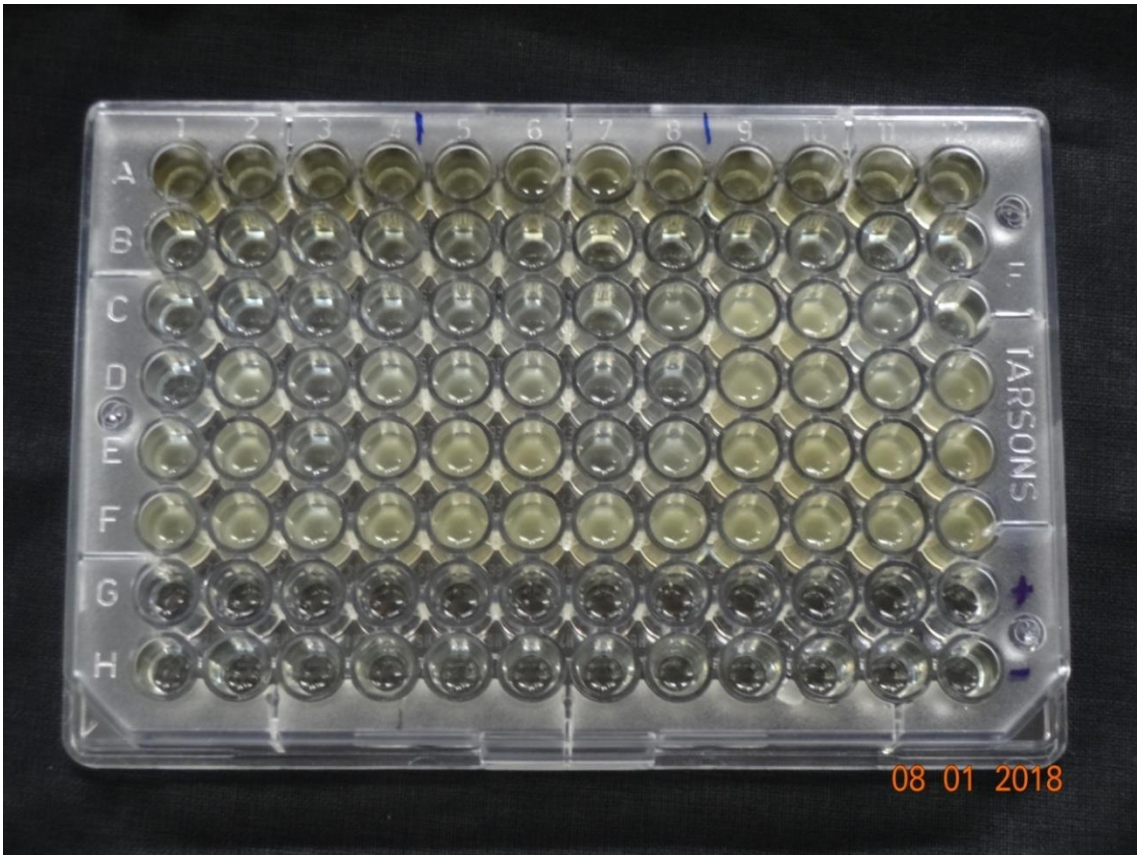

Plate 12: Minimum inhibitory concentration of leaf ethanol, leaf methanol, callus ethanol and callus methanol extract against *B. subtilis, S. aureus, P. aeruginosa.* The column 1, 2,3, 4 represents leaf ethanol, leaf methanol, callus ethanol and callus methanol extract against *B.subtilis*. Similarly,column5, 6,7,8 and9, 10, 11,12 represents the extracts against *S. aureus* and *P. aeruginosa* respectively. Column A, B, C,D, E, F consists of 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.039 mg/ml concentration and G , H represents positive and negative control respectively.

