PLANT REGENERATION OF *Coscinium fenestratum* (Gaertn.) Colebr. THROUGH AXENIC SEED CULTURE AND AXILLARY BUD CULTURE

By

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(2018-12-004)

THESIS

Submitted in the partial fulfilment of the requirements for the degree of

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(Plantation Crops and Spices)

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DECLARATION

I, **Abhaya M. C. (2018-12-004)**, hereby declare that the thesis entitled **"Plant regeneration of *Coscinium fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other university or society.

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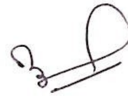

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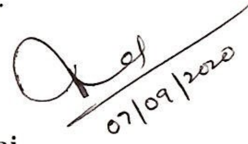

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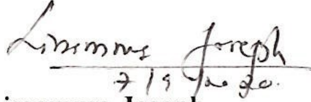

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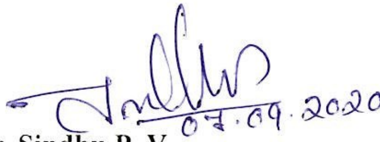

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ABBREVIATIONS

$HgCl_2$ - Mercuric chloride

NaOCl - Sodium hypochlorite

2,4-D - 2,4-dichlorophenoxy acetic acid

BA - Benzyl adenine

BAP - 6-Benzylaminopurine

IAA - Indole-3- acetic acid

IBA - Indole-3- butyric acid

KNO_3 - Potassium nitrate

TDZ - Thidiazuron

NAA - Naphthalene acetic acid

GA_3 - Gibberellic acid

WPM - Woody Plant Medium

MS - Murashige and Skoog

LAF - Laminar air flow

UV - Ultravilolet

v/v - volume in volume

w/v - weight in volume

µM - micromolar

mg L^{-1} - milligram per litre

g L^{-1} - gram per litre

min - minute

INTRODUCTION

INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr., a medicinally important, perennial woody climber belonging to the family Menispermaceae, is a large dioecious plant with yellow wood and sap. The plant commonly known as Tree turmeric, False calumba or Colombo weed in English and locally called as *Maramanjal* in Kerala and Tamil Nadu. This plant is endemic to Indo - Malayan region (Thriveni, 2015) and mainly distributed in Western Ghats region of Southern India and Sri Lanka. Berberine, a yellow crystalline, isoquinoline alkaloid is the main active principle compound in the stem and roots of this woody liana (Rojsanga *et al*., 2009).

In the traditional Ayurveda and Siddha systems of medicine, the plant has been used mainly for treating diabetes mellitus. In ethano medicine, stem is used as poultice for cuts and sores, ulcers, fever, jaundice, snakebite, piles, etc. Roots are used as a bitter tonic, for dressing wounds, treating ulcers and dysentery, and also used as stomachic and antiseptic (Rai *et al*., 2013).

On the point of traditional knowledge, the stem pieces of *C. fenestratum* are used against jaundice, rheumatism and skin diseases by Kaadar tribes of Thrissur district and Oorali tribes of Idukki district of Kerala, and powdered bark is used for treating problems in eye by traditional healers in the Gandai region of Chhattisgarh. The yellow dye obtained from the wood is used as a fabric dye in Malaysia (Tushar *et al*., 2008).

Pharmacological studies have revealed that *C. fenestratum* extract has antiacne, antiinflammatory, antioxidant, hypotensive, antiplasmodial, antibacterial, antidiabetic, antiproliferative, antihepatotoxic, CNS depressant and analgesic properties (Rai *et al*., 2013).

Owing to its therapeutical potential, this plant has attained significance as one among the highly traded medicinal plants, which has resulted in the unsustainable, over exploitation of the plant from its natural habitats. In addition to this habitat destruction due to deforestation has led to reduction of natural population of this species. Moreover, long pre-bearing age, seed dormancy and viability and regeneration related problems have led to the extinction of this species, and now the plant is listed as a critically

endangered species in the IUCN red list of threatened species. *C. fenestratum* is a slow growing woody plant which takes almost 15 years to attain reproductive stage (Tushar *et al*., 2008). Seed germination of this plant is very low under normal conditions since it exhibits both chemical and mechanical dormancy. In addition to the above factors, dioecious nature of the plant is another issue which demands the presence of male and female plants in close proximity coupled with their synchronized flowering to get fruiting. These factors in turn have led to the insufficient availability of the planting material for conservation and mass propagation of the species.

In conventional methods, the species is chiefly propagated through seeds and vegetative perennial stem cuttings. However, these conventional methods are not adequate to meet the demands of conservation and sustainable utilization of this species.

Plant propagation by tissue culture is widely recommended as a biotechnological tool for mass multiplication and conservation of endangered species. Therefore, the development of an *in vitro* protocol for the production of planting material is important to conserve this valuable endangered species and also to ensure the germplasm conservation. Hence, the present study was undertaken to develop a feasible and reproducible *in vitro* protocol for mass propagation of *C. fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture.

Experiments were designed to meet the following objectives with the aim of conservation and mass propagation of *C. fenestratum* (Gaertn.) Colebr.

1. Identification of best surface sterilization procedure for establishment of seeds in aseptic cultures
2. Identification of best *in vitro* seed germination medium
3. Standardization of shoot regeneration medium for nodal explants (axillary bud from poly house-grown plants)
4. Standardization of shoot regeneration medium for cotyledonary nodal explants from seedlings
5. Standardization of suitable embryo culture method

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. DESCRIPTION OF THE PLANT *C. fenestratum*

C. fenestratum (Gaertn.) Colebr., commonly known as tree turmeric, is a large woody climber belonging to the family Menispermaceae. It is a medicinally important, highly traded perennial dioecious plant which will grow up to 10 m long, and has a cylindrical brown coloured, very hardy stem with a spiral phyllotaxy. The main feature of this plant is that, its internal stem colour of both young and mature stage is found to be yellow in colour due to the presence of an alkaloid known as berberine. At the younger stage, branchlets are brown and tomentose, later at mature stage, it is found to be glabrous with disciform petiole scars (Tushar *et al*., 2008; Shenoy, 2015).

Leaves are simple, alternate, exstipulate and broadly ovate, rounded, truncate or shallowly cordate at the base, acuminate at the apex (Tushar *et al*., 2008). Leaf lamina has dark green colour on the glabrous adaxial surface and light green on the minutely tomentose abaxial surface. Leaf characters have slight variations in both sex form and it can be used as an indicator for identification of the sex of the plant. Leaf lamina is narrowly ovate in male plants with 21.13 cm length and 15.47 cm breadth whereas it is broadly ovate in female plants with 17.95 cm length and 17.21 cm breadth. Petioles are tomentose with a pulvinus, its length in the female plant (12.02 cm) is significantly higher than that in male plant (10.28) (Shenoy, 2015).

The inflorescence of *C. fenestratum* is a compound raceme, with globose heads borne on long peduncle developing on the old leafless stem in the axils of the fallen leaf as cauliflorous clusters, and female florets have a larger size (0.32 cm) than the male florets (0.22 cm) (Shenoy, 2015).

The fruit is a one seeded drupe, with globular shape, dark brown colour and tomentellous nature. An average fruit has 8.53 g weight, 2.36 cm length and 2.36 cm breadth respectively. It was reported that 150 days were taken from flower opening to fruit maturity (Shenoy, 2015).

Thriveni (2015) reported that fruits mature in August to September with a peak in the first week of September. The ideal stage for fruit collection is reported to be that,

mature drupes start showing the visual signals like a globose shape, attain a size of about 3 cm across. And fruits turn to slightly pale yellow colour at this stage from deep brown colour with a greenish tinge and possess dense, minute and brown coloured hairs. At this stage, the fruit rind will be thin with the bright yellow inner wall.

The seed is kidney shaped, greenish brown colour, and it is surrounded by dark brown pulp. The seed is subglobose with divaricate, much folded, and divided cotyledons. An average seed has 1.7 g weight, 1.52 cm length, and 1.14 cm breadth. In the fully matured seed, an adaxial intrusion of the endocarp known as condyle was present. The bright yellow coloured embryo has very thin divaricate cotyledons, and it is located in a region near to the hilum (Shenoy, 2015).

2.2. MEDICINAL USES OF *C. fenestratum*

Roopa and Hosetti (2017) reported that in the local health traditions of Karnataka dried stem is used to treat pimples, psoriasis, allergy, lump on the surface of the body (subcutaneous) especially limbs without pain and movable, backache, pain reliever and snake bite poison. Stem and bark both are used for treating itching with dryness of the skin, appendicitis and ulcer. The dried roots are used for eczema, secondary infections in old wounds due to cuts and burns, boils filled with water on the skin, small patches of scaly skin, crack in the heel, piles (hemorrhoids), hernia, diabetes, pain in joints, asthma, thyroid and debility. Bark alone used for blood purification, gastro-intestinal purification, skincare, and kidney stones. Leaves used for treating an obstruction in the urinary tract and dimness of eye due to conjunctivitis.

In classical Ayurveda 35 disease conditions are treated using *C. fenestratum*, some examples are tvakdosha (pimples and skin diseases), kushta vrana (psoriasis), vrana (wounds), vipadika (cracks in the heel), premeha (diabetics), svasa (asthma), blood purifier (raktha vikara), varnya (skincare), visha (snake bite) (Roopa, and Hosetti, 2017).

In the traditional system, *C. fenestratum* used to treat inflammations, jaundice, wounds, burns, skin diseases, ulcers, abdominal disorders, fever, general debility, diabetes and used against bites of snake and monkeys (Tushar *et al*., 2008).

C. fenestratum is a future potent drug source, formulation from this plant is very effective and safe because it has antiacne activity, antiinflammatory activity, antioxidant activity, hypotensive activity, antiplasmodial activity, antibacterial activity, antidiabetic activity, antiproliferative activity, antihepatotoxic activity, central nervous system (CNS) depressant and analgesic activity and anticancer activity (Rai *et al*., 2013; Taher *et al*., 2019).

Furthermore, many clinical trial and phytochemical investigation proved the medicinal activity of the *C. fenestratum*, for example, berberine, the active principle in *C. fenestratum* have the antibacterial property (Nair *et al*., 2005; Goveas and Abraham, 2013), alcoholic stem extract reported to have antidiabetic activity (Shirwaikar *et al*., 2005), methanolic stem and leaf extract showing antioxidant activity (Goveas and Abraham, 2013), aqueous stem extract have antihyperglycemic activity (Shirwaikar *et al*., 2005). Ethanolic extract ointment 10 % w/w concentration reported being having wound healing property (Thangathirupathi and Santharam, 2011).

2.3. MAJOR CONSTITUENTS IN *C. fenestratum*

Malhotra *et al*. (1989) reported that *C. fenestratum* had a minor alkaloid 12,13-dihydro-8-oxo-berberine along with berberine, oxyberberine, tetrahydro berberine (canadine), sitosterol and stigmasterol.

Pinho *et al*. (1992) reported berberine as the main alkaloidal constituent in the stem of *C. fenestratum* and smaller amounts the protoberberine alkaloids like oxyberberine, oxypalmatine, (-)-8-oxocanadine, (-)-8-oxo-tetrahydro thalifendine, (-)-oxoisocorypalmine and (-)-8-oxothaicanine or (-)-8-oxo-3-hydroxy-2,4,9,10-tetra methoxy berberine also present.

Madhavan *et al*. (2014) reported the presence of significant amounts of ecdysterone in the stem (0.22 %) and leaves (0.12 %), in addition to berberine.

2.4. VEGETATIVE PROPAGATION PROPAGATION METHODS IN *C. fenestratum*

Thriveni (2015) reported that vegetative propagation was successful in *C. fenestratum* using mature healthy stem cuttings with 3 - 4 cm diameter. Stem cuttings

without any treatment reported only 5.5 per cent sprouting and 3.5 per cent of rooting, whereas rooting treatments like liquid formulation of IBA resulted in 24 per cent sprouting and 20 per cent rooting. Powder formulation of IBA resulted in 12.5 per cent sprouting and 8.5 per cent rooting. Pre-treatment with commercial quick root solution resulted in 50.5 per cent sprouting and 45 per cent rooting.

Thriveni (2015) reported that vegetative propagation through air layering was also successful in *C. fenestratum*. 70 per cent success was reported with the use of commercial quick root solution and sphagnum moss treatment.

2.5. SEED PROPAGATION AND SEED DORMANCY BREAKING METHODS

A propagation study conducted by Thriveni (2015) revealed that poor seed germination in *C. fenestratum* was primarily due to its hard seed coat. The tetrazolium test conducted showed 85 per cent viability for fresh seeds, which means seeds have a viable embryo, even though impermeable hard seed coat reduced seed germination by imparting mechanical dormancy. Only 5 per cent of *C. fenestratum* seeds sown without any pregermination treatment showed emergence at 70 days.

Treatment with GA_3 at 2000 mg L^{-1} (soaked overnight), treatment with 10 per cent sulphuric acid (H_2SO_4) for half an hour along with GA_3 at 500 mg L^{-1} (overnight) and accelerated ageing treatment for 7 days (seeds were subjected to alternate wetting and drying cycles for 12 hours each) were reported to be best treatments for breaking the hard seed coat and embryo dormancy of *C. fenestratum* (Thriveni, 2015).

Seeds of *C. fenestrartum* had a reasonably long period of dormancy (40-364 days) and germination under natural conditions is less than 20 per cent. As per seed anatomical studies, the average seed coat thickness was 1.62 mm. The embryo was located deeply within the invaginations of the endospermic tissues. The thick, stony seed coat (integument) and the dry and hard endospermic tissues act as mechanical barriers to the developing embryo. The seed coat consisted of a compactly arranged lignified strongly water repellent macrosclereid layer that impedes the imbibition process (Ariyarathna *et al*., 2001).

Anilkumar *et al*. (2010) reported that the germination of *C. fenestratum* increased when fresh seeds were pre-treated with 2 to 10 per cent KNO_3 or GA_3 3000 mg L^{-1}. The maximum germination of 93 per cent was recorded in GA_3 3000 mg L^{-1} treatment for 24 hours, followed by GA_3 4000 mg L^{-1} (90 %). The higher and lower concentration of GA_3 reduced the germination percentage. Also, pre-treatments with 2, 5 and 10 per cent solution of KNO_3 for 24 hours gave only 63 to 67 per cent germination among all the treatments. They also studied the effect of light and pulp on germination percentage and found that only 7 per cent of the seeds with pulp germinated even after one year of incubation, whereas depulped seeds showed higher germination percentage (20 %). When exposure to light conditions was compared, it was noticed that dark condition favoured the germination (36.7 %).

Warakagoda and Subasinghe (2013) studied different pre-treatments in *ex vitro* seed germination and the highest germination rate (97.9 %) was achieved when seeds subjected to sun cracking for 6 hours followed by 2250 mg L^{-1} GA_3 treatment for 24 hours.

Warakagoda and Subasinghe (2014) reported that under *in vitro* condition dipping seeds in 2250 mg L^{-1} GA_3 solution for 24 hours after sun cracking achieved 60 per cent germination followed by 44 per cent germination in seeds dipped in sterilized distilled water for 24 hours after sun cracking. Whereas sun cracking alone or GA_3 treatment alone do not favoured the germination. The sun cracking helped to remove the mechanical barrier created by the hard seed coat for imbibition of water whereas GA_3 treatment and dipping in water allowed the dissolving and leaching out of inhibitory substances present inside the seed. Breaking the hard seed coat coupled with either GA_3 treatment or water dipping enhanced the germination.

2.6. *IN VITRO* SEED GERMINATION OF *C. fenestratum* THROUGH AXENIC SEED CULTURE

Under *in vitro* condition, aseptically maintained sand: coir dust (1:1) or water agar media fortified with 1g L^{-1} activated charcoal (both are nutrient free) were reported to be better for seed germination of *C. fenestratum* than nutrient supplementing MS media fortified sand: coir dust. Sand: coir dust media (without MS medium) reported

39 per cent seed germination whereas water agar media fortified with 1 g L^{-1} showed 35 per cent germination during 12 months of the observation period. Total dark conditions and 32±2˚C temperature reported to be favorable for enhancing the *in vitro* germination of *C. fenestratum* seeds (Warakagoda and Subasinghe, 2015).

2.7. DIFFERENT STERILANTS USED FOR SURFACE STERILIZATION UNDER *IN VITRO* CONDITION

Even though different aseptic techniques are employing strictly in tissue culture, explants will act as the main contaminating agent. Therefore standardization of surface sterilization of explant is one of the most critical steps in tissue culture, to get the culture free of microbial contaminants and thereby to ensure fully aseptic condition. To achieve this different sterilizing agents like calcium hypochlorite, sodium hypochlorite and mercuric chloride and ethanol are mainly used as sterilants. A wrong concentration of sterilant will have a detrimental effect on plant cells and their growth. Therefore, the selection of suitable sterilant, standardization of its concentration and exposure time is important in plant tissue culture.

Mercuric chloride ($HgCl_2$) is a white crystalline solid, widely used as a surface sterilant, because of its strong bactericidal action (Das *et al*., 2012). Warakagoda and Subasinghe (2014) reported that mature seeds of *C. fenestratum* can be successfully surface sterilized by dipping explants in 0.1 per cent mercuric chloride ($HgCl_2$) solution for two minutes.