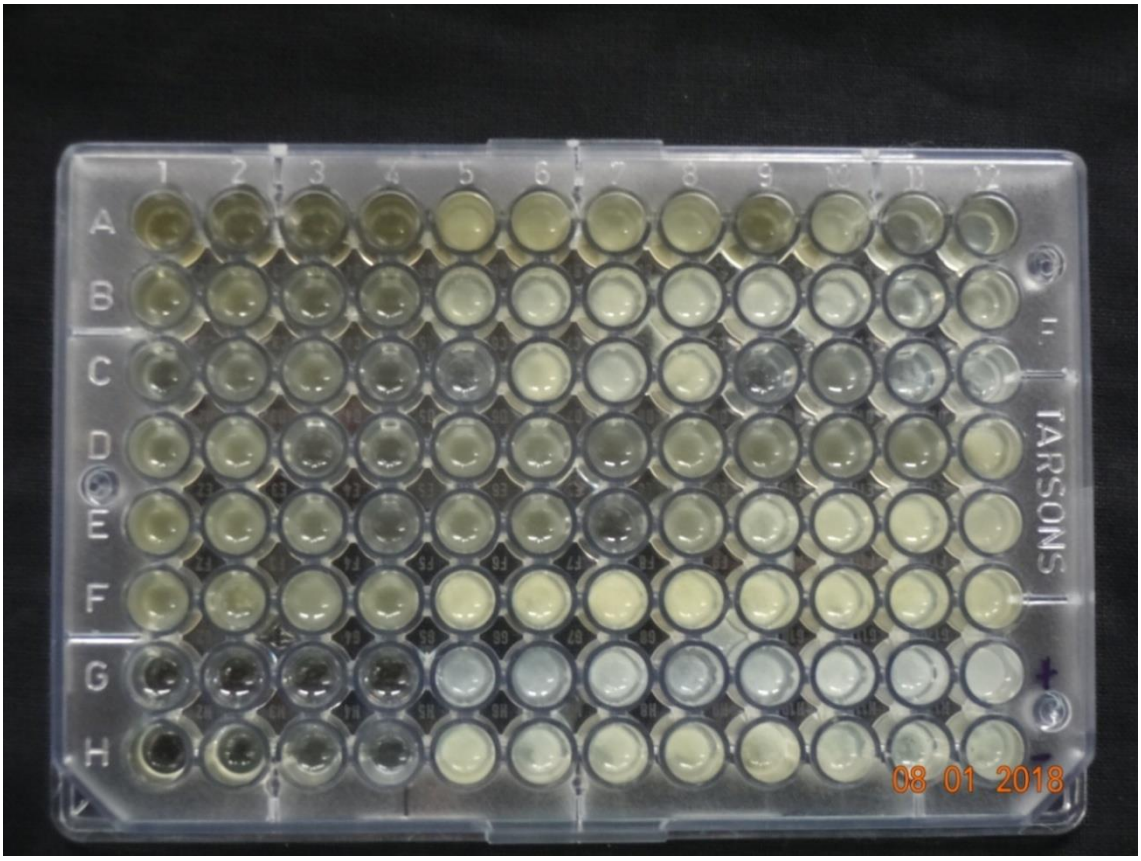

Plate 13: Minimum inhibitory concentration of leaf ethanol, leaf methanol, callus ethanol, callus methanol extract against *S. marcescens*, *A. fumigatus and A. niger*. The column 1, 2, 3, 4 consists of leaf ethanol, leaf methanol, callus ethanol and callus methanol extracts against *S. marcescens*. Similarly, 5,6,7,8 and 9, 10, 11, 12 represents the extracts against *A. fumigates* and *A. niger* respectively. Column A, B, C,D, E, F represents 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.039 mg/ml concentration and G , H represents positive and negative control respectively.

DISCUSSION

Northeast India is one of the mega biodiversity hotspots of the world with plethora of plant diversity. Plants are rich source of pharmaceutically active compounds and for which plants have been used in different systems of treatments worldwide (Rabe and Van Stoden, 2000). Some of these plants have been used in traditional medicinal system, some as folk medicines and many in pharmaceutical industries for use in modern medicine (Salie *et al.*, 1996). As a result, many of the medicinal plants have been collected from wild habitats which results in depletion of their population. In addition to anthropogenic factors, natural calamities like landslides, floods and climate changes also directly or indirectly contributed to the risk of their survival.

The genus *Brucea* comprises of 10 species occurring in several parts of tropical eastern hemisphere which possess a number of phytochemical constituents to cure diseases like malaria, colonic diseases, parasitosis, gastrointestinal cancer and dysentery. In India two species *viz.*, *B.mollis* and *B. javanica* are occurring and of these two species, *Brucea mollis* has been used traditionally in Northeastern region of India. It is a popular antimalarial plant used by several ethnic groups of Karbi Anglong district of Assam. Its fruit and root decoction are used to cure malaria. The plant has immense biological properties as antiplasmodial, cytotoxic, pesticide, antimalarial, anticancer and antitumor activities. In the traditional systems of medicine in India and China it has been used in the treatment of malaria and cancer which has been affirmed by the

presence of chemical compounds like Brucein B, Brucine D, Yadanziolide A, Yadanziolide B, Yadanziolide D, Yadanziolide T-V, Brusatol. Inspite of its importance in folk medicine the population of the species is decreasing due to many anthropogenic factors like destruction of the habitat, harvesting from wild, etc. It has been enlisted as an endangered plant during the CAMP survey in 2003. In *B. mollis*, it has been reported that the conventional technique is inefficient due to limited seed availability and low rate of seed germination (Kakati and Borthakur, 2017). As a result, the plant population becomes rare in its natural habitats. Therefore, there is a need to develop an efficient protocol for mass propagation and conservation of this important endangered medicinal plant. Further, no work has been carried out on the antimicrobial activity of *B. mollis.*

***In vitro* propagation**

Tissue culture is an important technique applied for micropropagation and conservation of medicinal plants through mass propagation either directly or indirectly from the explants. In recent years, plant tissue culture is receiving importance in the field of plant propagation, disease elimination, plant improvement and production of secondary metabolites. A single explant can produce numerous plants in relatively short period of time and space under controlled environmental condition. In present study, an *in vitro* propagation protocol has been established for *B.mollis* using leaf, node and internode explants through callus induction. The results obtained suggested that *in vitro* plant propagation using leaf, node and internode explants can be used for mass propagation of this endangered medicinal plant. Two different media MS and B_5 media were used in the study and both the media showed good response in callus induction,

shoot proliferation and root initiation. However, the overall better performance was shown on MS media.

In the present study three plant growth regulators BAP, NAA and 2,4-D were used in different sets of hormonal combination on leaf, node and internode explants and examined their effects on callus color, texture, number of days taken to initiate callus and percentage of callus induction. Callus was found to be obtained from all the source explants. Leaf explants showed the highest percentage of callus induction *i.e.*, 100% than the other explants. This may be due to the endogenous hormone present in the explants responsible to determine the ability of callus induction (Lane, 1978).

The specific concentration needed for callus induction varies from species to species and depends on the source explants (Charriere *et al*., 1999). An intermediate ratio of auxin and cytokinin is very important for inducing callus and the synergistic effect of BAP with NAA and BAP with 2,4-D used for callus induction have been reported by number of workers (Islam *et al*., 2014; Arif *et al*, 2014; Debnath, 2013). In the present study callus induction took place in all the concentrations of BAP + NAA. The difference in callus response among the explants in BAP + NAA concentrations in the media could be a reflection of differences in endogenous growth regulators in the source explants or different tissue sensitivities to these growth regulators (Lisowska and Wysokinska, 2000). In BAP + NAA combination, the least time taken to initiate callus was found at BAP (2 mg/L) and NAA (0.5 mg/L) in nodal and internodal explants and was found to be the optimum concentration for induction of callus on MS media which showed similar trends recorded by Ardestani *et al*.(2015) on *Scrophularia striata* Boiss. In BAP + 2,4-D concentrations, the percentage of callus induction was maximum (100%) at BAP (2 mg/L) + 2,4-D (1 mg/L) in the leaf explants on MS media similar to

the findings of Chowdhury *et al*. (2011) on *Vitex negundo*. The significant effect of 2,4-D on callus induction have also been reported by a number of workers (Sen *et al.*, 2014; Mungole *et al.*, 2009; Choudhury *et al.*, 2011; Nakasha *et al.*, 2016; Biradar, 2017).The percentage of callus induction was found to be maximum at 1 mg/L of 2,4 - D in both nodal and internodal explants and the percentage of callus induction decreased with increase in concentration which is corroborated by the findings of Sen *et al*.(2014) on *Achyranthes aspera.* According to Cheng *et al*. (2006) plant growth regulators are the key factor for callus initiation and development in plant tissue culture. However, the optimal concentration of the plant growth regulators depend on a number of factors, such as genotype of the source plant, explants origin, etc. (Mathur and Shekhawat, 2013). In the study, it was observed that MS medium without plant growth regulators did not initiate callus which was also reported by Ray *et al*. (2011) and Mathur and Shekhawat (2013). The callus of leaves were friable and pale yellowish, a similar type of response were reported by Hussein *et al*. (2005a) in *Eurycoma longifolia,* Kakuturu *et al*. (2014) in *Simarouba glauca* and by Ahmad *et al*. (2010) in *Ruta graveolans*. In the present study, the callus from node and internode explants were recorded to be compact and creamy in color at 2,4-D concentrations a similar observation was also recorded by Dudhare *et al.*(2014) in *Simarouba glauca*. In the present study, BAP with NAA and BAP with 2,4-D concentrations, the callus were recorded to be compact and light greenish in colour. It was also recorded that when the callus were transferred for shoot regeneration at BAP + Kinetin, the texture of callus changed into hard and green in color after 20 days. This may be due to the differential of growth regulators during subculturing for shoot regeneration.