Daud *et al*. (2012) observed that treatment with 0.2 per cent $HgCl_2$ for 12 minutes was very effective for controlling microbial contamination in seed explant of *Aquilaria malaccensis* collected from the natural environment, whereas treatment with 0.1 per cent $HgCl_2$ for 15 and 30 seconds gave the best results for leaf and nodal explants collected from shade house. Treatment with clorox bleach (5.25 % sodium hypochlorite as the active ingredient) even at high concentration (50 % clorox) was ineffective to control microbes in all three explants used in the experiment.

Treatment with $HgCl_2$ 0.1 per cent for 2 minutes gave 55.55 per cent culture survival and treatment with $HgCl_2$ 0.1 per cent for 4 minutes gave 84.44 per cent survival in the *in vitro* seed germination of *Cucumis sativus*, whereas higher

concentration and longer exposure time showed a detrimental effect. They were reported that the action of $HgCl_2$ might be due to bleaching action of two chloride atoms and also ions that combines strongly with proteins and cause the death of organisms (Firoz *et al*., 2016).

Yadav and Singh (2011) reported that in an *in vitro* study on *Albizia lebbeck*, seed treated with 0.1 per cent $HgCl_2$ for 5 minute gave maximum of 83.3 per cent germination and healthy seedlings while less germination and more percentage of contamination was observed with 0.05 per cent $HgCl_2$. They also reported that with the increase of exposure time and concentration, contamination was significantly reduced, however it affected the germination of seeds.

In *Dendrocalamus membranaceus*, an edible bamboo, combination of 0.1 per cent $HgCl_2$ for 10 minutes and 15 per cent sodium hypochlorite (NaOCl) for 10 minutes was reported to be effective in *in vitro* seed sterilization (Brar *et al*., 2013).

Mercuric chloride was reported to be as a best surface sterilizing agent in many other explants, for example, for shoot tip explants of guava, $HgCl_2$ at 0.05 per cent for 5 minutes plus 70 per cent ethanol gave maximum survival percentage of 67 per cent (Zamir *et al*., 2004). $HgCl_2$ 0.1 per cent treatment for the duration of 12 minutes was reported to be the best with 52.84 per cent survival in young sucker explant of banana (Dangi *et al*., 2009). Surface sterilization of nodal segments of *Bambusa wamin* with 0.15 per cent HgCl2 for 15 minutes yielded 63.33 per cent aseptic cultures (Arshad *et al*, 2005).

Ethanol is a strong phytotoxic sterilizing agent. It is reported that alcohols are rapidly bactericidal rather than bacteriostatic against bacterial vegetative cells, they are also tuberculocidal, fungicidal, and virucidal, however it do not destroy spores. Therefore its treatment alone is ineffective to destroy the bacterial spores. There are reports that ethanol alone is ineffective to control microbes. It was reported that to increase the efficiency of sterilization 70 per cent ethanol treatment was mainly given along with other sterilants. For example Zamir *et al*. (2004) reported that for shoottips of *Psidium guajava* L. Cv. Safeda, mercuric chloride (HgCl2) at 0.05 per cent for 5 minutes plus 70 percent ethanol was found to give maximum survival percentage (67

%). Firoz *et al*. (2016) reported that in the *in vitro* seed germination of *Cucumis sativus* 70 per cent ethanol alone was ineffective in seed surface sterilization.

Sodium hypochlorite was reported to be an antimicrobial agent, due to its mechanism that can cause biosynthetic alterations in cellular metabolism and phospholipid destruction, the formation of chloramines that interfere in cellular metabolism, oxidative action with irreversible enzymatic inactivation in bacteria, and lipid and fatty acid degradation (Estrela *et al*., 2002).

The antimicrobial activity of NaOCl depends on the concentration of undissociated hypochlorous acid (HClO) in the solution. HClO exerts its germicidal effect by an oxidative action on sulfydryl groups of bacterial enzymes. As essential enzymes are inhibited, important metabolic reactions are disrupted, resulting in the death of the bacterial cell. In a study, it was found that 5.25 per cent NaOCl killed microorganisms within seconds (Vianna *et al*., 2004).

In *Althaea officinalis*, an important medicinal herb, seed surface sterilization with 4 per cent NaOCl for 5 minutes was reported to be the best (Younesikelaki *et al.*, 2016).

In tomato seed surface sterilization with 5 per cent NaOCl for 20 minutes was reported to be more effective resulting in high germination rateand contamination free cultures (Himabindu *et al*., 2012).

An *in vitro* study on potato tuber, when sprout of 0.5 to 0.1 was used as explant, NaOCl surface sterilization treatment was found better for controlling the infection and also reported that it had no adverse effect on explants even treated for a long duration. Sodium hypochlorite (NaOCl) for 8 minutes was reported to be effective (Badoni and Chauhan, 2010).

2.8. MICROPROPAGATION

Micropropagation is the technique for large scale vegetative propagation of the plants from living plant cells or parts of the plant, known as explant in an artificially supplied nutrient media, with controlled atmosphere and a fully aseptic condition (George *et al*., 2008).

Theoretically through three methods micropropagation can be achieved, either by axillary bud culture and shoot culture or by adventitious shoots or through adventitious somatic embryos production i.e either direct or indirect organogenesis (George *et al*., 2008).

Direct organogenesis and indirect organogenesis are the two basic terms used in the micropropagation of plants. Direct organogenesis means the production of organs directly from the explant, which does not include intermediary callus formation. It is a more reliable source to get large number of true to type plants. Where explants directly give rise to shoots and roots through direct organogenesis (Einset, 1991; Bhatia *et al.*, 2015).

While indirect organogenesis is the callus mediated organ formation which may induce somaclonal variation in the repeated subculturing cycle. Both direct and indirect organogenesis will produce adventitious shoots, which depend on the explant type, medium, growth hormone types and concentration (Einset, 1991; Bhatia *et al*, 2015).

It is reported that among the different tissue culture techniques shoot culture or axillary bud culture is the most applicable for woody species to get true to type plants (Zimmerman, 1988).

2.8.1. Steps in micropropagation

The micropropagation steps can be divided in to five phases (Kumar and Reddy, 2011).

Phase 0: Production of mother plants for explant collection, under proper hygienic conditions such as inside the polyhouse to minimize the contamination percentage.

Phase 1: Initiation of culture. It includes the selection of suitable explants, sterilization, and culture under the aseptic condition to get an axenic culture.

Phase 2: Shoot regeneration and proliferation and subculturing cycles.

Phase 3: Shoot elongation and root initiation

Phase 4: Acclimatization of plantlets.

2.9. *IN VITRO* SHOOT CULTURE AND AXILLARY BUD CULTURE

In vitro shoot culture means culturing of explants containing an intact shoot meristem, which includes both shoot tips and nodal segments containing axillary buds and lateral meristems. Axillary buds are formed in between the junction of shoots and leaf (George *et al*., 2008).

In axillary bud culture nodal explant containing dormant axillary bud and lateral meristem are cultured *in vitro* and induced to proliferate and give rise to microshoots by use of plant growth regulators like cytokinins. These microshoots produced can either be subdivided in to shoot tips or nodal segments which will serve as the secondary explant for further proliferation or that can be used for the rooting and plantlet production (George *et al*., 2008).

This technology does not involve cell differentiation but includes the development and growth of new shoots from pre-existing meristems by exploiting the normal ontogenetic route for plant development by lateral meristems (Phillips and Hubstenberger, 1995; Ngezahayo and Liu, 2014).

Axillary bud culture is also widely used in the conservation of the endangered plant species through mass clonal propagation. Genetic variations are almost absent in this culture method, which helps to preserve and conserve the main features of the endangered plants (Ngezahayo and Liu, 2014).

2.9.1. Effect of explant type on *in vitro* shoot regeneration

The success of *in vitro* regeneration depends on the type of explant selection and suitable culturing medium optimization. A variety of explants such as leaves, shoots, anthers, ovules, epicotyls, hypocotyls, etc. are used for the multiplication of plant species in different culture methods. In which, there are several reports of successful use of nodal segments containing an axillary bud for shoot regeneration in many woody species and medicinal plants.

Lakshmanan *et al*. (1997) reported that compared to shoot tips, nodal segments (3 node cuttings) were best for *Ixora coccinea* proliferation. They reported that in 2.5 μM BA, about 4 times more shoots developed on stem cuttings when compared to shoot

tips after 6 weeks of culture. The result showed that shoot tip meristem exerts strong apical dominance even in the presence of BA whereas nodal segments gave a high shoot proliferation. Persistence of strong apical dominance in the shoot tip explant was a major constraint in the development of efficient *in vitro* procedures for clonal propagation of woody species.

A woody aromatic and medicinal shrub *Vitex negundo* induced bud break at an optimal concentration of 2.0 mg L^{-1} BA by *in vitro* culture of nodal segments from mature plants (Sahoo and Chand, 1998).

Maerua oblongifolia, a rare ornamental from semi-arid regions of Rajasthan, India can be proliferated using nodal explants (Rathore and Shekhawat, 2011).

Preetha *et al*. (2012) showed that a vulnerable woody medicinal climber, *Embelia ribes* Burm. F. could be clonally multiplied by using nodal segments in MS media.

Warakagoda *et al*. (2017) reported that in *C. fenestratum* only mature nodal cuttings produced multiple shoots, whereas shoot tip and immature shoot did not produced any shoots. They also reported that compared to single nodal cuttings mature double nodal cuttings resulted in the highest shoot proliferation rate when cultured in the WPM medium.

When explants from mature plants shows difficulty in shoot proliferation and regeneration, seedling explants can be used. Many authors tried to develop a feasible protocol using seedling explants in medicinal plants, due to its high frequency regeneration capacity. Shoot tips, nodal segments, epicotyl and hypocotyl portion and cotyledonary nodes can be used as explants from the seedling. The shoots produced from these cultures will be true to type to the seedling but not to mother plant from which seeds are collected.

Komalavalli and Rao (2000) reported that in *Gymnema sylvestre* when micropropagation was carried out a maximum number of shoots (57.2) were induced from 30 day old seedling axillary node explant on MS medium.

Jeyakumar and Jayabalan (2002) reported that *Psoralea corylifolia* L. a medicinal plant belongs to Leguminosae, could be *in vitro* multiplied using cotyledonary explant.

Faisal *et al*. (2006) reported that in *Mucuna pruriens*, an important medicinal plant in India, shoot proliferation was achieved when cotyledonary nodal explant cultured on MS medium supplemented with 5.0 µ*M* BA.

Faisel *et al*. (2007) reported that an endangered medicinal plant, *Tylophora indica* Burm. f. Merrill, can be multiplied *in vitro* via enhanced axillary bud proliferation from nodal explants collected from young shoots of a two year old plant.

Nayak *et al*. (2007) reported that high-frequency plantlet regeneration can be achieved in cotyledonary nodes of one month old *Aegle marmelos* seedlings by growing seedlings *in vitro* on MS medium supplemented with 6.6 µM BA and 1.14 µM IAA. Siddique and Anis (2007) reported that *Cassia angustifolia* could be successfully regenerated through nodal explants excised from 14 day old aseptic seedlings.

Husain *et al*. (2008) reported that *Pterocarpus marsupium*, a valuable multipurpose forest tree can be *in vitro* multiplied using nodal explants obtained from *in vitro* raised 18 day old axenic seedlings in MS media by supplementing BA and IAA as growth regulators. Sharma and Shahzad (2008) reported that *in vitro* shoot production in *Abelmoschus moschatus* Medik. L. (muskdana plant) cotyledonary nodal explant, when cultured on MS medium supplemented with 0.01 mg L^{-1} TDZ.

Parveen and Shahzad (2010) reported that *in vitro* shoot production of *Cassia sophera* was successfully done using cotyledonary nodal explant excised from 21 days old axenic seedlings, when cultured on MS medium supplemented with 2.5 µM TDZ. Senarath, (2010) reported that multiple shoots of *C. fenestratum* were formed in MS medium when seedling epicotyl was used as explant.

2.9.2. Effect of medium type on *in vitro* shoot regeneration in different plants

The type of medium used for tissue culture depends on the plant species. Salt and plant growth regulator requirements are specific to each species and also largely vary from different explants used. Growth and development of explant under *in vitro*

condition depends on its genetics, surrounding environmental conditions and tissue culture medium components. Standardization of the tissue culture medium for a particular species and its particular explant include series of trial and error experiments (Trigiano and Gray, 2011).

Tissue culture medium generally consist of water, macro and micronutrients, vitamins, organic compounds, sugar, agar and plant growth hormones.

Macronutrients like nitrogen, potassium, magnesium, calcium, phosphorus, and sulfur are major contents in the medium which are required more than micronutrients like boron, iron, manganese, molybdenum, copper, and zinc. These nutrients have different roles in plant growth and development which is shown in Table 1 (Trigiano and Gray, 2004; Bhatia, 2015).

Table 1. Roles of different nutrients in plant growth and development

Nutrients	**Functions in plant cell**
Nitrogen	Protein and nucleotide synthesis
Potassium	Regulate osmotic potential
Phosphorus	Protein and nucleotide synthesis
Sulfur	Protein and nucleotide synthesis
Magnesium	Membrane integrity, enzyme cofactor
Calcium	Cell wall synthesis
Zinc	Enzyme cofactor, chlorophyll biosynthesis
Molybdenum	Enzyme cofactor, component of nitrate reductsase.
Boron	Involved in different enzymatic activity
Copper	Enzyme cofactor, electron transfer reaction
Iron	Electron transfer
Manganese	Enzyme cofactor

Sugar is the most important carbon and energy source in the media which is applied to compliment the carbon available to plant through photosynthesis since cultured tissue has less photosynthesis efficiency. Sucrose 30 g L^{-1} is generally used in tissue culture media as sugar source. Instead of sucrose mono or disaccharides and sugar alcohols such as glucose, fructose, sorbitol, and maltose can also be used (Trigiano and Gray, 2004).

In addition to this, tissue culture medium is also supplemented with vitamins. Vitamins are the organic supplements required for various metabolic processes of the plant as cofactor or parts of the enzymes. Thiamine is the major one, which is involved in the carbohydrate metabolism and biosynthesis of some amino acids, needed to supply exogenously as thiamine hydrochloride. Other vitamin like nicotinic acid, which play role in the respiratory coenzymes, pyridoxine which plays a role as a coenzyme in metabolic reactions and biotin also supplemented to the tissue culture mediums to improve the growth (Trigiano and Gray, 2004; Bhatia, 2015).

Myoinocitol is a plant vitamin, which is sugar alcohol involved in thesynthesis of phospholipids, cell wall pectins and membrane systems in cell cytoplasm also supplemented to the tissue culture medium for proper growth and development (Trigiano and Gray, 2004).

Among the different mediums so far developed, Murashige and Skoogmedium (1962) is the most commonly used tissue culture medium, it was originally developed for tobacco callus culture. This medium was well accepted because of the content of nitrogen both in ammonium and nitrate form. Even though, these standard media generally applicable to herbaceous plants found to be not successful for the majority of the woody species, due to the total high ionic strength of the media (Trigiano and Gray, 2011; Phillips and Garda, 2019).

Woody plant medium (WPM), which was developed by Lloyd and McCown (1980) with less salt concentration was widely used for salt sensitive woody species culture. Compared to MS, the WPM has less total nitrogen and less ammonium content. There are many reports that woody plant medium is better for woody species for the morphogenic response (Trigiano and Gray, 2011; Phillips and Garda, 2019).

2.9.3. Effect of plant growth regulators on *in vitro* shoot regeneration in different plants

Plant growth regulators can be either naturally occuring substances or synthetic chemicals, which includes auxins, cytokinins, gibberellic acid, abscisic acid and ethylene, which either promote or inhibit the plant growth and development. These are widely used in micropropagation for the morphogenic response. The level of organogenesis depends on the kind of growth regulator used, its concentration, combination or absence of other growth regulators. A growth hormone that elicits a morphogenic response in a particular concentration may fail to give the same response in a different concentration for the same explant (Minocha, 1987).

In tissue culture when small explants are inoculated to culture media, they are not able to synthesis plant growth regulators by its own. However the supply of exogenous plant growth hormones will help to make a desirable developmental and growth response from explant (Trigiano and Gray, 2004; Bhatia, 2015).

In tissue culture, cytokinins play roles like alleviate apical dormancy, allowing shoot proliferation, and to stimulate the cell division and differentiation. Cytokinins divided into two groups, adenine type cytokinins and phenyl urea type cytokinins. Kinetin, zeatin, benzyl adenine are the adenine type cytokinins. TDZ is the phenyl urea type cytokinins. The most widely used cytokinins are BA and kinetin, both are synthetic cytokinins. Several reports are proving that the exogenous supply of these cytokinins help in shoot proliferation (Trigiano and Gray, 2011). The applied cytokinins break the apical dominance and help to axillary bud proliferation and shoot multiplication from the lateral meristem. The response of the explant to different cytokinins also shows variation (Trigiano and Gray, 2004).