According to Xu *et al.* (2008), the ratio of auxin and cytokinin is a critical factor to determine organogenesis in plant tissue culture. Endogenous plant hormones that exist in the plant tissues along with exogenous plant hormones combine together to induce shoot organogenesis. Hence, the level of endogenous hormone in cultured explants and derived callus may be the most significant factor in shoot organogenesis (Lee and Huang, 2013). Plant growth regulators BAP in combination of auxin such as NAA, IBA, IAA have been used for shoot regeneration and multiplication of a number of plant species by a number of workers which include Faisal *et al*. (2005) in *Ruta graveolans,* Namli *et al.*(2010) in *Hypericum retusum,* Hossain *et al*. (2013) in *Achyranthes bidentata,* Dudhare *et al*. (2014) in *Simarouba glauca* and other species, Venkatachalam *et al*. (2015) in *Bambusa arundinacea,* etc. Blakesley and Constantine (1992) opined that BAP alone or in combination with NAA are effective shoot inducing agents in different species. In the present study, combinations of BAP and NAA with Kinetin or IBA in shoot organogenesis among the explants were used. The effect of BAP + NAA + Kinetin on shoot proliferation were also studied in *Aloe vera* by Daneshvar *et al*. (2013), in *Psoralea corylifolia* by Pandey *et al.*(2013), in *Abru sprecatorius* by Biswas *et al.*(2007) and in *Simarouba glauca* by Lavanya *et al*. (2016). Among all the plant growth regulators used in the present study, BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L) was found to be best in terms of number of shoot regenerated on MS media which is corroborated by the findings of Lavanya *et al.*(2016) in *Simarouba glauca* where maximum number of shoots with 100% response was obtained from cotyledonary node explants at BAP (3 mg/L)+NAA (0.5 mg/L) + Kinetin (0.5 mg/L). However, in the present findings, the percentage of shoot regeneration was found to be 73.33±11.54 % at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L)

from leaf derived callus. The combination of BAP+NAA + Kinetin was also found to be effective from cotyledonary node explants at BAP (3 mg/L)+ NAA (0.5 mg/L) + Kinetin (0.5 mg/L) by Arockiasamy *et al.*(2000) in *Pterocarpus santalinus.*

The effect of BAP + Kinetin on shoot regeneration has been studied earlier by some workers (Biswas *et al.,* 2007; Muhammad *et al.,* 2007; Sen *et al.,* 2013). In the present study, the combination of BAP + Kinetin was also used for shoot organogenesis and it was found that with increasing concentration of BAP, the percentage of shoot regeneration increased in B_5 media. Though it showed 100% shoot regeneration at all the concentrations in MS media, it exhibited best effect at BAP 4 mg/L + Kinetin 0.5 mg/L in both MS and B_5 media. A similar trend was also recorded by Sen *et al*. (2014) in *Achyranthes aspera.*

Successful rooting is a prerequisite for the *in vitro* regenerated shoots in order to facilitate their establishment in soil. In the present study IBA and NAA were used for root initiation and IBA was found to be more effective than NAA. This is due to the fact that IBA is less quickly destroyed by autoclaving or light than other auxins (Gaspar and Coumans, 1987). IBA exhibited better performance in terms of percentage of root initiation, root length and root number. The roots were thick and strong at IBA 3 mg/L. While at IBA 0.5 and 1 mg/L, they were thin and long. Similar findings were also recorded by Biswas *et al*. (2007), Shekhawat *et al*. (2015), Arikat *et al*. (2004) and Faisal *et al*. (2005). It was also recorded that in *B.mollis* root induction, number of roots initiated and root length increased with increasing concentrations of auxins. Similar findings were also reported in *Carum copticum* L.by Salehi *et al.* (2014) and in *Populus euphratica* Oliv. by Cai *et al.*(2015). Lavanya *et al.* (2016) while studying the effect of NAA at concentrations of 0.5, 1, 2,3 and 4 mg/L on rooting in *Simarouba glauca*, found

that NAA 3 mg/L was the best concentration in terms of rooting response, root number and root length which was also recorded in the present study that NAA 3 mg/L exhibited best performance among all the concentrations used for rooting in *B. mollis*.