Another group of PGR used in tissue culture is auxins. IAA is the natural auxin while 2, 4-dichlorophenoxyacetic acid (2, 4-D), Indolyl-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are the widely used synthetic auxins. Auxin help in root and callus production, shoot elongation and help in cell elongation and cell division. Along with cytokinins which also help to the differentiation of phloem and xylem (Trigiano and Gray, 2004).

Cytokinin to auxin ratio also play a crucial role in plant tissue culture. A high ratio of auxin to cytokinin induces root formation while a low ratio induces axillary shoot production in axillary bud culture (Trigiano and Gray, 2004).

The morphogenic response of a woody plant in micropropagation is difficult compared to other herbaceous species. The principle of micropropagation via cytokinin-controlled shoot growth is not applicable to all the woody plants.

Einset (1991) reported that the ability of shoots to be manipulated by cytokinin treatments under *in vitro* appears to vary from different taxa of woody species. According to the study woody species are classified into three groups.

1) Species that exhibit an inherent response as explants to cytokinin
2) Species that respond sporadically as explants or only after repeated subculturing
3) Species that do not show response to cytokinin treatments

They also reported that the cytokinin response also depends on the maturity of the shoot explant. Sometimes juvenile shoots become responsive but adult shoots become unresponsive to cytokinin. So it is important to standardize a suitable medium and plant growth hormone concentration for a particular plant species for successful *in vitro* culture.

2.9.4. Effect of media and plant growth regulators on *in vitro* shoot regeneration in different plants

Warakagoda *et al*. (2017) reported that *in vitro* clonal propagation of *C. fenestratum* was successful in the WPM. Double nodal cuttings explant cultured on WPM supplemented with 2.0 mg L^{-1} BAP and 1mg L^{-1} TDZ and 0.4 mg L^{-1} 2, 4-D gave the highest shoot proliferation rate of 3.90 shoots per explant.

Senarath (2010) described a protocol for *in vitro* propagation of *C. fenestratum* (Gaertn.) Colebr. An average of 5 multiple shoots were obtained in 75 days during the culture period, when epicotyl explants were cultured on MS medium supplemented with 1.0 µM kinetin and 0.25 µM 2, 4-D.

Lakshmanan *et al*. (1997) established a rapid *in vitro* multiplication method for *Ixora coccinea* L. using stem cuttings (3 nodal cuttings) as explant. Cytokinin, BA at a concentration of 2.5 µM in WPM gave the maximum multiple shoots up to 27 shoots per explant after 6 weeks in culture.

Preetha *et al*. (2012) showed that for woody medicinal climber *Embelia ribes* Burm. f. MS medium supplemented with 0.5 mg L^{-1} BA was found to be most effective for shoot bud induction resulting in a maximum of 6 shoots from nodal explants after 4 weeks of culture.

Anjusha and Gangaprasad (2016) reported the micropropagation of *Gynochthodes umbellata* on MS medium using nodal explants. Among different cytokinins at different concentrations used, BA promoted high multiple shoot proliferation, at 2 mg L^{-1} BA concentration. An average of 6.8270 ± 0.28 shoots/nodes were obtained.

Komalavalli and Rao (2000) developed a protocol for *Gymnema sylvestre* shoot proliferation, in MS media. Maximum number of shoots (57.2) were induced from 30 day old seedling axillary node explants, when supplemented with 1 mg L^{-1} BA, 0.5 mg L^{-1} kinetin, 0.1 mg L^{-1} IAA, 100 mg L^{-1} malt extract and 100 mg L^{-1} citric acid.

Faisel *et al*. (2007) reported that an endangered medicinal plant, *Tylophora indica* (Burm. f.) Merrill, can be *in vitro* multiplied using nodal explants on MS media. In MS medium supplemented with 2.5 µM BA, 0.5 µM NAA and 100 mg L^{-1} IAA at pH 5.8, maximum number of shoots (8.6 ± 0.71) and the maximum average shoot length (5.2 ± 0.31 cm) were obtained.

Axillary bud proliferation and direct organogenesis have been developed for *Salacia reticulate*. Nodal segments were cultured on MS media supplemented with different growth regulators. The most efficient shoot multiplication was obtained with the supplementation of BA 3, 5 mg L^{-1} and IAA 0.5 mg L^{-1} (Dhanasri *et al*., 2013).

A woody aromatic and medicinal shrub *Vitex negundo* induced bud break at an optimal concentration of 2.0 mg L^{-1} BA in MS medium by *in vitro* culture of nodal segments from mature plants (Sahoo and Chand, 1998).

Husain *et al*. (2008) reported that *Pterocarpus marsupium* could be micro propagated through seedling explants on MS medium supplemented with 4.0 µM BA, 0.5 µM IAA and 20 µM adenine sulphate.

2.9.5. Effect of pH of the medium for *in vitro* shoot regeneration

pH is the negative logarithm of hydrogen ion activity. The pH of tissue culture media highly affect the absorption of ions and solidification of culture media. Optimum pH for culture media is 5.6 to 5.8, before sterilization. The higher pH (>6) gives a hard media and reduces the absorption of ions, and lower pH (< 5) value does not allow the gelling of the agar. Higher or lower pH of the medium affects the gelling efficiency of agar, uptake of ingredients, solubility of different salts, and chemical reactions in the medium (Bhatia, 2015).

2.9.6. Effect of activated charcoal as an additive on tissue culture medium

Activated charcoal has very fine pores with a large inner surface area, which helps to absorb many molecules both in the liquid and gas phases. It is added to tissue culture media to improve plant growth and development, absorption of phenolic compounds exudated from the explant and to reduce explant browning. Activated charcoal help to improve shoot multiplication and elongation, rooting, somatic embryogenesis, protoplast culture, anther and microspore culture and synthetic seed production. Even though it is helpful to reduce toxic substances, it may also absorb plant growth hormones (Thomas, 2008).

It was reported that in *C. fenestratum* tissue culture, the addition of activated charcoal reduced the phenolic compound released to the culture media by shoot tip and nodal explant (Warakagoda *et al.*, 2017).

2.9.7. Effect of solidifying agents in the media for *in vitro* shoot regeneration

Solidifying agents are materials, added to tissue culture media for getting a semisolid consistency to the media, which in turn enable explants to be placed on the culture media. Agar, a high molecular weight polysaccharide obtained from seaweeds, is a main solidifying agent used in tissue culture. It is added to the concentration ranging from 0.5 to 1 % (6-8 g L^{-1}). Agar is preferred over other gelling agents like agarose and

phytagel because it will not react with plant hormones and will not get digested by plant enzymes (Bhatia, 2015).

2.9.8. Effect of media and plant growth regulators on *in vitro* root production

Senarath, (2010) reported that in *C. fenestratum in vitro* propagation, multiple shoots obtained from seedling epicotyl explants shown 100 per cent *in vitro* rooting in half-strength MS media supplemented with 2.5 µM IBA.

Maerua oblongifolia, a rare ornamental from semi-arid regions, was proliferated in MS media using nodal explants and micro shoots obtained were rooted in half-strength MS media containing 3.0 mg L^{-1} of IBA (Rathore and Shekhawat, 2011).

Komalavalli and Rao (2000) reported that in *Gymnema sylvestre* micropropagation, high-frequency rootings were obtained when axillary node explant derived shoots were cultured on half-strength MS media supplemented with IBA (3 mg L^{-1}).

Faisel *et al*. (2007) reported that in micropropagation of an endangered medicinal plant, *Tylophora indica* (Burm. f.) Merrill, *in vitro* regenerated shoots rooted on half strength MS media supplemented with 0.5 µM IBA.

2.10. ENDOPHYTES IN *C. fenestratum*

All microbes which colonize within the plant organs is commonly known as the endophytes, which also include both beneficial bacterial prokaryotes and fungal eukaryotes whose infections are internal and not visible, and in which the infected host tissues are at least transiently symptomless (Schulz and Boyle, 2006).

Goveas *et al*. (2011) isolated 41 endophytic fungi belonging to sixteen different taxa from 195 samples of both leaves and stem of *C. fenestratum*. The identified species of the endophytic fungi mainly belongs to Ascomycota. The isolated genera of endophytes were; *Phomopsis jacquiniana, Alternaria alternata, Aspergillus tamarii, Aspergillus fumigatus, Drechslera, Fusicladiella, Penicillium senticosam, Cladosporium cladosporioides, Asperisoprium caricae, Staphylotrichum cocosporium, Nigrospora oryzae, Mycelia sterilia, Gliocladium roseum, and Cladosporium sp.*

2.11. EMBRYO CULTURE

The isolation of immature or mature zygotic embryos from the ovules and seeds of higher plants and their culture in a defined media under controlled and fully aseptic conditions to get a viable plantlet is known as embryo culture. It is mainly divided into two categories, mature and differentiated seed embryo culturing and immature early division phase proembryo culturing (Raghavan, 1961; Bridgen, 1994).

The embryo has a bipolar structure, with primordial root radicle meristem on one side and primordial shoot plumule meristem on another side. Immature embryo culture is mainly used to rescue the embryos before abortion by supplementing nutrients for proper growth development. Mature embryos can be easily cultured in the medium supplemented with mineral salts and sucrose compared to immature embryos. Mature embryo culture is mainly used when seed germination inhibitors interfere with the seed germination (Raghavan, 1961; Bridgen, 1994).

The nutritional requirement of the embryo depends upon its developmental stages. During the developmental process of an embryo, it has two stages, heterotropic early stage and autotropic later stage. In a heterotropic immature stage it requires exogenous supplementation of plant growth regulators for the development in the culture media, while when autotropic mature embryos are excised and cultured, it does not need an exogenous supply of plant growth regulators. During the autotropic phase, embryos are capable of synthesizing substances for their growth and development from salts and sugars. Hence they can be easily cultured on the basal media supplemented with sugar as carbon source (Raghavan, 1961; Bridgen, 1994).

The main applications of embryo cultures are in plant breeding works and in plant conservation areas. Embryo culture used to understand the nutritional requirement of an embryo in different growth stages, rescue the immature embryos before abortion in wild crosses and wide hybridization, helps to reduce breeding cycle and overcome the seed dormancy. It can be used as an explant for micropropagation (Bridgen, 1994).

Sucrose is the carbon energy source in the culture media which also maintains the osmotic potential of the media, which is required in the range of 2 per cent to 3 per cent for mature embryo culture. While when immature embryos were cultured higher

sucrose concentration up to 8 to 12 percent was needed. Agar was the main solidifying agent used in embryo culture (Bridgen, 1994).

Embryo culture plays an important role in the conservation of endangered species, which is showing difficulty in seed germination due to dormancy. There are many reports of successful embryo culture. Kaveri and Rao (2015) reported mature embryo culture of *Nothapodytes foetida* (Wight) Sleumer in MS medium.

Kagithoju *et al*. (2013) reported the mature embryo culture of an endangered forest tree species *Strychnos potatorum* on full strength MS media containing 20 g L^{-1} sucrose.

Green zygotic embryos excised from forest tree *Boswellia serrata* could be cultured on MS medium containing 3 per cent sucrose together with 200 mg L^{-1} PVP (Ghorpade *et al*., 2010).

Mature embryo culture of *Khaya grandifoliola*, an endangered tree species on MS medium supplemented with growth hormones was reported (Okere and Adegeye, 2011).

Gibberellic acid increased the embryo growth and conversion of embryos into plantlets in the *in vitro* germination of coconut zygotic embryos (Ake *et al.*, 2007).

Plate 1a. *Coscinium fenestratum* plant maintained at College of Horticulture, KAU medicinal plant field

Plate 1b. Female plant leaves

Plate 1c. Male plant leaves

Plate 1. *Coscinium fenestratum* plant

Plate 2a. *Coscinium fenestratum* female flowers

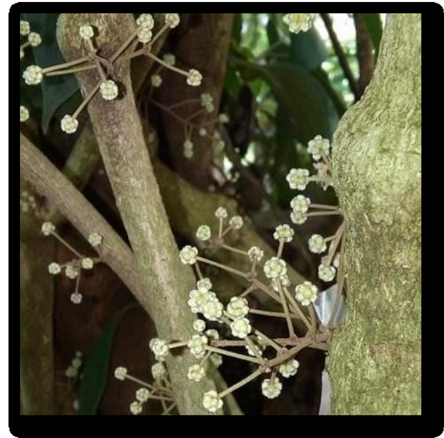


Plate 2b. *Coscinium fenestratum* male flowers

Plate 2c. *Coscinium fenestratum* fruits

Plate 2d. *Coscinium fenestratum* seeds

Plate 2. *Coscinium fenestratum* plant

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Plant regeneration of *Coscinium fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture" was carried out at the tissue culture laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the academic year 2018-2020. Details of materials used and the methods followed are presented below.

3.1. PREPARATION OF THE CULTURE MEDIA

3.1.1. Murashige and Skoog (MS) Medium

3.1.1.1. Preparation of MS medium stock solution

Concentrated stock solutions for preparation of MS medium were made and stored in the refrigerated condition at 4 ˚C. MS medium was prepared as five separate stocks, the contents in the stock solutions of MS medium is depicted in Table 2. It consisted of major salts stock (Stock 1, 50x concentration), calcium chloride stock (Stock 2, 50x concentration), EDTA and iron stock (Stock 3, 100x concentration), minor salts stock (Stock 4, 100x concentration) and vitamins and organic supplements stock (Stock 5, 100x concentration). These stock solutions were prepared separately by dissolving the required amounts of contents in a measured volume of distilled water and stored in amber coloured bottle in refrigerated condition and regularly checked for contamination.

3.1.1.2. Preparation of MS medium and sterilization

To prepare one litre of MS medium 20 ml each of stock solution I and II and 10 ml each of stock solution III, IV and V were pipetted out from the respective stock solutions into a beaker. Then 30 g of sucrose was added and dissolved completely by adding distilled water. To this 100 mg of inositol was added, and stirred until it dissolved completely. Volume was then made up to one litre by using distilled water. After that required quantity of the plant growth hormones were added. Then medium pH was adjusted between 5.6 to 5.8, with 1N NaOH or HCl. To this, gelling agent agar 7.5 g L^{-1} and 1 g L^{-1} activated charcoal were added and mixed thoroughly. The medium was boiled to dissolve the agar and the required quantity (15 to 20 ml medium) was

poured into each test tubes (25×150mm). All the test tubes were cotton plugged tightly, and made into bundle of seven test tubes. Top of the bundles was wrapped with thick paper and autoclaved at 121°C for 20 minutes. After sterilization, test tubes were arranged in test tube stands and kept overnight for solidification in sterile area of tissue culture lab and used within a week. These sterilized test tubes with medium were kept inside the laminar airflow chamber under UV radiation for 20 minutes before use.

3.1.2. Woody Plant Medium (WPM)

3.1.2.1. Preparation of WPM stock solution

Concentrated stock solutions for preparation of WPM were made and stored in the refrigerated condition at 4 ˚C. WPM was prepared as six separate stocks. The contents in the stock solutions of WPM are given in Table 3. It consisted of major salts stock (Stock 1, 50x concentration), calcium nitrate stock (Stock 2, 50x concentration), calcium chloride (Stock 3, 50x concentration), EDTA and iron stock (Stock 4, 100x concentration), minor salts stock (Stock 5, 100x concentration) and vitamins and organic supplements stock (Stock 6, 100x concentration). These stock solutions were prepared separately by dissolving the required amounts of contents in a measured volume of distilled water and were stored in amber coloured bottle in refrigerated condition and regularly checked for contamination.

3.1.2.2. Preparation of WPM and sterilization

To prepare one litre of medium 20 ml of each of stock solutions I, II, III and 10 ml each of stock solutions, IV, V and VI were pipetted out from the respective stock solutions into a beaker. To this 30 g of sucrose was added and dissolved it completely by adding little amount of distilled water. 100 mg of inositol was added to this solution, and stirred until it dissolved completely. The volume was made up to one litre using distilled water. After that required quantity of the plant growth hormones were added. Then media pH was adjusted to 5.6 to 5.8 with 1N NaOH or HCl. After that gelling agent agar 7.5 g L^{-1} and 1 g L^{-1} activated charcoal were added and mixed thoroughly. The medium was then boiled to dissolve agar and the required quantity (15 to 20 ml medium) was poured into test tubes (25×150 mm capacity culture tubes). Sterilization of medium was done as described in 3.1.1.2.

Table 2. Composition of Murashige and Skoog (MS) Medium

Chemicals	mg L^{-1}	Stock	Stock g L^{-1}
Stock 1			
Ammonium nitrate ($(NH_4)_2NO_3$)	1650	50x	82.5
Potassium nitrate (KNO_3)	1900		95
Potassium dihydrogen phosphate (KH_2PO_4)	170		8.5
Magnesium sulphate ($MgSO_4.7H_2O$)	370		18.5
Stock 2			
Calcium chloride ($CaCl_2.2H_2O$)	440	50x	22
Stock 3			
Na_2EDTA	37.3	100x	3.7
$FeSO_4.7H_2O$	27.8		2.8
Stock 4			
Manganese sulphate ($MnSO_4.4H_2O$)	22.3	100x	2.23
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6		0.86
Boric acid (H_3BO_3)	6.2		0.62
Potassium iodide (KI)	0.83		0.083
Copper sulphate ($CuSO_4.5H_2O$)	0.25		0.025
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25		0.025
Copper chloride ($CoCl.6H_2O$)	0.02		0.002
Stock 5			
Glycine	2.0	100x	0.2
Pyridoxine	0.5		0.05
Nicotinic acid	0.5		0.05
Thiamine	0.1		0.01

Table 3. Composition of Woody plant medium (WPM)

Chemicals	**mg L^{-1}**	**Stock**	**Stock g L^{-1}**
Stock 1			
Potassium sulphate ($(NH_4)_2SO_4$)	990	50x	49.5
Ammonium nitrate NH_4NO_3	400		20.0
Potassium dihydrogen phosphate (KH_2PO_4)	170		8.5
Magnesium sulphate ($MgSO_4.7H_2O$)	370		18.5
Stock 2			
Calcium nitrate $Ca(NO_3)2.4H_2O$	556	50x	27.8
Stock 3			
Calcium chloride ($CaCl_2.2H_2O$)	96	50x	4.8
Stock 4			
Na_2EDTA	37.3	100x	3.7
$FeSO_4.7H_2O$	27.8		2.8
Stock 5			
Manganese sulphate ($MnSO_4.4H_2O$)	22.3	100x	2.23
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6		0.86
Boric acid (H_3BO_3)	6.2		0.62
Copper sulphate ($CuSO_4.5H_2O$)	0.25		0.025
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25		0.025
Stock 6			
Glycine	2.0	100x	0.2
Pyridoxine	0.5		0.05
Nicotinic acid	0.5		0.05
Thiamine	1.0		0.1

3.2. PREPARATION OF THE PLANT GROWTH REGULATORS (PLANT GROWTH HORMONE) STOCK SOLUTION

For preparing 1000 mg L^{-1} (1000 mg / 1000 ml) stock solution of growth hormones, 100 mg of growth hormone was taken in a sterilized small beaker and dissolved with few drops of 1 N NaOH. Then it was poured into a volumetric flask of 100 ml and the volume was made up by using distilled water and then stored in the refrigerated condition. In the present study, five plant growth hormones namely synthetic cytokinins like 6-Benzylaminopurine and kinetin, auxins like 2,4-D and IBA, and Gibberellins were used in the different concentrations for different purpose.

The volume of stock solution needed was calculated using the formula $N1V1=N2V2$

Where N1 = Concentration of stock solution

V1 = Volume of stock solution to be pippeted

N2 = Concentration of growth hormone needed

V2 = Volume of final medium

3.3. STERILIZATION OF GLASSWARES AND OTHER ACCESSORIES

All glasswares and other accessories used were cleaned using detergent and dried in the oven before use. The scalpels, forceps, knife, plates and beakers and other accessories needed were wrapped in an aluminum foil and sterilized in an autoclave at 121 ˚C for 20 minutes before use.

Contaminated test tubes were autoclaved with cotton plugs intact, then washed using detergent and dried in an oven for reusing the test tubes.

3.4. MAINTENANCE OF ASEPTIC CONDITION

3.4.1. Maintenance of aseptic condition during inoculation

All inoculation procedures were carried out inside a laminar airflow chamber (LAF). Prior to inoculation, the base portion of the LAF was wiped with 95 per cent ethanol to disinfect the surface area. All sterilized culture vessels, equipments and other materials needed for inoculation and culture media were placed inside, and LAF was

closed and UV light was switched on for 20 minutes before culturing. After UV treatment, switched on the light and airflow, then pretreated explants were kept inside the LAF. Hands were washed using soap water and wiped with 70 per cent alcohol before the work to check contamination from hand. The instrument such as knife, forceps and scalpels were dipped in absolute alcohol, followed by flaming and cooling before every use. Test tube in which inoculation has to be carried out was taken and cotton plug was removed by placing near the flame in LAF. The rim of the test tubes were flamed for few seconds and sterilized explants were inoculated quickly by using a sterilized forceps. Then again the rim of the test tube was heated and cotton replugged. After completing the inoculation of explants all the used equipments were removed from LAF and again wiped with absolute alcohol.

3.4.2. Maintenance of aseptic condition in tissue culture room

Tissue culture room was fumigated with potassium permanganate and formaldehyde (In pertriplates 5 ml of formaldehyde solution was taken and to this added 500 mg of potassium permanganate) at bimonthly intervals to remove the contaminants from the environment.

3.5. *IN VITRO* SEED GERMINATION STUDIES

3.5.1. Identification of best surface sterilization procedure for establishment of seeds in aseptic cultures

3.5.1.1. Collection of seeds and preliminary treatments in the field

Healthy ripened fruits were collected from the mother plant maintained in the medicinal plant garden of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the ripening season of *C. fenestratum* i.e., from July to August. Fruits were collected directly by hand plucking. Seeds were extracted from the fruits and adhering pulp was removed. After that seeds were rubbed with a sterilized stone, to smoothen the hard seed coat. Then seeds were thoroughly washed, packed inside a clean polythene cover and brought into the laboratory.

3.5.1.2. Pre-treatments in the laboratory

To reduce fungal contamination seeds were pre-treated with broad spectrum fungicide bavistin at 0.3 per cent (w/v) for 20 minutes with continuous shaking using a shaker. After this seeds were washed thrice with distilled water.

To minimize the bacterial contamination seeds were pre-treated with streptocycline 0.1 per cent (w/v) for 20 minutes with continuous shaking by a shaker. After this seeds were washed three times with distilled water. Later seeds were immersed in 4000 mg L^{-1} GA_3 solution for 72 hours inside a sterilized bottle. These seeds were used for further surface sterilization studies.

3.5.1.3. Surface sterilization and inoculation of pretreated seeds

Different surface sterilization treatments were carried out inside the laminar air flow chamber under fully aseptic conditions. After each exposure to chemicals, seeds were washed three times with the sterilized water. Surface sterilization treatments studied for decontamination of seeds were listed in the Table 4.

3.5.1.4. Inoculation of seeds

After surface sterilization treatments seeds were kept for drying for 30 minutes inside the laminar air flow chamber, and then inoculated in the basal MS media without growth hormones. The experiment was conducted in a completely randomized block design (CRD) with five replications per treatment, each with 15 seeds. The observations were taken for a period of one month at three days intervals. Percentage of culture establishment were made and recorded and statistically analysed.

Table 4. Surface sterilization treatments used for decontamination of seeds

Treatments No.	Surface sterilization method
T_1	20 % (v/v) clorox solution for 5 min followed by 70 % (v/v) ethanol for 2 min
T_2	20 % (v/v) clorox solution for 3 min followed by 70 % (v/v) ethanol for 1 min
T_3	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 2 min
T_4	20 % (v/v) clorox solution for 10 min followed by 70 % (v/v) ethanol for 5 min
T_5	50 % (v/v) clorox solution for 3 min followed by 70 % (v/v) ethanol for 1 min
T_6	50 % (v/v) clorox solution for 5 min followed by 70 % (v/v) ethanol for 2 min
T_7	4 % (v/v) sodium hypochlorite solution (NaOCl) for 3 min followed by 70 %(v/v) ethanol for 1 min
T_8	4 % (v/v) sodium hypochlorite (NaOCl) solution for 5 min followed by 70 % (v/v) ethanol for 2 min
T_9	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 5 min
T_{10}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 10 min
T_{11}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 15 min
T_{12}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 20 min
T_{13}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 30 min

3.5.2. Identification of best *in vitro* seed germination media

Attempts were made to germinate the *C. fenestratum* seeds under *in vitro* condition.

3.5.2.1. Prepararion of the media

Three sets of media were studied to select the best one for seed germination. The first set used was MS basal medium. It was prepared as per standard method with 1 g L^{-1} activated charcoal to absorb the toxic exudates.

The second and third sets were the sand and coir pith media. For preparing it, sand and coir pith were washed thoroughly and removed the dirt and contaminants. It was then sterilized for 1 hour to remove the soil borne pathogens. After that, both mixed equally in a 1:1 ratio and used to fill $1/4^{th}$ portion of large test tubes. Medium in one set of test tubes was soaked with liquid MS media and other with distilled water. All test tubes were cotton plugged and sterilized for 20 minutes.

3.5.2.2. Inoculation and incubation of seeds in different media

The GA_3 pre-treated and surface-sterilized seeds were cultured in different media under the laminar airflow chamber as shown in Table 5. Seeds were incubated in the culture room under 25±2˚C temperature and dark conditions. Total darkness was provided by covering the culture racks with black cloth.

Table 5. Media for seed germination studies

Treatment No.	**Media composition**
T_1	MS media with 1 g L^{-1} Activated charcoal
T_2	Sterilized sand : coir pith (1:1) soaked with liquid MS media
T_3	Sterilized sand: coir pith (1:1) soaked with distilled water

The experiment was conducted in a completely randomized design (CRD) with five replications per treatment, each with 15 seeds. The observations were taken for 6 months and the data's were statistically analyzed. Observations on the germination percentage and time taken for germination were recorded.

3.6. SHOOT INITIATION AND MULTIPLICATION STUDIES

3.6.1. Shoot initiation and multiplication from cotyledonary nodal explants

Ex vitro germinated 1 to 2 month old seedlings were collected from polyhouse and washed thoroughly to remove adhering mud and contaminants and brought soon into the laboratory. Cotyledonary nodal portion from the seedling was cut in such a way that a slanting cut was given in the lower portion of seedling and a horizontal cut was given in the upper portion, and taken as explant. Explants were washed with liquid detergent for 10 minutes with continuous shaking by a shaker and then washed under running tap water for 30 minutes.

Explants were pre-treated with 0.3 per cent (w/v) bavistin for 20 minutes with continuous shaking by using shaker to remove the surface fungal contaminantion load, and then washed three times with distilled water. Explants were then pretreated with 0.1 per cent (w/v) streptocyclin for 20 minutes with continuous shaking to reduce the bacterial contaminants and followed by distilled water washing for 3 times.

After pre-treatment, explants were surface sterilized with 0.1 per cent (w/v) mercuric chloride for 2 minutes and ethanol 70 per cent (v/v) for 1 minute inside the laminar airflow chamber. After each treatment, the explants were rinsed three times with sterilized water to remove adhering sterilants. Then the cut ends of nodal segments were trimmed to remove dead cells and cultured on each treatment media, which was listed in Table 6.

Experiment was laid out in completely randomized design (CRD) with three replications per treatment, each with 5 explants. Organogenic response of explants were recorded for three months. Observations on the percentage of culture establishment and shoot induction, time taken for shoot induction, number of multiple shoot/culture were recorded and the data were statistically analysed.

Table 6. Media and plant growth regulators combinations for shoot induction from cotyledonary nodal explants

Treatment No.	Media composition
T_1	Control (MS basal)
T_2	MS + BA 0.1 mg L^{-1}
T_3	MS + BA 0.2 mg L^{-1}
T_4	MS + BA 0.4 mg L^{-1}
T_5	MS + Kinetin 0.1 mg L^{-1}
T_6	MS + Kinetin 0.2 mg L^{-1}
T_7	MS + Kinetin 0.4 mg L^{-1}
T_8	MS + BA 0.1 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}
T_9	MS + BA 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}
T_{10}	MS + BA 0.4 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}
T_{11}	MS + Kinetin 0.1 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}
T_{12}	MS + Kinetin 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}
T_{13}	MS + Kinetin 0.4 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}

3.6.2. Shoot initiation and multiplication from nodal segments with axillary bud taken from poly house grown plants

3.6.2.1. Maintenance of mother plants

Mother plants were maintained inside the polyhouse and regularly sprayed with fungicides.

3.6.2.2. Collection of explant and pretreatments

The cuttings were collected using a sharp knife or secateur from the *C. fenestratum* mother plants maintained inside the polyhouse and brought soon into the laboratory. Leaves were trimmed from these cuttings and single nodal cuttings of about 2 to 2.5 cm length with a bud was taken as explant. A slanting cut was given in the lower portion of nodal cuttings (explants) and transverse cut was given in the upper portion to identify the bud region. Then hairs present in the nodal segments were removed as much as possible by placing each node under running water and with gentle scrubbing by hand, care was taken not to damage the bud region. Then explants were washed with liquid detergent for 10 minutes with continuous shaking, followed by running tap water for 30 minutes.

Nodal segments were then pretreated with 0.3 per cent (w/v) bavistin for 20 minutes with continuous shaking by using a shaker, to minimize fungal load in the explant, followed by washing with distilled water for three times. After that explants were treated with 0.1 per cent (w/v) streptocycline for 20 minutes with continuous shaking by using a shaker to remove the bacterial contaminants. Then washed with distilled water for 3 times.

These nodal segments were surface sterilized with 0.1 per cent (w/v) mercuric chloride for 2 minutes and ethanol 70 per cent (v/v) for 1 minute inside the laminar airflow chamber. After each treatment, the explants were rinsed three times with sterilized water to remove adhering sterilants. The cut ends of the nodal segments were trimmed to remove dead cells and cultured on media, listed in Table 7. The basal culture medium consisted either of Murashige and Skoog's medium or Woody plant medium supplemented with different growth regulators. All the media were

supplemented with 30 per cent sucrose as carbon source, 0.1g L^{-1} inositol and 1g L^{-1} activated charcoal as an antioxidant and toxic absorbent, and 7.5 g L^{-1} of agar as a gelling agent. The cultures were maintained at 25±2 ˚C inside the culture room.

The experiment was laid out in CRD with three replicationsper treatment, each with 15 explants. Internode explant organogenic response was recorded for three months. Observations on the percentage of culture established and shoot induction, time taken for shoot induction, number of multiple shoot/culture were recorded and statistically analysed.

Table 7. Media and plant growth regulators for shoot induction from nodal explants

Treatments No.	Media composition
T_1	MS basal
T_2	MS+ BA 0.1 mg L^{-1}
T_3	MS+ BA 0.2 mg L^{-1}
T_4	MS+ BA 0.4 mg L^{-1}
T_5	MS + Kinetin 0.1 mg L^{-1}
T_6	MS + Kinetin 0.2 mg L^{-1}
T_7	MS + Kinetin 0.4 mg L^{-1}
T_8	WPM basal
T_9	WPM + BA 0.1 mg L^{-1}
T_{10}	WPM + BA 0.2 mg L^{-1}
T_{11}	WPM + BA 0.4 mg L^{-1}
T_{12}	WPM + Kinetin 0.1 mg L^{-1}
T_{13}	WPM + Kinetin 0.2 mg L^{-1}
T_{14}	WPM + Kinetin 0.4 mg L^{-1}

3.7. STANDARDIZATION OF MATURE EMBRYO CULTURE IN *C. fenestratum*

The present study was conducted to identify the influence of GA_3 presoaking in embryo development and plantlet growth in embryo culture. Healthy ripened fruits were collected from the mother plant maintained in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the ripening season of *C. fenestratum* i.e., from July to August. Fruits were collected directly by hand plucking. Seeds were extracted from the fruits and adhering pulp was removed. Then seeds were thoroughly washed, packed inside a clean polythene cover and brought into the laboratory.

Inside the laboratory, seeds were cleaned with liquid detergent for 10 minutes, and then washed under running tap water for half an hour. After that seeds were pretreated with fungicide bavistin 0.3 per cent (w/v) for 20 minutes, followed by washing with distilled water for three times. Seeds were further treated with streptocycline 0.1 per cent (w/v) for 20 minutes, and again washed three times with distilled water. Later seeds were separated into two sets, one set immersed in 4000 mg L^{-1} GA_3 solution for 72 hours inside a sterilized bottle and another set without GA_3 treatment. Embryo was excised from each set for further study.

Before the excision of the intact embryo, these pretreated seeds were sterilized with $HgCl_2$ for 10 minutes inside the laminar airflow chamber and then rinsed three times with sterile water. Seeds were cut into two halves and bright yellow coloured mature embryos located near the hilum region were excised and cultured on the basal MS media containing 1g/l activated charcoal, 3 per cent sucrose, 0.75 per cent agar, but free of plant growth regulators.

Embryos excised from the seeds treated with GA_3 and non-GA_3 treated seeds were separately cultured, as per details furnished in Table 8. Culture tubes were kept in dark condition for 2 weeks, after that developing embryos were exposed to light.

Observations on the percentage of culture establishment, percentage of plumule emergence, percentage of radicle emergence and percentage of seedling development were taken and the data were analysed.

Table 8. Pre-treatments for *C. fenestratum* seeds prior to embryo culture

Treatment No.	Pretreatments
T_1	Embryo excised from the seeds without GA_3 treatment cultured on basal MS media
T_2	Embryo excised from the seeds treated with 4000 mg L^{-1} GA_3 for 72 hours cultured on basal MS media

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSIONS

The experiment entitled "Plant regeneration of *Coscinium fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture" was conducted at tissue culture laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the academic year 2018 - 2020. The results obtained from the experiment and discussions are presented in the following sections.

4.1. *IN VITRO* SEED GERMINATION

4.1.1. Identification of best surface sterilization procedure for establishment of seeds in aseptic cultures

Seeds were subjected to different sterilization treatments and observations were carried out for 30 days. The results are presented in Table 9. Results indicated significant differences in culture establishment percentage in different sterilization treatments.