The ultimate success of *in vitro* propagation of plants lies on their successful establishment in the soil (Saxena and Dhawan, 1999). In present study, well rooted plantlets were transferred to plastic cups containing sterilized soil rite for hardening and kept in culture room under controlled condition. Plantlets were covered with polybags for maintaining humidity. After 2 weeks, new leaves appeared from the apical portion of shoots. The plantlets were successfully hardened in 30-40 days. The polybags were finally removed and transferred to soil rite: garden soil: farmyard manure (1:1:1). The survival rate was 60%. Yahya *et al*. (2015) successfully acclimatized *Eurycoma longifolia* with 100% survival of plantlets grown in jiffy 7. In *Simarouba glauca*, Lavanya *et al*. (2016) reported 81.5 % survival percentage during acclimatization while Dudhare *et al*. (2014) reported only 72% survival during secondary hardening.

The present study is the first report of plant regeneration of *B. mollis* through indirect organogenesis from leaf, node and internode explants. Among the three explants used in the study, the best organogenic differentiation from callus was recorded from internode explants which exhibited 100% shoot regeneration with minimum days taken for shoot regeneration *i.e.,* 21.20±0.23 and maximum shoot number 7.60±0.40 at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L) in MS media.

Antimicrobial activity

In the present study, antimicrobial activities of plant extracts of leaves and callus derived from leaves explants were carried out in aqueous, methanol and ethanol extract

against two gram positive, gram negative bacteria and two fungal strains. Leaves and callus derived from the leaves of *B. mollis* were used for the extracts for testing antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Serratia marcescens, Aspergillus fumigatus* and *Aspergillus niger*. For bacterial strains, the highest zone inhibition was found to be 17.33±0.33 mm inhibition against *B. subtilis* in ethanol extract of callus as compared with the positive control Ceftadizime with 12.33±0.88 mm inhibition. For fungal strains, ethanol extract of leaf showed more potency against *Aspergillus niger* at 20 mg/ml concentration with highest zone inhibition 18.33±0.33 mm inhibition as compared to the positive control Amphotericin B which showed 15.66±0.33 mm inhibition. In general, the ethanol extract showed more potency than the methanol extract.

The presence of phytochemical constituents such as tannins, flavanoids, alkaloids, saponins and other compounds present in plants are the secondary metabolites and served as defence mechanism against microorganisms, insects and herbivores (Castello *et al*., 2002; Johnson and Babu, 2010). There are number of reports on isolation of a number of phytochemical compounds with different biological activities from *B. mollis* (Bharati and Singh, 2012). Tung *et al*. (2013) isolated ten bioactive compounds from leaves, stems and root extracts of *B. mollis* and demonstrated to have cytotoxic activity against cancer cell lines. The tirucallane triterpenoids isolated from the stems of *B. mollis* has been reported to have significant cytotoxic activity against cancer cells (Chen *et al.*, 2013). Another study on*B. mollis* conducted by Chen *et al.* (2011) has not only exhibited cytotoxic activity but also confirmed the presence of indole alkaoloids and quassinoids in ethanol extract. An antifungal activity of *Brucea javanica* was studied by Nordin *et al.* (2013) and established fungistatic and growth inhibiting effect of the plant

extract against seven *Candida* strains and for which it is considered to be a potent antifungal agent in oral health products. An antimicrobial study was conducted by Manikandan *et al.* (2015) in *Ailanthus excelsa*(a member of Simaroubaceae) against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillusniger, A. fumigatus* and *Penicillium chrysogenum* and exhibited broad spectrum activity against all the pathogens with highest zone inhibition in ethanol extracts. So far, no work has been carried out on antimicrobial activity of *B. mollis* and in the present work, it was found that the alcoholic extracts of *B. mollis* have the potential to be used as antimicrobial agent against all the bacterial and fungal strains. It was also found that with the increase in concentration of the extract, there was also increase in antimicrobial activity against all the tested bacterial and fungal strains which is corroborated by the findings of Viswanad *et al.* (2011). There was no antimicrobial activity in the water extract as compared to methanol and ethanol extract. This may be due to the fact that the active components responsible for the antimicrobial activity may dissolve better in alcohol than in water. Similar findings were also recorded by Taye *et al*. (2011) that water extract of *B. antidysenterica* exhibited poor antimicrobial activity as compared to methanol extract. Ethanol extracts of callus showed more activity than ethanol extracts of leaves. Similar findings were also recorded by Johnson*et al.* (2011) in *Mentha arvensis* which corroborate the fact that antibacterial efficacy of ethanol extract of leaf derived callus was better than the other solvents used. Similarly, ethanol extracts of leaf exhibited more antifungal activity against *A. niger* with highest zone inhibition 18.33±0.33 mm. The differences in the inhibitory effect of both leaf and leaf derived callus extract against the bacterial and fungal strains may be due to the qualitative and quantitative differences in the phytochemical compounds present in them.

The microdilution method was used to determine the minimum inhibitory concentration (MIC) of the plant extracts and was found to be effective in the evaluation of MIC. The MIC of leaf derived callus extract was found to be lowest (0.156 mg/ml) against *B. subtilis*, *S. aureus* and *A.fumigatus* in ethanol extract and *S. marcescens* in methanol extract. On the other hand, the MIC of leaf methanol extract was found to be minimum (0.156 mg/ml) against *A. niger*. In general, the methanol and ethanol plant extracts showed activity against all the tested bacterial and fungal strains which indicates the broad spectrum antimicrobial potential of the plant. This study provides evidence that the secondary bioactive compounds present in the plant might have synergistic impact on inhibition of the tested pathogens. Therefore, further studies are necessary to identify the bioactive compounds and evaluate their antimicrobial activity against a wide range of pathogens in order to confirm their efficacy as antimicrobial agent.

Significant findings

- Of the two media, *i.e.*MS and B_5 media used for callus induction from leaf, node and internode explants as well as for shoot proliferation and root initiation, MS media was found to exhibit better response than B_5 media.
- 100% callus induction was recorded at BAP (2 mg/L) + NAA (0.3 and 0.5 mg/L) both from leaf and internode explants and only from leaf explants at BAP (2 mg/L) + 2,4-D (0.5 and 1 mg/L) in MS media. Since leaf explants exhibit 100% response in both the combinations it is inferred from the present study that leaf explants are the best for callus induction in *B. mollis*.

- 100% shoot regeneration was recorded from the callus of all the explants *viz.*,leaf, node and internode explants at BAP + Kinetin and BAP + NAA + IBA in MS media with minimum number of days 21.20±0.23 and maximum shoot number 7.60±0.40 at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L) from internodal callus.
- 100% root initiation was recorded at 0.5, 1.0, 2.0 and 3.0 mg/L concentration at IBA and NAA in MS media. The best response for root initiation exhibited at 3.0 mg/L IBA in MS media with minimum number of days taken for root initiation *i.e.,* 21.80±0.20 days, highest number of roots (26.46±1.62) and root length 1.82±0.20 cm.
- Well developed plantlets with roots of about 1.5-3 cm were successfully acclimatized in soilrite: garden soil: farmyard manure in the ratio 1:1:1 in culture room and 60% of fully acclimatized plants survived when transferred to field.
- From the present study for successful micropropagation of *B. mollis,* it recommended the use of internode explants for callus induction at BAP (2 mg/L) + NAA (0.3 and 0.5 mg/L), shoot initiation at BAP (2 mg/L) + NAA (0.3 mg/L) + IBA (1 mg/L) in MS media and for root initiation at IBA 3 mg/L.
- Ethanol extracts of callus exhibited better antimicrobial activity than the leaf extracts against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Serratia marcescens*. The highest zone inhibition 17.33±0.33 mm was recorded against *B. subtilis* at 20 mg/ml.
- Leaf extracts exhibit better antifungal activity than callus extracts with 18.33±0.33 mm inhibition against *A. niger* at 20 mg/ml concentration in ethanol extract and 17.66±0.33 mm inhibition against *A. fumigatus* at 20 mg/ml in methanol extract.

- The MIC of ethanol callus extract was found to be lowest, *i.e.*, 0.156 mg/ml against *B. subtilis*, *S. aureus* and *A. fumigatus* and methanol leaf extract exhibited lowest MIC at 0.156 mg/ml against *A. niger*.

SUMMARY

Brucea mollis Wall. ex Kurz belonging to the family Simaroubaceae is an endangered medicinal plant confined to Northeastern region of India. The plant popularly known as '*koinine*' and has been used in traditional healthcare practices against malaria in Karbi Anglong district of Assam. Its fruit and root decoction has been used by indigenous people of Karbi Anglong district to cure malaria. The powdered dried seeds are taken alongwith water, thrice daily to cure malaria and stomach complaints. Besides, the plant is also reported to have anticancer, antitumor, cardiovascular and diuretic activity. The species is listed as endangered during the CAMP survey in 2003. Moreover, in *B. mollis,* the pollination as well as seed dispersal mechanism is inefficient and for which the population of the species is depleting in its natural habitats.

The present study was focused with an aim to establish a micropropagation protocol and to determine its antimicrobial activity. Leaf, node and internode were taken as explants for *in vitro* callus induction and regeneration in micropropagation. For the study of antimicrobial activity, extracts of leaf and callus obtained from the leaves were tested against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Serratia marcescens, Aspergillus niger* and *Aspergillus fumigatus*.