4.1.1.1. Effect of surface sterilization treatments on the culture establishment of C. fenestratum seeds

The percentage of culture establishment without contamination under different treatments tried varied from 0 to 49.33 per cent after a month of inoculation in growth hormone free MS media.

Clorox, which is a household sterilant solution containing sodium hypochlorite (6 %), used as bleaching agent, was tested as seed surface sterilant. Its two concentrations (20 % and 50 %) were used in combination with 70 per cent ethanol, with different exposure time. None of them gave any good result. In all these combinations tested cultures were contaminated within a period of two weeks, with zero per cent culture establishment.

As well, in this study, lab-grade sodium hypochlorite chemical solution 4 per cent was tested with 70 per cent ethanol combination, with different exposure time. It was also found to be ineffective to control surface microbes and resulted in zero per cent culture establishment when observed for two weeks.

In all clorox and sodium hypochlorite containing treatments, the main contaminants noticed were fungus. Within a week, fungal contamination was observed as a white mycelial growth. In lower concentration tested cultures, fungal hyphae growth was observed all over the seed surface, while when concentration and time of exposure increased, fungal hyphae growth mainly started from the micropylar region of the seed and gradually spread to all the surface and media, indicating that the microbe may be an endophyte (Ganley and Newcombe, 2006; Goveas *et al*., 2011). Even though the fungus was the predominant contaminant in these combinations, bacterial growth was also found with slimy growth of white and creamy colour in some cultures.

Mercuric chloride ($HgCl_2$) at a lower concentration (0.1 %) significantly reduced the fungal contaminations. However bacterial endophytes remained to be the main contaminant. The effectiveness of $HgCl_2$ increased with an increase in exposure time. When seeds were treated with 0.1 per cent (w/v) $HgCl_2$ solution for 2 minutes and 5 minutes, only 5.33 per cent culture establishment was noticed. Whereas, when exposure time was extended to 10 minutes, culture establishment increased to 10.67 per cent and 25.33 per cent with 15 minutes. With higher exposure time of 20 and 30 minutes, contamination levels got reduced in both the treatments. It may be due to the toxic nature of $HgCl_2$ that penetrated into the seeds. In 20 minutes exposure time, 36 per cent of culture establishment, and in 30 minutes exposure time, 49.33 per cent of culture establishment was observed within a period of one month. But higher exposure time (20 and 30 minutes) may increase the mortality rate of the seed. So, further studies are needed to check the viability of the seeds in these two higher exposure time of 0.1 per cent $HgCl_2$. Hence, in the present experiment among the different sterilants tested for surface sterilization of seeds, 0.1 per cent $HgCl_2$ (15 min) was found to be the best agent for the culture establishment with minimum contamination.

Similar reports were given by Warakagoda and Subasinghe (2014) in *C. fenestratum*. In seed surface sterilization study authors reported that clorox was ineffective to control microbes. They tried 20 per cent clorox solution for 5 minutes followed by 70 per cent ethanol for 2 minutes and 70 percent ethanol for 1 minute followed by 75 percent clorox solution for 3 minutes. The whole culture was lost within

eight weeks. The authors further mentioned that 0.1 per cent $HgCl_2$ was the best surface sterilant for *C. fenestratm* seeds.

When seeds were cultured in potato dextrose agar media, it was found that the seeds were the living house for many endophytic bacteria as depicted in Plate 3.

Based on these observations finally it was concluded that *C. fenestratum* seeds harboured fungal and bacterial endophytes. In spite of strong surface sterilization treatments given, the endophytes living inside the seeds survived and persisted inside the seeds. Hence after inoculation endophytes came out and proliferated within the nutrient rich MS medium. Even though endophytes were not harmful as such, they interfered with the culture establishment by contaminating the media.

There are reports of the presence of endophytes in the seeds of plants such as *Pinus monticola* (Ganley and Newcombe, 2006) *Lolium multiflorum, Festuca arundinacea and F. pratensis* (Latch *et al*., 1987).

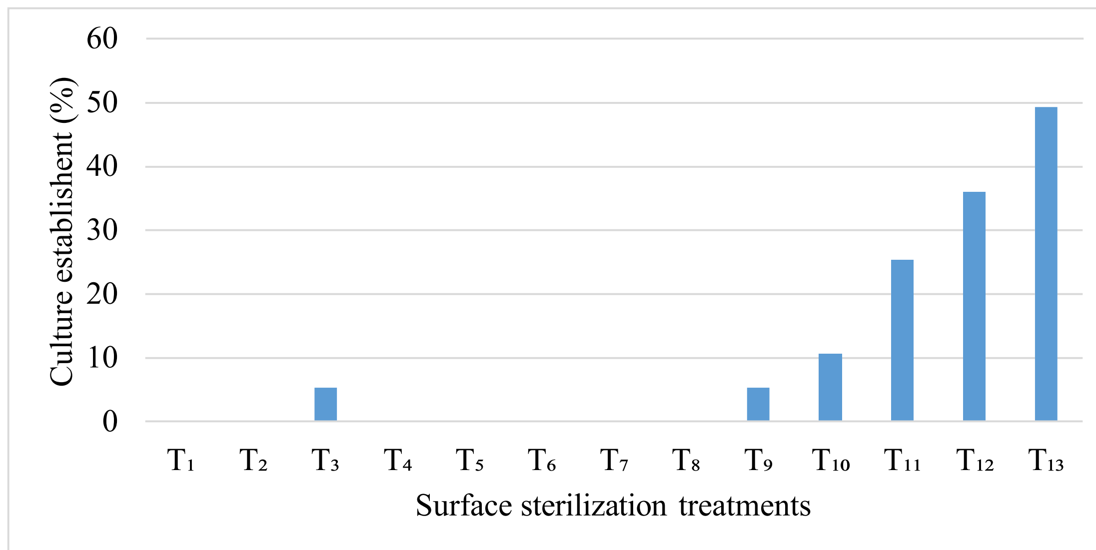
So far, no studies are available regarding the presence of endophytes in seeds of *C. fenestratum*, whereas Goveas *et al*., (2011) isolated 41 endophytic fungi belonging to sixteen different taxa from both leaves and stem of *C. fenestratum*. The identified species of the endophytic fungi were reported to belong to Ascomycota and isolated genera of endophytes were *Penicillium senticosam, Phomopsis jacquiniana, Aspergillus tamarii, Alternaria alternata, Aspergillus fumigatus, Drechslera, Fusicladiella, Asperisoprium caricae, Cladosporium cladosporioides, Staphylotrichum cocosporium, Mycelia sterilia, Nigrospora oryzae, Gliocladium roseum* and *Cladosporium sp*. which also represents the chance of presence of endophytes in the *C. fenestratum* seeds. Hence further studies are needed for identifying the endophytes in the seed and for controlling the culture loss by endophytes with the incorporation of antibiotics and fungicides in the culture media.

Table 9. Effect of surface sterilization treatments in the culture establishment of *C. fenestratum* seeds

Treatment No.	Sterilization methods	Culture establishment (%)	Type of contamination
T_1	20 % (v/v) clorox solution for 5 min followed by 70 % (v/v) ethanol for 2 min	0	F
T_2	20 % (v/v) clorox solution for 3 min followed by 70 % (v/v) ethanol for 1 min	0	F
T_3	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 2 min	5.33	F, B
T_4	20 % (v/v) clorox solution for 10 min followed by 70 % (v/v) ethanol for 5 min	0	F
T_5	50 % (v/v) clorox solution for 3 min followed by 70 % (v/v) ethanol for 1 min	0	F
T_6	50 % (v/v) clorox solution for 5 min followed by 70 % (v/v) ethanol for 2 min	0	F, B
T_7	4 % (v/v) sodium hypochlorite solution (NaOCl) for 3 min followed by 70 % (v/v) ethanol for 1 min	0	F
T_8	4 % (v/v) sodium hypochlorite (NaOCl) solution for 5 min followed by 70 % (v/v) ethanol for 2 min	0	F
T_9	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 5 min	5.33	F, B

T_{10}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 10 min	10.67	F, B
T_{11}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 15 min	25.33	B
T_{12}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 20 min	36.00	B
T_{13}	0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 30 min	49.33	B

B - Bacteria, F - Fungus



T_1 - 20 % clorox (5 min) + 70 % ethanol (2 min)

T_2 - 20 % clorox (3 min) + 70 % ethanol (1 min)

T_3 - 0.1 % $HgCl_2$ (2 min)

T_4 - 20 % clorox (10 min) + 70 % ethanol (5 min)

T_5 - 50 % clorox (3 min) + 70 % ethanol (1 min)

T_6 - 50 % clorox (5 min) + 70 % ethanol (2 min)

T_7 - 4 % NaOCl (3 min) + 70 % ethanol (1 min)

T_8 - 4 % NaOCl (5 min) + 70 % ethanol (2 min)

T_9 - 0.1 % $HgCl_2$ (5 min)

T_{10} - 0.1 % $HgCl_2$ (10 min)

T_{11} - 0.1 % $HgCl_2$ (15 min)

T_{12} - 0.1 % $HgCl_2$ (20 min)

T_{13} - 0.1 % $HgCl_2$ (30 min)

Fig 1. Effect of surface sterilization treatments on culture establishment of *C. fenestratum* seeds

Plate 3. Bacterial growth in seed cultured on potato dextrose agar media

Plate 4. Surface sterilised seeds inoculated in MS media containing activated charcoal

Plate 5. Bacterial contamination starting from mycropylar region of surface sterilised seeds

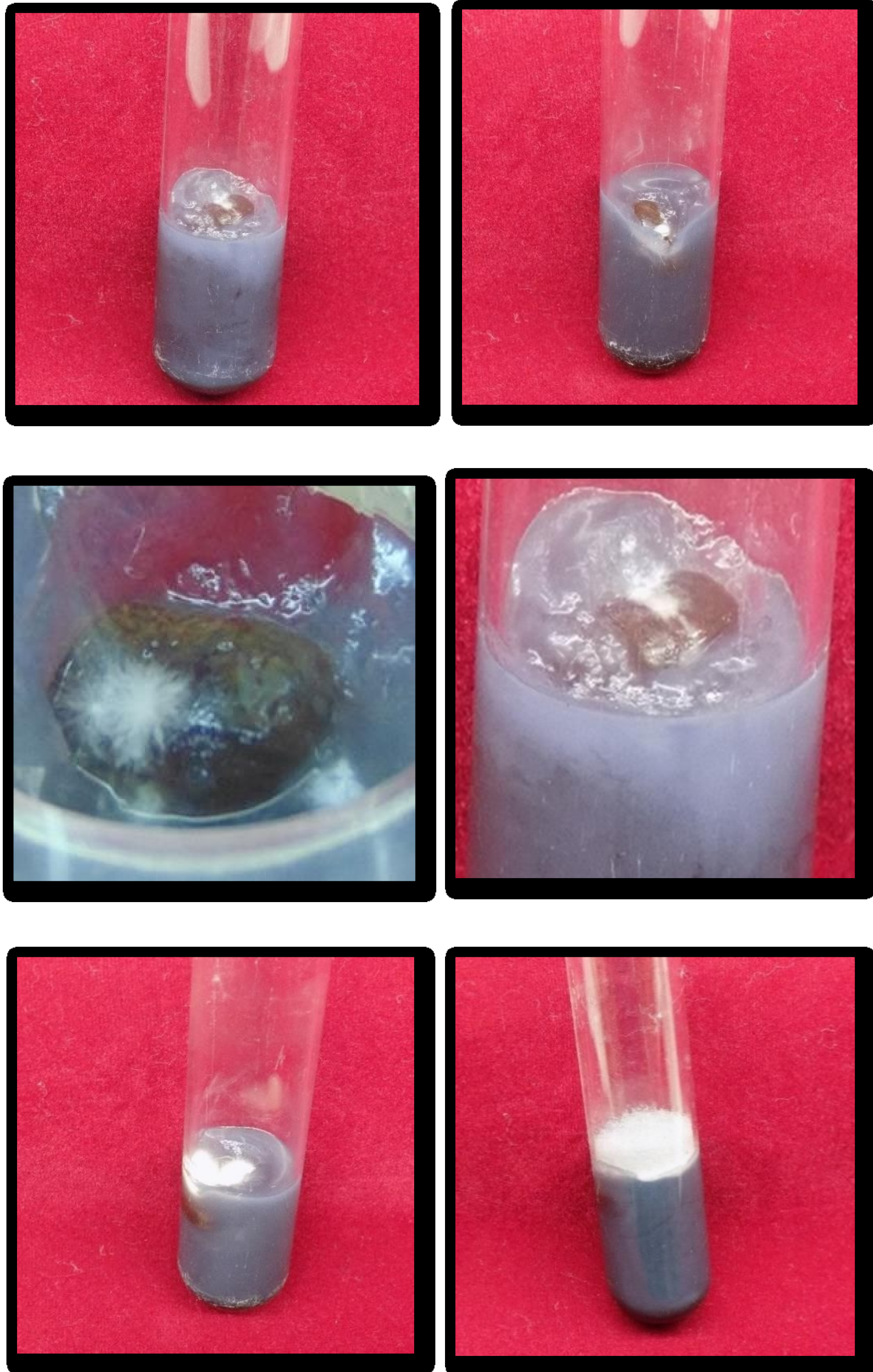


Plate 6. Fungal contamination starting from mycropylar region of surface sterilised seeds

4.1.2. Identification of best *in vitro* seed germination media

4.1.2.1. Effect of different media on the percentage of in vitro seed germination and time taken for germination of seeds

C. fenestraum seed has both chemical and physical dormancy. After exposure to pre-treatments like bavistin and streptocyclin, seeds were soaked in GA_3 for 72 hours and surface sterilized inside the laminar airflow chamber using 0.1 per cent $HgCl_2$ for 15 minutes and cultured in different media. Observations were taken for a period of one year and results on the percentage of seed germination and time taken for germination are presented in the Table 10.

Experiment on standardization of best media for seed germination revealed that, among the different media tried, sterilized sand: coir pith (1:1) media soaked with distilled water was found to be the best for *in vitro* seed germination, as it showed the highest germination percentage (22.95 %) and lowest germination time (55.68 days). The other two treatments were not found to be suitable for seed germination, because of high contamination percentage during culture establishment. Sterilized sand: coir pith (1:1) media soaked with liquid MS medium showed, a low germination rate (13.91 %). It took an average of 79.80 days for initiation of germination. MS media with 1 g L^{-1} activated charcoal was not found to be a favourable media for seed germination, because of high contamination of fungal and bacterial endophytes full culture lost within a period of one month.

This report is in line with report of Warakagoda and Subasinghe (2014), who observed sand: coir dust media as best for seed germination (39.0 %), whereas, seeds cultured on MS media containing either activated charcoal or sand: coir pith (1:1) exhibited no germination due to fungal contaminations.

Coir pith is a suitable media for seed germination due to its water holding capacity. The present study revealed that the sterilized sand and coir pith soaked with distilled water will act as a good germination media under *in vitro* condition, whereas MS medium cannot be used due to contamination with endophytes. The use of properly sterilized coir pith soil media soaked with distilled water will hold the moisture for a long time and acts as a good substrate for seed germination and help to replace the costly MS media and thereby reducing the media cost and expenses in tissue culture.