For callus induction, cultures were established on MS and B_5 media at BAP and NAA, BAP and 2,4-D and 2,4-D singly in different concentrations. Among the explants,

the leaf explants showed better effect on callus inductions which exhibited 100% callus induction at BAP (2 mg/L) + NAA (0.3 and 0.5 mg/L) and BAP (2 mg/L) + 2,4-D (0.5,1 mg/L) combination on MS media. The internode explants showed 100% callus induction at BAP (2 mg/L) + NAA (0.3 and 0.5 mg/L) concentration on MS media. Though the percentage of callus induction was less in 2,4-D concentrations, however, the minimum time (7.06±0.61 days) was taken by the leaf explants to initiate callus on MS media. While in case of B_5 media, the leaf and internode explants showed maximum 93.33±11.54% at BAP+NAA and BAP+2,4 -D combination. Among the explants, the node explants took least time *i.e.*, 7.16±0.57 days to initiate callus at BAP (2 mg/L) + NAA (2 mg/L) concentration. The leaf callus was friable whereas node and internode callus were compact.

The callus were subcultured for shoot regeneration in three different sets of combination BAP + NAA + Kinetin, BAP + Kinetin and BAP + NAA + IBA at different concentrations in both MS and B_5 media respectively. The MS media showed better effect than B_5 media. All the explants exhibited 100% shoot initiation at all the concentrations of BAP + Kinetin and BAP + NAA + IBA on MS media. Among the explants, the leaf callus exhibited minimum time duration (17.80±1.03 days) to initiate shoots on MS media at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L). Though, the maximum number of shoots (7.86±0.50) initiation occurred from the leaf callus, the percentage of shoot regeneration was found to be 73.33±11.54 %. While, the internode callus showed 100% with maximum shoot initiation 7.60±0.40. In case of B_5 media, the maximum number of shoots (5.53±1.10) with 60% shoot initiation occurred from the leaf callus at BAP (2 mg/L) + NAA (0.5 mg/L) + Kinetin (3 mg/L) concentration. However, 100% shoot regeneration occurred from nodal callus at BAP (2 mg/L) + NAA

(0.5 mg/L) + Kinetin (0.5 mg/L) with 3.86±2.04 shoot number in B_5 media. There was no significance in the number of leaves in all the concentrations of growth regulators used in both the media.

The *in vitro* raised shootlets were transferred for rooting in half strength MS and B_5 media augmented with IBA and NAA at 0.5-4 mg/L concentration. It was observed that MS media was more effective than B_5 media. The shootlets initiated 100% root initiation at 0.5,1,2 and 3 mg/L concentration IBA and NAA respectively in 21-44 days. Among all the concentrations studied, IBA 3 mg/L concentration was found to be optimum for percentage of root initiation, time duration, root length and root number. Though NAA could enhance 100% root initiation, IBA was found to be more effective in the parameters that evaluated. The highest number of roots (26.46±1.62) initiated at IBA 3 mg/L as compared to NAA 3 mg/L which showed maximum 19.80±1.40 in root number. The maximum root length (1.82±0.20 cm) was obtained in 30 days at IBA 3 mg/L as compared to 1.68±0.20 cm which was maximum in NAA 3 mg/L concentration. In case of B_5 media, similar response exhibited among the concentrations in IBA and NAA. IBA was found to be more effective than NAA in the parameters studied. Though similar kind of response in terms of percentage of root initiation could be observed between IBA and NAA, the former is more effective in terms of the parameters studied.

The well developed rooted plantlets of about 1.5-3 cm approx. were acclimatized in soilrite. They were covered with poly bag in order to maintain humidity for one week after which the poly bag was perforated to lower down the atmospheric humidity. Gradually, the poly bag was removed and during alternate day they were watered in very less amount. When the plantlets began to grow new leaves, they were transferred to

soilrite: garden soil: farmyard manure in the ratio of 1:1:1. About 60% of plantlets survived under natural environment.