Table 10. Effect of different media on *in vitro* seed germination

Treatment No.	Media composition	*In vitro* seed germination (%)	Time taken for *in vitro* seed germination (Days)
T_1	MS media with 1 g L^{-1} activated charcoal	-	-
T_2	Sterilized sand : coir pith (1:1) containing soaked with liquid MS media	13.91	79.80
T_3	Sterilized sand: coir pith (1:1) containing soaked with distilled water	22.95	55.68

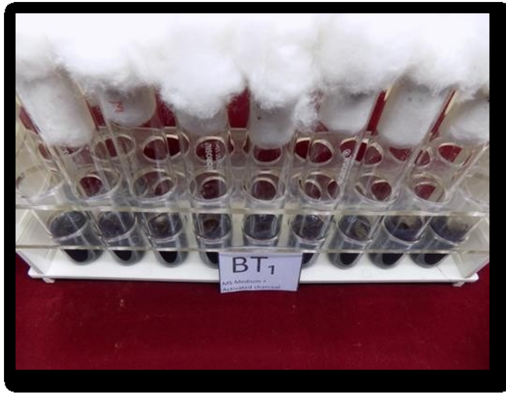
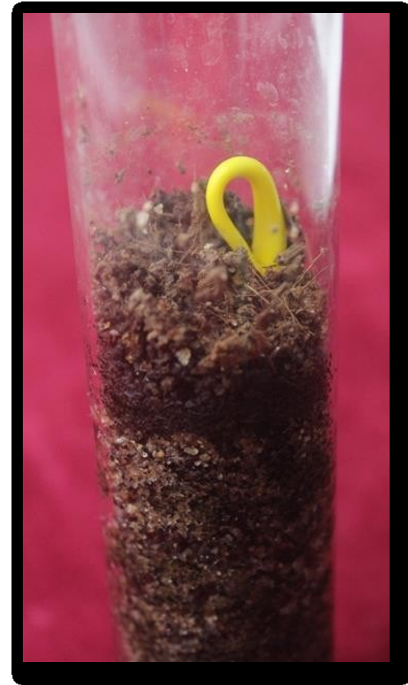


Plate 7a. Seeds inoculated in MS media with 1 g L^{-1} activated charcoal

Plate 7b. Seeds inoculated in sterilized sand: coir pith (1:1) soaked with liquid MS medium

Plate 7c. Seeds inoculated in sterilized sand: coir pith (1:1) soaked with distilled water

Plate 7. *In vitro* seed germination media



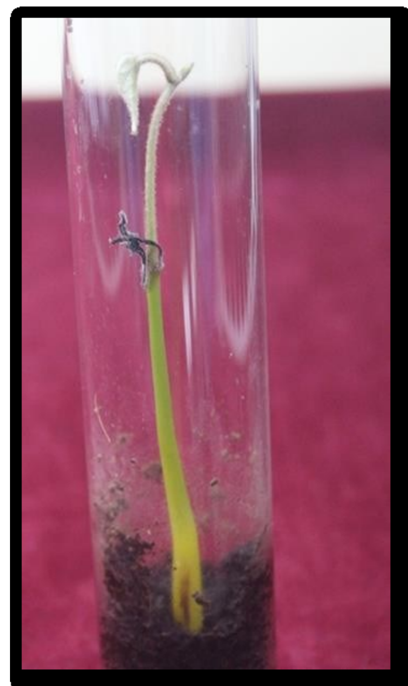


Plate 8. Seed germination in the sterilized sand: coir pith (1:1) soaked with liquid MS media

Plate 9. Seed germination in the sterilized sand: coir pith (1:1) soaked with distilled water

4.2. STANDARDIZATION OF SHOOT REGENERATION PROTOCOL USING COTYLEDONARY NODAL EXPLANTS FROM *EX VITRO* GERMINATED SEEDLINGS AND NODAL SEGMENTS CONTAINING AXILLARY BUDS AS EXPLANTS FROM POLY HOUSE-GROWN PLANTS

4.2.1. Shoot initiation and multiplication from cotyledonary nodal explants from *ex vitro* germinated seedlings

In the present study *ex-vitro* grown seedling cotyledonary nodal explants were used to estimate shoot proliferation rate in MS medium supplemented with different growth hormones. Observations and results obtained are discussed in the following section.

4.2.1.1. Percentage of culture establishment

In the present study, seedlings were pre-treated with bavistin, streptocyclin and surface sterilized with $HgCl_2$ and ethanol. Results showed that, when the cotyledonary nodal portion was used as explant from *ex-vitro* polyhouse grown seedlings, culture establishment was very low due to high microbial contamination. Even though explant was exposed to strong sterilant $HgCl_2$ 0.1 per cent for 2 minutes followed by 70 percent ethanol for 1 minutes for surface sterilization treatment, culture establishment was found to be very low due to the high contamination rate. Duncan's multiple range test was used to comparing the treatment means. Results are depicted in Table 11.

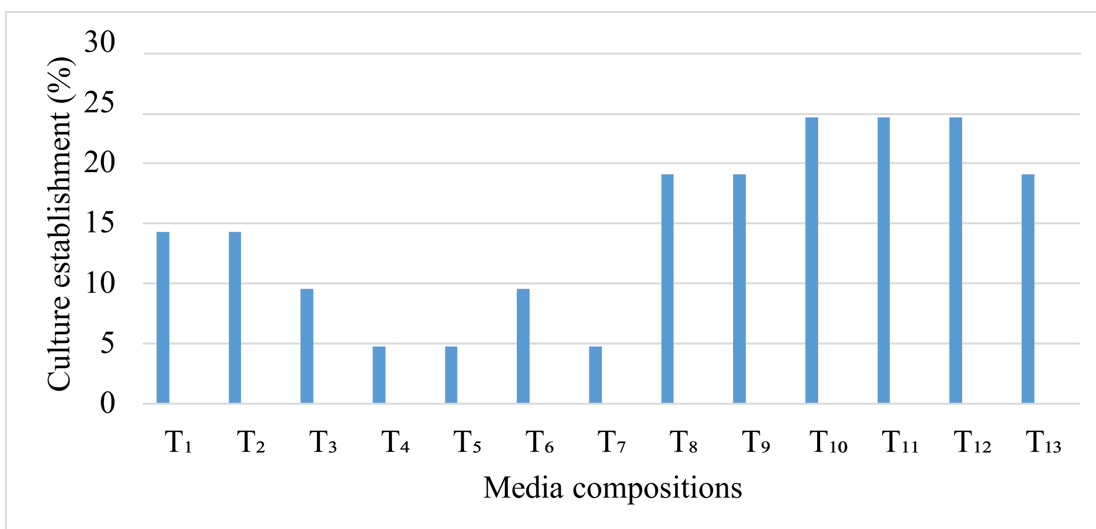
Maximum culture establishment (23.81 %) was noticed in three treatments, MS media supplemented with 0.1 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D, MS media supplemented with 0.2 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D and MS media supplemented with 0.4 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D, whereas least culture establishment (4.76 %) was noticed in three treatments. $HgCl_2$ has strong bactericidal and fungicidal effect, so it is widely used as a sterilant in tissue culture, where as other sterilants were ineffective. Mercury present in $HgCl_2$ is a hazardous chemical, which when exposed to aqueous solution form numerous organic toxic compound and the chloride compound oxidize the peptide linkage and denatures the protein of microbes (Das *et al*., 2012). Even though it is having strong sterilization activity, its higher concentration and higher time of exposure will lead to the death of the explant, especially young delicate seedling explants. Due to this property in the present study,

higher exposure time of $HgCl_2$ was not attempted. Hence, culture establishment was found to be a limiting factor here, for the shoot regeneration from the seedling explant. Further studies are needed to eliminate the explant contamination and to get higher culture establishment.

Table 11. Culture establishment on different media compositions of cotyledonary nodal explants

Treatment No.	Media composition	Culture establishment (%)
T_1	Control	14.29 (1.22)^{abc}
T_2	MS+ BA 0.1 mg L^{-1}	14.29 (1.22)^{abc}
T_3	MS+ BA 0.2 mg L^{-1}	9.53 (1.05)^{bc}
T_4	MS+ BA 0.4 mg L^{-1}	4.76 (0.88)^{c}
T_5	MS + Kinetin 0.1 mg L^{-1}	4.76 (0.88)^{c}
T_6	MS + Kinetin 0.2 mg L^{-1}	9.53 (1.05)^{bc}
T_7	MS + Kinetin 0.4 mg L^{-1}	4.76 (0.88)^{c}
T_8	MS + BA 0.1 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	19.05 (1.34)^{ab}
T_9	MS + BA 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	19.05 (1.34)^{ab}
T_{10}	MS + BA 0.4 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	23.81 (1.46)^{a}
T_{11}	MS + Kinetin 0.1 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	23.81 (1.46)^{a}
T_{12}	MS + Kinetin 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	23.81 (1.46)^{a}
T_{13}	MS + Kinetin 0.4 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	19.05 (1.34)^{ab}
CV = 19.33		
CD (0.05) = 0.390		

Square root transformed values are given in parenthesis.



T_1 - Control

T_2 - MS + BA 0.1 mg L^{-1}

T_3 - MS + BA 0.2 mg L^{-1}

T_4 - MS + BA 0.4 mg L^{-1}

T_5 - MS + Kinetin 0.1 mg L^{-1}

T_6 - MS + Kinetin 0.2 mg L^{-1}

T_7 - MS + Kinetin 0.4 mg L^{-1}

T_8 - MS + BA 0.1 mg L^{-1} + 2, 4 - D 0.06 mg L^{-1}

T_9 - MS + BA 0.2 mg L^{-1} + 2, 4 - D 0.06 mg L^{-1}

T_{10} - MS + BA 0.4 mg L^{-1} + 2, 4 - D 0.06 mg L^{-1}

T_{11} - MS + Kinetin 0.1 mg L^{-1} + 2, 4 - D 0.06 mg L^{-1}

T_{12} - MS + Kinetin 0.2 mg L^{-1} + 2, 4 - D 0.06 mg L^{-1}

T_{13} - MS + Kinetin 0.4 mg L^{-1} + 2, 4 - D 0.06 mg L^{-1}

Fig 2. Culture establishment on different media compositions of cotyledonary nodal explants

4.2.1.2. Percentage of shoot induction in different media

In the present study, MS media supplemented with 2, 4-D along with cytokinin i.e. 0.2 mg L^{-1} BA, 0.2 mg L^{-1} kinetin and 0.4 mg L^{-1} kinetin showed response for shoot initiation with 100 per cent shoot induction in established cultures, whereas in other treatments, established cultures failed to give the response. The effect of different growth hormone concentrations in shoot initiation is presented in Table 12.

Table 12. Effect of different media compositions on shoot induction of cotyledonary nodal explants

Treatment No.	Media composition	Shoot induction (%)
T_1	Control	0
T_2	MS + BA 0.1 mg L^{-1}	0
T_3	MS + BA 0.2 mg L^{-1}	0
T_4	MS + BA 0.4 mg L^{-1}	0
T_5	MS + Kinetin 0.1 mg L^{-1}	0
T_6	MS + Kinetin 0.2 mg L^{-1}	0
T_7	MS + Kinetin 0.4 mg L^{-1}	0
T_8	MS + BA 0.1 mg L-1 + 2,4 - D 0.06 mg L^{-1}	0
T_9	MS+ BA 0.2 mg L-1 + 2,4 - D 0.06 mg L^{-1}	100
T_{10}	MS+ BA 0.4 mg L-1 + 2,4 - D 0.06 mg L^{-1}	0
T_{11}	MS + Kinetin 0.1 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	0
T_{12}	MS + Kinetin 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	100
T_{13}	MS + Kinetin 0.4 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	100

4.2.1.3. Time taken for shoot induction in different media

The number of days taken for shoot initiation was found to be the same without significant difference in the responded cultures. On an average, bud initiation took place within 14 days in responded treatments. The result are depicted in the Table 13.

Table 13. Effect of different media compositions on time taken for shoot induction of cotyledonary nodal explants

Treatment No.	**Media composition**	**Time taken for shoot induction (days)**
T_9	MS + BA 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	13.67
T_{12}	MS + Kinetin 0.2 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	14.67
T_{13}	MS + Kinetin 0.4 mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	14.33

4.2.1.4. Multiple shoot production in different media

Multiple shoot initiation was noticed in the cotyledonary nodal explant (Table 14). Significantly higher shoots were produced in cotyledonary nodal explant inoculated in MS media supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D (4.5). Whereas BA and 2, 4 - D supplemented other treatments did not responded. When MS media was supplemented with 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D, an average of 3.833 shoots per culture was produced and MS media supplemented with 0.2 mg L^{-1} kinetin + 0.06 mg L^{-1} 2, 4 - D produced an average of 2 shoots per culture.

From the responded cultures, it was observed that the shoots produced in MS media supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D were stout and bigger than that obtained from kinetin supplemented media and also produced larger and broader leaves within a month (Plate 12). In 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D supplemented media shoots were very thin and slender and leaves were smaller when compare to that of 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D supplemented media (Plate 14).

It was noticed that, among the cytokinin supplemented media, 0.2 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4-D supplemented media produced larger leaves and smaller multiple shoots than 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D supplemented media, which showed that when kinetin concentration increased there was a reduction in leaf size.

However, Cytokinin alone failed to give a response in the present study. Results revealed that the addition of a little amount of auxin with cytokinin enhanced the response for shoot multiplication. Senarath (2010) reported that *C. fenestratum* seedling epicotyl explant gave the best response in 1.0 μM kinetin and 0.25 μM 2,4-D and Joshi and Dhar (2003) reported that 1.0 mM kinetin in combination with 0.25 mM NAA gave the best response for *Saussurea obvallata* seedling epicotyl explant culture.

Table 14. Effect of different media compositions on multiple shoot production of cotyledonary nodal explants

Treatment No.	Media composition	Number of multiple shoots
T_9	MS + BA 0.2mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	4.50
T_{12}	MS + Kinetin 0.2mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	2.00
T_{13}	MS + Kinetin 0.4mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	3.83

Plate 10. Cotyledonary nodal explant

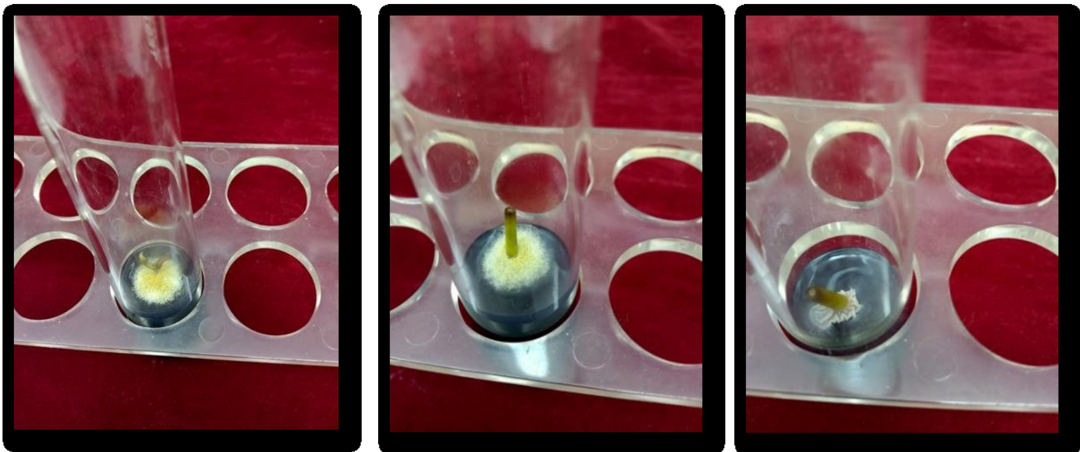


Plate 11. Contamination in seedling cotyledonary nodal explants

Plate 12. Multiple shoot induction in cotyledonary nodal explant in MS media supplemented with 0.2 mg L^{-1} BA + 0.06 mg L^{-1} 2, 4 - D

Plate 13. Multiple shoot induction in cotyledonary nodal explant in MS media supplemented with 0.2 mg L^{-1} kinetin + 0.06 mg L^{-1} 2, 4 - D

Plate 14. Multiple shoot induction in cotyledonary nodal explant in MS media supplemented with 0.4 mg L^{-1} kinetin + 0.06 mg L^{-1} 2, 4 - D

4.2.2. Shoot initiation and multiplication from nodal segments with axillary bud as explant from poly house-grown plants

In this experiment nodal segments with axillary bud collected from the mother plants maintained inside the polyhouse were used as explant.

The shoot induction response of the nodal explant containing axillary bud explant in two different media (MS and WPM) supplemented with different concentrations of two types of cytokinins (BA and kinetin) were tested in the present study and results are discussed in the following section.

4.2.2.1. Percentage of culture establishment in different media

There was no significant difference in the percentage of culture establishment from nodal explants in different media (Table 15). On an average, 70 to 80 per cent culture establishment was obtained in all shoot initiation treatments when shoots were pretreated with 0.3 per cent (w/v) bavistin and 0.1 per cent (w/v) streptocyclin, each for 20 minutes followed by surface sterilization with 0.1 per cent (w/v) mercuric chloride for 2 minutes and ethanol 70 per cent (v/v) for 1 minute. Higher culture establishment was noticed in WPM supplemented with 0.4 mg L^{-1} BA (82.22 %) followed by 80 per cent in WPM supplemented with 0.1 mg L^{-1} BA, WPM supplemented with 0.4 mg L^{-1} kinetin, MS media supplemented with 0.2 mg L^{-1} kinetin and MS media supplemented with 0.4 mg L^{-1} kinetin. The explant was the main source of contamination in micropropagation, the hairs present in the nodal segment in *C. fenestratum* acted as the carrier of bacterial and fungal inoculum, and this could be almost eliminated by pretreatments followed in the present study. The spraying of mother plants with fungicides, removal of hairs of nodal explant by gentle rubbing with hand under the running tap water, fungicidal and bactericidal pretreatments and $HgCl_2$ surface sterilization followed in the present study reduced the percentage of contamination drastically and helped to get a higher culture establishment.

Table 15. Culture establishment on different media compositions of nodal explants

Treatment No.	Media composition	Percentage of culture establishment (%)
T_1	MS basal	71.11
T_2	MS + BA 0.1 mg L^{-1}	73.33
T_3	MS + BA 0.2 mg L^{-1}	77.78
T_4	MS + BA 0.4 mg L^{-1}	71.11
T_5	MS + Kinetin 0.1 mg L^{-1}	73.33
T_6	MS + Kinetin 0.2 mg L^{-1}	80.00
T_7	MS + Kinetin 0.4 mg L^{-1}	80.00
T_8	WPM basal	73.33
T_9	WPM + BA 0.1 mg L^{-1}	80.00
T_{10}	WPM + BA 0.2 mg L^{-1}	77.78
T_{11}	WPM + BA 0.4 mg L^{-1}	82.22
T_{12}	WPM + Kinetin 0.1 mg L^{-1}	77.78
T_{13}	WPM + Kinetin 0.2 mg L^{-1}	77.78
T_{14}	WPM + Kinetin 0.4 mg L^{-1}	80.00
CV (%) = 9.930		
NS		

4.2.2.2. Percentage of shoot induction in different media

Among the two different media tested, when supplemented with plant growth hormones, WPM found to be better than the MS media for bud break and shoot induction. Among the two cytokinins tested kinetin was found to be more effective than the BA with more percentage of shoot initiation in WPM. Duncan's multiple range test was used to comparing the treatment means. Results are presented in Table 16.

Both basal MS media and WPM devoid of plant growth hormones did not show any bud break. In MS media supplemented with different concentrations of kinetin and BA, shoot initiation was found to be either nil or very low. Treatments like 0.1 mg L^{-1} BA, 0.1 mg L^{-1} kinetin and 0.2 mg L^{-1} kinetin when supplemented in MS media did not showed any response to shoot induction. Whereas in MS media treatments like 0.4 mg L^{-1} BA, 0.2 mg L^{-1} BA, 0.4 mg L^{-1} kinetin showed an average of 40.97 per cent, 11.36 per cent and 22.55 per cent of shoot initiation respectively.

In the present study, when WPM was used as the basal media all growth hormones treated cultures showed a positive response for shoot initiation. In WPM supplemented with 0.4 mg L^{-1} kinetin 91.63 per cent cultures responded for bud breaking, whereas in MS media supplemented with the same concentration of kinetin (0.4 mg L^{-1}) response noticed was only 22.55 per cent.

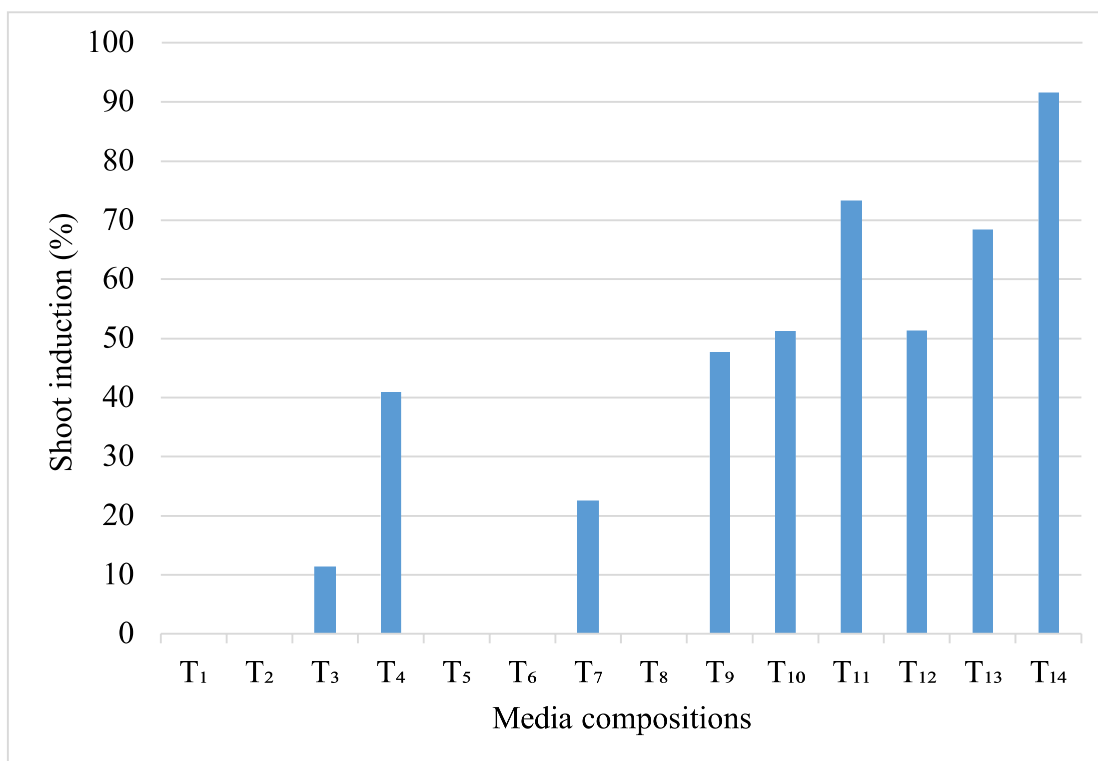
The suitability of WPM over MS media on high-frequency shoot initiation was reported by Lakshmanan *et al*. (1997) in nodal cuttings of *Ixora coccinea*, Lu (2005) in shoot tip culture of *Vitis thunbergii* Sieb. et Zucc., Raghu *et al*. (2006) in nodal explant culture of *Tinospora cordifolia*, Alatar *et al*. (2012) in the nodal segment of *Rauvolfia serpentine* and Warakagoda *et al*. (2017) in *C. fenestratum.*

Among cytokinins, 0.4 mg L^{-1} kinetin was found to be superior for shoot induction than other treatments with 91.63 per cent of shoot induction in WPM, followed by WPM supplemented with 0.4 mg L^{-1} BA with 73.29 per cent shoot induction and WPM supplemented with 0.2 mg L^{-1} kinetin with 68.44 per cent shoot induction.

Table 16. Effect of different media compositions on shoot induction of nodal explants

Treatment No.	Media composition	Shoot induction (%)
T_1	MS basal	0 (1.654) [g]
T_2	MS + BA 0.1 mg L^{-1}	0 (1.654) [g]
T_3	MS + BA 0.2 mg L^{-1}	11.36 (19.473) [f]
T_4	MS + BA 0.4 mg L^{-1}	40.97 (39.789) [d]
T_5	MS + Kinetin 0.1 mg L^{-1}	0 (1.654) [g]
T_6	MS + Kinetin 0.2 mg L^{-1}	0 (1.654) [g]
T_7	MS + Kinetin 0.4 mg L^{-1}	22.55 (28.190) [e]
T_8	WPM basal	0 (1.654) [g]
T_9	WPM + BA 0.1 mg L^{-1}	47.67 (43.646) [cd]
T_{10}	WPM + BA 0.2 mg L^{-1}	51.26 (45.728) [c]
T_{11}	WPM + BA 0.4 mg L^{-1}	73.29 (59.192) [b]
T_{12}	WPM + Kinetin 0.1 mg L^{-1}	51.28 (45.734) [c]
T_{13}	WPM + Kinetin 0.2 mg L^{-1}	68.44 (55.884) [b]
T_{14}	WPM + Kinetin 0.4 mg L-1	91.63 (73.193) [a]
CV (%) = 10.939		
CD (0.05) = 5.476		

Angular transformed values are given in paranthesis



T_1 - MS basal

T_2 - MS + BA 0.1 mg L^{-1}

T_3 - MS + BA 0.2 mg L^{-1}

T_4 - MS + BA 0.4 mg L^{-1}

T_5 - MS + Kinetin 0.1 mg L^{-1}

T_6 - MS + Kinetin 0.2 mg L^{-1}

T_7 - MS + Kinetin 0.4 mg L^{-1}

T_8 - WPM basal

T_9 - WPM + BA 0.1 mg L^{-1}

T_{10} - WPM + BA 0.2 mg L^{-1}

T_{11} - WPM + BA 0.4 mg L^{-1}

T_{12} - WPM + Kinetin 0.1 mg L^{-1}

T_{13} - WPM + Kinetin 0.2 mg L^{-1}

T_{14} - WPM + Kinetin 0.4 mg L^{-1}

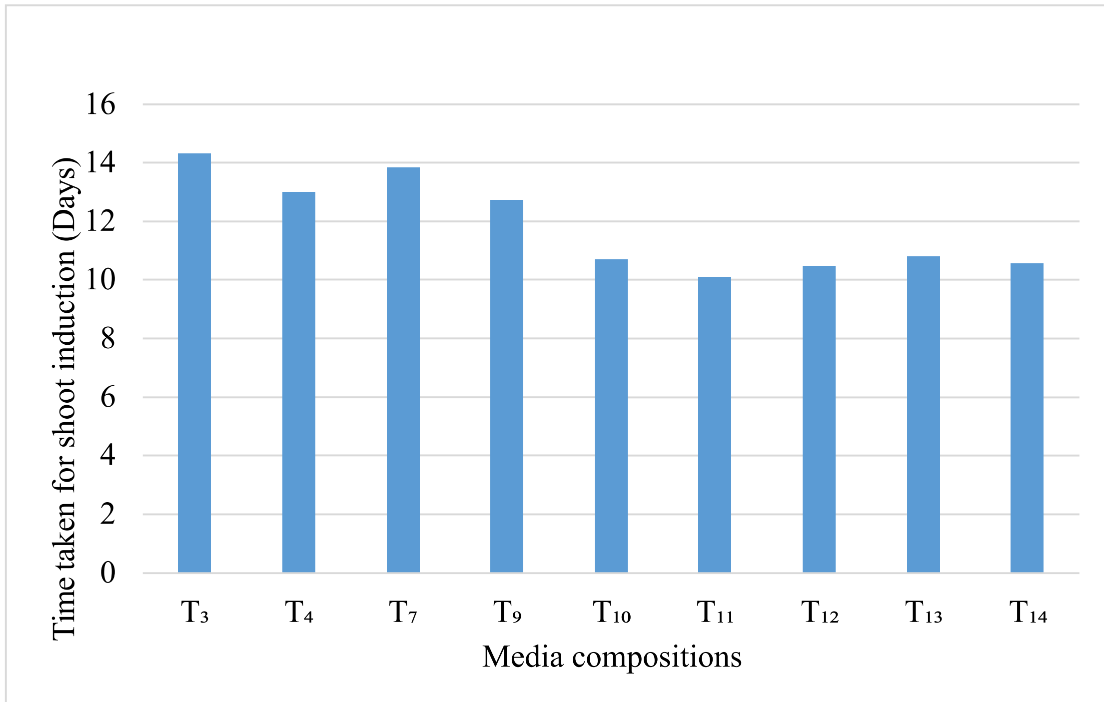
Fig 3. Effect of different media compositions on shoot induction of nodal explants

4.2.2.3. Time taken for shoot induction in different media

Period of morphogenic response for shoot induction was found to be faster in WPM whereas in MS medium cultures took more time for shoot initiation. Duncan's multiple range test was used to comparing the treatment means. Results are depicted in Table 17. When WPM supplemented with plant growth hormone (0.2 mg L^{-1} BA, 0.4 mg L^{-1} BA, 0.1 mg L^{-1} kinetin, 0.2 mg L^{-1} kinetin and 0.4 mg L^{-1} kinetin), it took only on an average of 10 days to initiate culture response by swelling of dormant bud and bud initiation. Whereas when supplemented with 0.1mg L^{-1} BA it took more days (12.72 days) for shoot initiation. However in MS media on an average of 13 to 14 days was required for shoot initiation.

Table 17. Effect of different media compositions on time taken for shoot induction of nodal explants

Treatment No.	Media composition	Time taken for shoot induction (days)
T_3	MS+ BA 0.2 mg L^{-1}	14.33 [a]
T_4	MS+ BA 0.4 mg L^{-1}	13.01 [bc]
T_7	MS + Kinetin 0.4 mg L^{-1}	13.83 [ab]
T_9	WPM + BA 0.1 mg L^{-1}	12.72 [c]
T_{10}	WPM + BA 0.2 mg L^{-1}	10.70 [d]
T_{11}	WPM + BA 0.4 mg L^{-1}	10.10 [d]
T_{12}	WPM + Kinetin 0.1 mg L^{-1}	10.47 [d]
T_{13}	WPM + Kinetin 0.2 mg L^{-1}	10.81 [d]
T_{14}	WPM + Kinetin 0.4 mg L^{-1}	10.57 [d]
CV (%) = 4.188		
CD (0.05) = 0.851		



T_3 - MS + BA 0.2 mg L^{-1}

T_4 - MS + BA 0.4 mg L^{-1}

T_7 - MS + Kinetin 0.4 mg L^{-1}

T_9 - WPM + BA 0.1 mg L^{-1}

T_{10} - WPM + BA 0.2 mg L^{-1}

T_{11}- WPM + BA 0.4 mg L^{-1}

T_{12} - WPM + Kinetin 0.1 mg L^{-1}

T_{13} - WPM + Kinetin 0.2 mg L^{-1}

T_{14} - WPM + Kinetin 0.4 mg L^{-1}

Fig 4. Effect of different media compositions on time taken for shoot induction of nodal explants

4.2.2.4. Number of multiple shoots per culture in different media

Even though shoot initiation was noticed, none of the treatments showed multiple shoot production within 4 months. This result was in confirmatory with Warakagoda *et al*. (2017).

Plate 15. Mother plant maintained inside the polyhouse

Plate 16. Nodal explants

Plate 17. Axillary bud culture in MS media

Plate 18. Bud initiation in MS media

Plate 19. Shoot produced in MS media

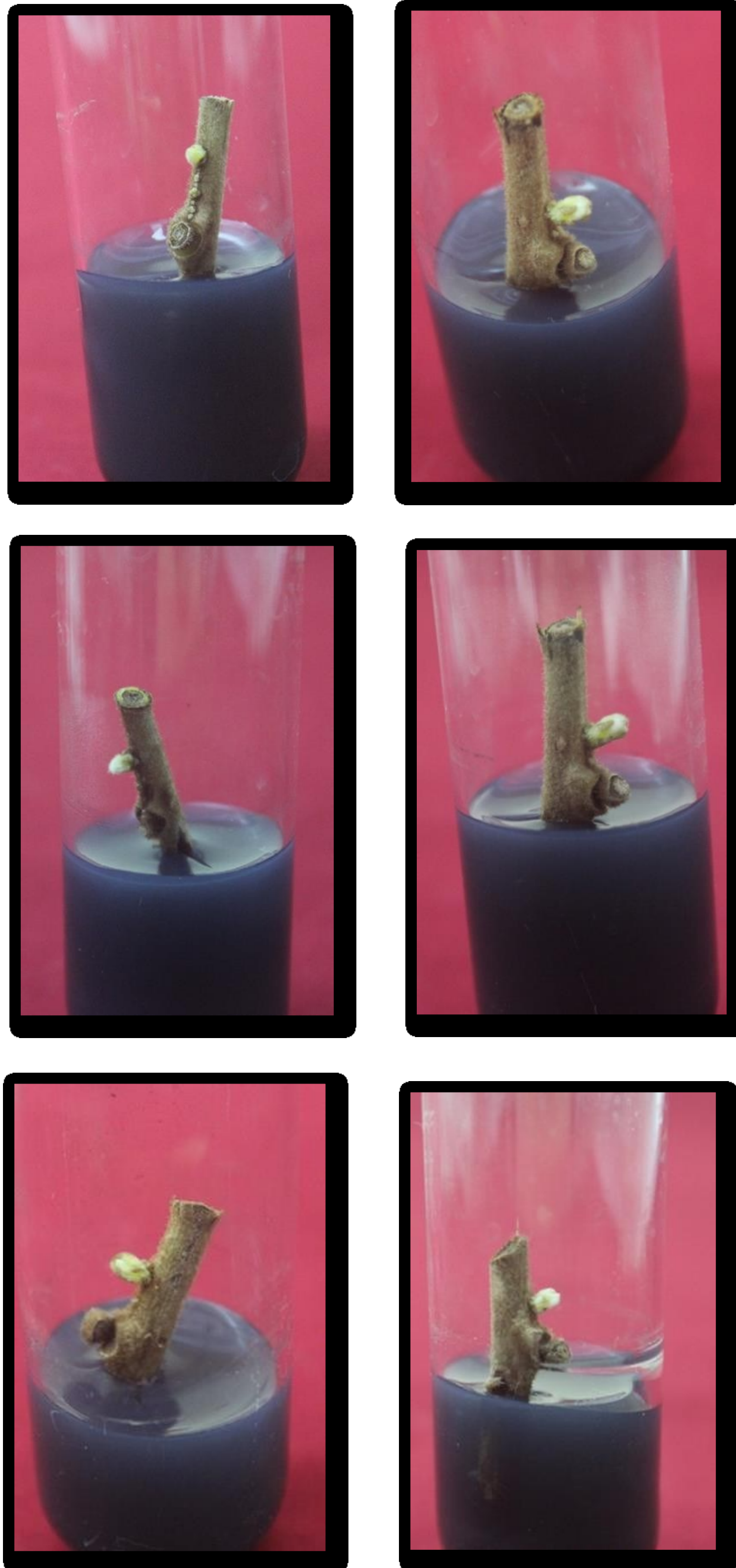


Plate 20. Bud initiation in WPM

Plate 21. Shoot produced in WPM

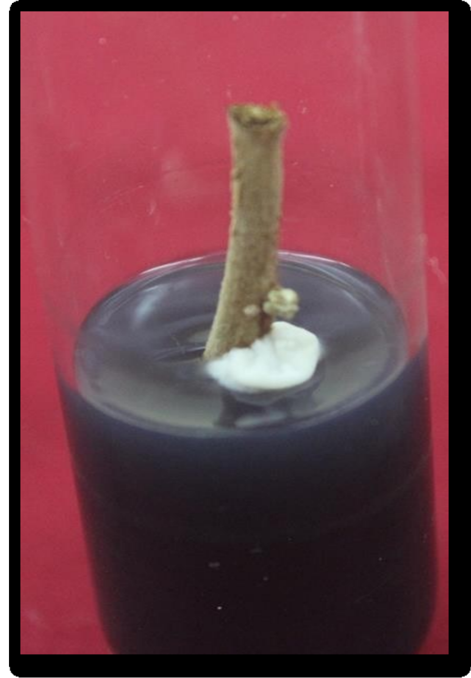


Plate 22. Fungal contamination noticed in the axillary bud culture

4.3. STANDARDIZATION OF EMBRYO CULTURE IN *C. fenestratum*

In this experiment, germination of excised embryos of the *C. fenestratum* were tried under *in vitro* condition. The effect of GA_3 pretreatment on seeds for overcoming the dormancy, embryo development and plantlet productions were studied. Results are presented in Table 18.