For antimicrobial activity of *B. mollis,* fresh leaves and callus derived from the leaves were taken as the plant sample for preparing crude extract. Water, methanol and ethanol were taken as the solvents for preparing the extract. The microbial strains *B. subtilis* MTCC441, *S. aureus* MTCC3160, *P. aeruginosa* MTCC424 and *S. marcescens* MTCC2645 were taken as the bacterial strains and *A. fumigatus* MTCC2550, *A. niger* MTCC282 were the two fungal strains used in the study. The antimicrobial activity was studied by disc diffusion method. Both leaf and callus derived from the leaf extracts showed antimicrobial activity against the microbial strains in methanol and ethanol extracts. The water extracts did not show any zone inhibition. Two positive control Ceftadizime (30 µg/disc) and Amphotericin B (20 µg/disc) were taken for antibacterial and antifungal activity respectively and DMSO was taken as the negative control. In general, the callus extracts showed better activity than the leaf extracts against all the bacterial strains. The highest zone inhibition 17.33±0.33 mm was observed against *B. subtilis* in callus ethanol extract as compared with positive control 12.33±0.88 mm. With increasing concentration of the extract, it was found to have more potential against the tested bacterial and fungal strains. In case of fungal strains, the leaf ethanol extract showed better effect than callus ethanol extract against *A. niger* with 18.33±0.33 mm inhibition at 20 mg/ml concentration compared with positive control Amphotericin B (15.66±0.33 mm).While the methanol leaf extract was more effective against *A. fumigatus* with 17.66±0.33 mm inhibition than the leaf ethanol extract.

The minimum inhibitory concentration (MIC) was determined using microdilution method and was found to be effective. The MIC of ethanolic callus extract was found to

be the lowest (0.156 mg/ml) against *B. subtilis*, *S. aureus* and *A. fumigatus*. On the other hand, the lowest MIC at 0.156 mg/ml was recorded against *A. niger* in methanol leaf extract.

In this investigation, micropropagation protocol for *B. mollis* was standardized in two different media with different explants through indirect organogenesis. Among the three explants used in the study, the highest potential for organogenic differentiation from callus was observed from internode explants on MS media. The protocol thus established will facilitate mass propagation and conservation of this important medicinal plant. Further, the determination of antimicrobial activities of the plant against the microorganisms has strengthened the potentiality of the plant in healthcare sector, suggesting for further analyses of the plant.

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ANNEXURE

A. Paper Published/Communicated in Peer Reviewed Journals

- Das, P., Tanti, B. and Borthakur, S.K. (2017). Effect of 2, 4-D on callus induction at nodal and internodal explants of *Brucea mollis* Wall. ex Kurz- an endangered plant of Northeast India. ***Bioscience Discovery***. **8**(3): 369-374.

- Das, P., Tanti, B. and Borthakur, S.K. (2018). *In vitro* callus induction and indirect organogenesis of *Brucea mollis* Wall. ex Kurz –a potential medicinal plant of Northeast India. ***South African Journal of Botany***. 119: 203-211.

- Das, P., Tanti, B. and Borthakur, S.K. (2018). Antimicrobial activity and antioxidant properties of *Brucea mollis* Wall. ex Kurz.- a medicinal plant of Northeast India- **Under Review.**

B. Book chapter published

- Das, P., Tanti, B. and Borthakur, S.K. (2018). Notes on *Brucea mollis* Wall. ex Kurz- Chemical Constituents and Biological Properties. In: *Advances in Botanical Research in Northeast India*. EBH Publishers, Guwahati, India. pp.379-386.

C. Research paper presented

- Das, P., Tanti, B. and Borthakur, S.K. (2017). *In vitro* propagation of *Brucea mollis* Wall. ex Kurz from nodal and internodal explants-an important medicinal plant of Northeast India. Paper presented at International Symposium on Plant Biotechnology for Crop Improvement organized by IIT Guwahati, Assam in 2017.

- Das, P., Tanti, B. and Borthakur, S.K. (2017). *In vitro* callus induction and shoot regeneration of an important medicinal plant *Brucea mollis* Wall. ex Kurz of NE India. Paper presented at the National Seminar on Science and Technology for Human Welfare organized by the Institute of Advanced Study in Science and Technology, Guwahati-35, Assam in 2017.

- Das, P., Tanti, B. and Borthakur, S.K. (2018). *In vitro* callus induction and indirect organogenesis of *Brucea mollis* Wall. ex Kurz- an endangered medicinal plant of Northeast India. Paper presented at International Symposium on Biodiversity and Biobanking organized by IIT Guwahati, Assam in 2018.

D. WORKSHOPS ATTENDED

- Two days Workshop on 'Application of Basic Statistical Tools in Life Science', organized by International Quality Assurance Cell (IQAC), Cotton College, Guwahati on Feb 18-19, 2016.

- One day Workshop on Writing Skill, on 12th August, 2016 at Gauhati University, Guwahati.
- One day Workshop on IPR and Ethics on Human Research, organized by Gauhati University, Guwahati, Assam on 14th September, 2016.
- Indo-Japan Workshop on Translational Agriculture Avenues for International Cooperation, jointly organized by DBT Program Support Centre, Indian Institute of Technology Guwahati and Gifu University, Japan on 29th March, 2017 at IIT Guwahati.