Compared to seed culture contamination loss in embryo culture was very low. Culture establishment without contamination was 75 to 83.33 per cent.

The embryos excised from the seeds without GA_3 treatment failed to give a good response. These embryos failed to give a proper development of radicles and plumules within a month of observation. Whereas, mature embryos excised from the seeds treated with GA_3 cultured on MS media showed a more positive response and faster growth in the basal MS media with 100 per cent embryo survival within a month. The yellow coloured small embryo started to enlarge within a week when kept inside the dark condition. After two weeks when it was exposed to light the colour changed gradually from yellow to green due to chlorophyll development. Within two weeks, radicle and plumule started to developed and got a seedling in 60 days with proper growth. Compared to seed germinated seedling, seedling developed from embryo culture found to be smaller in size. Ake *et al.* (2007) reported beneficial influence of gibberellic acid treatment on coconut embryos on increasing the growth and conversion of embryo into plantlet.

To get a complete plantlet, excision of an intact embryo without damage is necessary. However *C. fenestratum* is characterized by very hard seed coat which makes the excision of the seeds inside the laminar airflow chamber and subsequent extraction of embryo difficult. Utmost care is needed to take the embryo excision without injury. During the embryo culture, it was noticed that either shoot or root portion only developed from injured embryo. The embryo had bipolar nature and damage in any side lead to the development of either shoot or root.

Present study revealed that *C. fenestratum* mature embryos excised from the GA_3 pretreated seeds can be successfully cultured on the MS basal media. Zygotic embryos excised from GA_3 pretreated seeds (4000 mg L^{-1} GA_3 solution for 72 hours)

when cultured on MS media in dark condition for 2 weeks followed by exposure to light showed faster development of the embryo, radicle, and plumule emergence and seedling development. Plantlets produced so can be used as base material for further micropropagation and conservation of this valuable medicinal plant.

Table 18. Effect of GA_3 pre-treatment in the seed for overcoming the dormancy, development of embryos and plantlet production

Treatment No.	Culture establishment (%)	Plumule emergence (%)	Radicle emergence (%)	Seedling development (%)
T_1	83.33	-	-	-
T_2	75	100	77.78	44.44

Plate 23. Split seed with yellow coloured embryo near the hilum

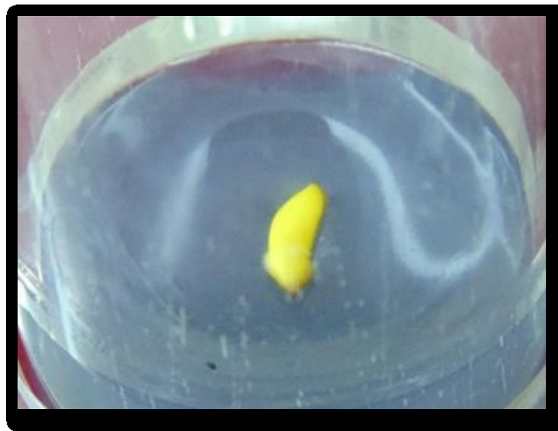


Plate 24. Excised embryo cultured in basal MS medium

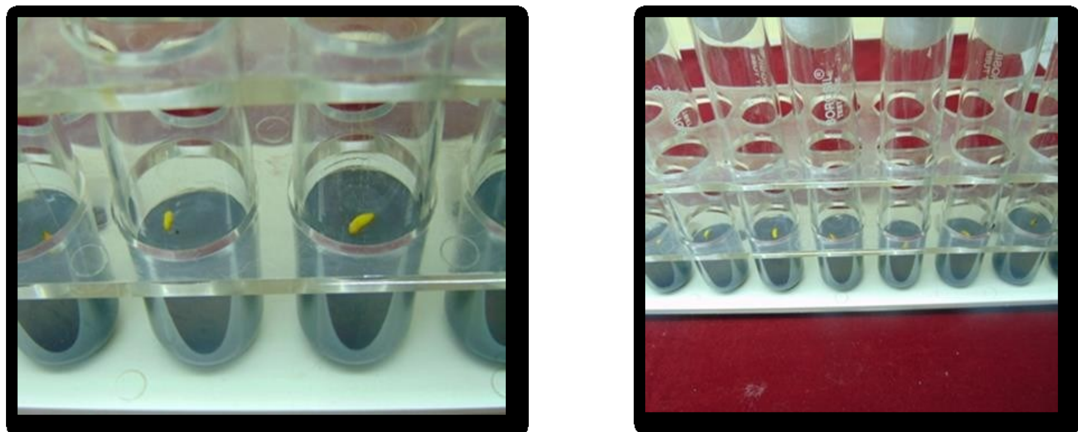
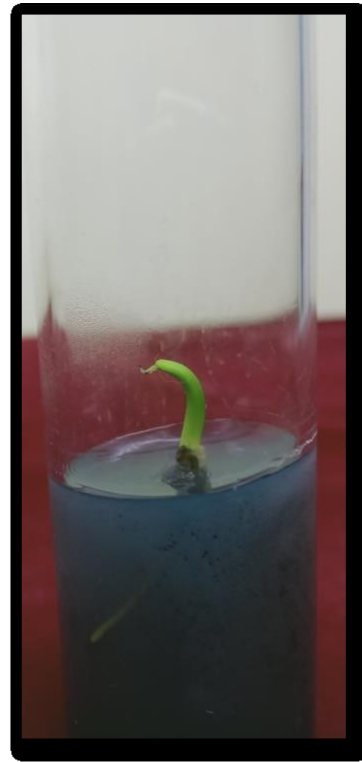


Plate 25. Effect of GA_3 pre-treatment on growth of embryos after 7 days

Plate 26. Effect of GA_3 pre-treatment on growth of embryo after 21 days

Plate 27. Colour change in embryo after exposure to light



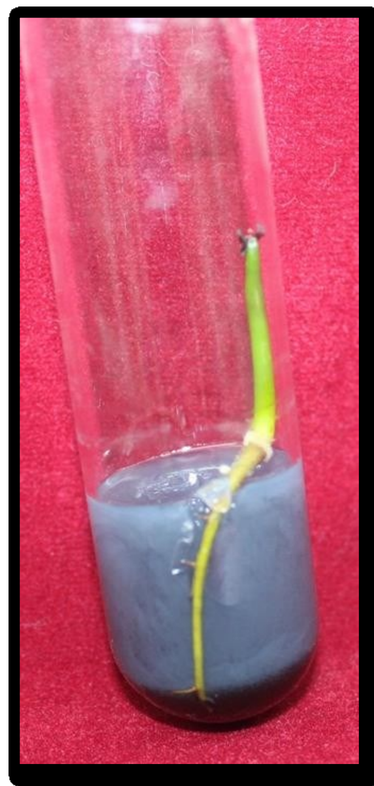


Plate 28. Effect of GA_3 pre-treatment on growth of embryo after 40 days

Plate 29. Effect of GA_3 pre-treatment on growth of embryo after 60 days

SUMMARY

SUMMARY

The present investigation entitled "Plant regeneration of *Coscinium fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture" was carried out to identify a suitable *in vitro* seed germination and shoot regeneration protocol for *C. fenestratum*. The experiment was conducted at the tissue culture laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the period 2018 - 2020.

The experiment consisted of treatments, for identification of best surface sterilization procedure, *in vitro* seed germination medium for the establishment of seeds in aseptic cultures, standardization of regeneration protocol using cotyledonary nodal explants from *ex vitro* germinated seedlings and nodal segments with axillary bud as explant from poly house grown plants. The salient features of the study are summarized below.

1. The culture establishment of *C. fenestratum* seeds under *in vitro* condition was found to be difficult with substantial culture loss due to endophytes. *C. fenestratum* seeds harbour fungal and bacterial endophytes. In spite of strong surface sterilization treatments given, the endophytes living inside the seeds survived and persisted inside the seeds. When inoculated these endophytes proliferated in the nutrient-rich culture medium. However endophytes were not harmful as such, they interfered with the culture establishment by contaminating the media.
2. Among the different sterilants tested for seed surface sterilization, either clorox or sodium hypochlorite along with ethanol combination, with different concentrations and exposure tried, found to be ineffective to control the contaminants with zero per cent culture establishment. In these cultures, fungal contamination started within a week. Fungal hyphal growth mainly started from the micropylar region and gradually spread to all over the surface of seed and media, confirming that microbes are endophytes.
3. The best sterilant for surface sterilization of seeds was 0.1 per cent $HgCl_2$. Treatment of seeds in liquid detergent for 10 min, followed by washing under running water for 30 min, then pre-treatment with bavistin (0.3 %) for 20 min

and streptocyclin (0.1 %) for 20 min outside the laminar airflow chamber and 0.1 per cent $HgCl_2$ (15 min) inside the laminar airflow chamber were found to be as suitable for reducing surface microbial load. In all $HgCl_2$ treatments even though surface microbial load and fungal endophytes reduced, bacterial endophytes found to be a major problem. When exposure time increased, bacterial endophytes also reduced, it may be due to the toxic nature of $HgCl_2$ that penetrated into the seeds. However higher exposure time may increase the mortality of the seed. So, further studies are needed to eliminate endophytes interaction by incorporating antibiotics inside the medium.

4. Among different media tried, sterilized sand: coir dust (1:1) media soaked with distilled water found to be best for the *in vitro* seed germination, which showed the highest germination percentage (22.95 %) and lowest germination time (55.68 days). Sterilized sand: coir dust (1:1) media soaked with liquid MS media showed, a low germination rate (13.91 %), an average of 79.8 days for initiation of germination. MS media with 1g/l activated charcoal was not a favorable media for seed germination, because of high contamination percentage due to fungal and bacterial endophytes, which led to full culture loss within a month.
5. When the cotyledonary nodal portions were used as explants from polyhouse grown seedlings, culture establishment was very low due to high microbial contamination. Even though explants were exposed to strong sterilant $HgCl_2$, 0.1 per cent for 2 min with 70 per cent ethanol for 1 min for surface sterilization treatment, culture establishment was found to be very low. The culture establishment percentage varied from 4.76 per cent to 23.81 per cent.
6. MS media supplemented with 0.06 mg L^{-1} 2, 4 - D along with cytokinin i.e., 0.2 mg L^{-1} BA, 0.2 mg L^{-1} kinetin and 0.4 mg L^{-1} kinetin showed response in shoot initiation in all established cultures. Whereas other treatments failed to gave the response in cotyledonary seedling explant culture.
7. The number of days taken for shoot initiation in cotyledonary nodal explant cultures was found to be the same without significant difference in the responded cultures. On an average bud initiation took place within 14 days.
8. Significantly more number of shoots were produced in cotyledonary explant inoculated in MS media supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2,

4 - D (4.5 shoots/ culture). When MS media supplemented with 0.2 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D an average of 3.83 shoots per culture and MS media supplemented with 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D an average of 2 shoots per culture were produced.

9. The shoots produced in MS medium supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D were stout and bigger than kinetin supplemented media and produced larger and broader leaves. In 0.4 mg L^{-1} kinetin with 0.06 mg L^{-1} 2, 4-D supplemented media shoots were very thin and slender and leaves were smaller than BA supplemented media.
10. Among the two different basal media tested for shoot initiation and multiplication from nodal segments from poly house grown plants, WPM was found to be good when compared to MS media.
11. In WPM supplemented with 0.4mg L^{-1} kinetin, almost 91.63 per cent of nodal explant culture responded for bud breaking. Whereas on MS media when supplemented with the same concentration of kinetin (0.4 mg L^{-1}) response noticed was only 22.55 per cent.
12. Among cytokinins tested for mature nodal explant bud culture, 0.4 mg L^{-1} kinetin was found to be superior for shoot induction compared to other treatments with 91.63 per cent of shoot induction in the basal WPM. WPM supplemented with 0.4 mg L^{-1} BA showed 73.29 per cent shoot induction and WPM supplemented with 0.2 mg L^{-1} kinetin reported in 68.44 per cent shoot induction.
13. Time required for morphogenic response of shoot induction in nodal axillary bud culture was found to be shorter in WPM (10 days), whereas in MS media the cultures took more time to shoot initiation (13 days).
14. Even though WPM basal medium supported better shoot initiation in mature nodal axillary bud, it failed to initiate multiple shoot production during a period of 4 months.
15. Embryo culture protocol was developed to get axenic seedlings in *Coscinium fenestratum*. Mature zygotic embryos excised from the GA_3 pretreated seeds (4000 mg L^{-1} GA_3 solution for 72 hours) cultured on MS media showed good response. Two week dark incubation followed by exposure to light showed

faster development of the embryo, radicle and plumule emergence and seedling development. These axenic seedlings can be used as the starting material for further micropropagation studies.

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PLANT REGENERATION OF *Coscinium fenestratum* (Gaertn.) Colebr. THROUGH AXENIC SEED CULTURE AND AXILLARY BUD CULTURE

By

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ABSTRACT OF THE THESIS

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Abstract

Coscinium fenestratum (Gaertn.) Colebr. is a medicinally important, perennial woody climber belonging to the family Menispermaceae. It is commonly known as Tree turmeric in English and locally as *Maramanjal* in Kerala and Tamil Nadu. Berberine, a yellow crystalline isoquinoline alkaloid is the main active principle compound present in the plant. The plant is a high volume traded one and its only source now is wild vegetation. Due to the combined impacts of high demand and over exploitation, the existence of this plant is under threat. Long pre-bearing age, seed dormancy, viability and regeneration problems also led to the extinction of this species and now the plant is listed as a critically endangered species in the IUCN red list of threatened species.

The present study entitled "Plant regeneration of *Coscinium fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture" was undertaken at tissue culture laboratory of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the academic year 2018 - 2020. The objective of the study was to develop a feasible and reproducible *in vitro* protocol for mass propagation of *Coscinium fenestratum*. Experiments included, identification of best seed surface sterilization procedure, identification of best *in vitro* seed germination medium, standardization of shoot regeneration medium for nodal explants and cotyledonary nodal explants and also standardization of embryo culture method.

Study revealed the presence of fungal and bacterial endophytes in the seeds of *Coscinium fenestratum*. Among the different sterilants tested for surface sterilization of seeds, 0.1% $HgCl_2$ (15 min) was found to be the best agent for the culture establishment with minimum contamination. Among the different media tested for *in vitro* seed germination, the sterilized sand: coir pith (1:1) media soaked with distilled water was found to be better with highest germination percentage (22.95 %) and lowest germination time (55.68 days).

An efficient shoot initiation and multiplication protocol was developed using seedling cotyledonary nodal explant. MS media supplemented with 0.06 mg L^{-1} 2, 4 - D along with different concentrations of cytokinin, 0.2 mg L^{-1} BA, 0.2 mg L^{-1} kinetin

and 0.4 mg L^{-1} kinetin responded to shoot initiation. On an average bud initiation was observed within 14 days after culture establishment. Significantly highest number of shoots were produced in MS media supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D (4.5 shoots/culture), followed by MS media supplemented with 0.2 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D (3.83 shoots/culture) and MS media supplemented with 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D (2 shoots/culture). The shoots produced in MS media supplemented with BA were stout and bigger than that obtained from kinetin supplemented media and they produced large and broad leaves.

Among the basal media tried for nodal explants, WPM was found to be better than the MS medium. Among the growth regulators, kinetin at 0.4 mg L^{-1} was found to be superior for shoot induction (91.63 %) with WPM basal medium. The period of morphogenic response for shoot induction was faster in the WPM medium (10 days). Even though shoot initiation was noticed in the WPM medium, all the treatments failed to give multiple shoot production.

Mature embryo of *Coscinium fenestratum* excised from the GA_3 pre-treated seeds could be easily cultured on the MS basal media. Zygotic embryo excised from GA_3 pre-treated seeds (4000 mg L^{-1} GA_3 solution for 72 hours) when cultured on MS medium in dark condition for 2 weeks followed by exposure to the light condition showed faster development of the embryo, radicle emergence (100 %), plumule emergence (77.78 %) and seedling development (44.44 %). These axenic seedlings without microbial contamination could be used as an explants for further micropropagation studies.

The study resulted in developing a feasible *in vitro* shoot regeneration protocol using seedling explant and axillary bud culture. The research results can be used as the stepping stone for further development of a high frequency plant regeneration protocol for the critically endangered medicinal plant *Coscinium fenestratum* and its conservation.