STANDARDIZATION OF MACRO AND MICRO PROPAGATION TECHNIQUES FOR MULBERRY (*Morus alba* L.)

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A Thesis

By

SURANJAN MONDAL

ROLL NO. MS 100808

Approved as to style and content by:

Prof. Dr. Md. Monirul Islam
(Supervisor)

Prof. Dr. Md. Abdul Mannan
(Co-supervisor)

Prof. Dr. Mohammad Bashir Ahmed
Chairman, Examination Committee
&
Head, Agrotechnology Discipline
Khulna University, Khulna

April, 2015

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ABBREVIATION

CAP	:	Cutting aid paste
BAP	:	N6 - benzylaminopurine
Kn	:	Kinetin
NAA	:	Naphthalene acetic acid
et al.	:	*et alli* and the rest
etc.	:	et cetra and the rest
i.e.	:	*id est*-which to say in other words
No.	:	Number
S.N.	:	Serial number
viz.	:	Namely
%	:	Percentage
CAP	:	Cutting aid paste
BAP	:	N6 - benzylaminopurine
IBA	:	Indole-3-butyric acid
ppm	:	Parts per million
g	:	gram
mg	:	milligram
mg/L	:	Milligram per litre
CD	:	Cowdung
VC	:	Vermicompost
cm	:	Centimeter
ANOVA	:	Analysis of variance
DMRT	:	Duncan's Multiple Range Test
EDTA	:	Ethylenediaminetetraacetic acid
mm	:	Millimeter
CRD	:	Completely Randomized Design
$HgCl_2$	:	Mercuric chloride
HCl	:	Hydrochloric acid
pH	:	Negative of logarithm to base 10 of the activity of hydrogen ion
NaOH	:	Sodium hydroxide
$FeSO_4$	:	Ferrous sulfate

ABSTRACT

Two experiments were conducted during March to September, 2014 in Khulna University campus. The first experiment was carried out to find a proper substrate for propagation of mulberry by stem cuttings in shed house of Plant Breeding and Biotechnology Laboratory, Agrotechnology Discipline, Khulna University. Fifteen types of different substrates were used. Cuttings from one year old mature branches with three potential buds were planted into media in 4"X5" size poly bag. From the study, the highest number of shoot was achieved from sand + CAP (2.0) and coconut coir (2.0). Average length of shoot was highest in sand (13.98 cm). Maximum average number of leaf was observed from sand media (8.4). The highest average number of root was count from cuttings planted in sand + CAP (3.48). Maximum average length of root was recorded from sand + Agronourish media (3.59 cm). For maximum average diameter of root, sand (2.43 mm), sand + CAP (2.46 mm), sand + Agronourish (2.44 mm), coconut coir (2.49 mm) and coconut coir + CAP (2.47 mm) were the best. Maximum root fresh weight was recorded from sand + Agronourish (1.43g). But the highest root biomass was achieved from sand + CAP (0.82 g). The highest fresh weight was obtained from sand (2.13 g), sand + CAP (2.23 g), sand + Agronourish (2.83 g), coconut coir (2.65 g) and coconut coir + CAP (2.52 g). Plants both in sand + CAP (0.58 g) and sand + Agronourish (0.6 g) produced the maximum average amount of shoot biomass. For leaf area index, sand + CAP (5.73), sand + Agronourish (5.74) and coconut coir + CAP (5.8) were the best.

In the second experiment, effect of different hormonal combination was tested for micropropagaton of mulberry. Direct shoot regeneration of mulberry was achieved by culturing nodal segment with axillary buds using ½ strength MS medium fortified with N 6 – benzylaminopurine (BAP), Kinetin (Kn) and naphthalene acetic acid (NAA). There were six different doses of BAP combined with NAA.Investigation was conducted onfrequency of proliferation of explants (%), average length of shoot (mm) and average number of leaf after 30 days of inoculation. From the statistical analysis of data, no significant difference found among the treatments for frequency of proliferation of explants (%). For the average length of shoot (mm), the doses of hormones showed highly significant effect. The highest average length of shoot (19.77) found from BAP 0.5 mgl^{-1} + NAA 0.5 mgl^{-1}. The maximum average leaf number (4.8) was achieved from Kn 1.0 mgl^{-1} in association with NAA 0.5 mgl^{-1}.

GENERAL INTRODUCTION

Mulberry (*Morus alba* L.) belongs to the genus *Morus*, family Moraceae, order Utricales. It's perennial in nature along with prolonged juvenile period. It is a cross-pollinated, heterozygous woody plant. On young vigorous mulberry shoots, leaves may be upto 30 cm long, with deeply and intricately lobed or with the lobes rounded. Leaves on older trees may be 5-15 cm long, unlobed, cordate at the base and rounded to acuminate at the tip, and serrate on the margins. Most of the types are found deciduous in temperate regions, but trees grown in tropical area can be evergreen. Mulberry flowers are single-sex catkins; male catkins are 2–3.5 cm long and female catkins 1–2 cm long. Male and female flowers are usually on separate trees although they may occur on same tree. Fruit of mulberry is generally 1–2.5 cm long. It is sweet but bland. Color of fruit in many cultivated plants varies from white to pink.

Most of the mulberry cultivars are believed to have originated in the area of China-Japan and in the Himalayan foothills. Over thousands of year, mulberry has been cultivated and adapted to a wide area of tropical, subtropical and temperate zones of Asia, Europe, North and South America, and Africa (Özgen *et al.*, 2009). Intensive production of mulberry and its utilization for animal production started in several countries in the late 1980's and early 1990's. It is widely distributed in India, China, Japan, North Africa, Arabia, South Europe etc. In the former Soviet Union, the most common species cultivated were *M. multicaulis, M. alba, M. tartarica* and *M. nigra* (Datta, 2000). In Indonesia (West Java), the mulberry species grown are *M. alba, M. nigra,M. multicaulis, M. australis,M. cathyana* and *M. mierovra*. The majority of the mulberry species are available in Asia, especially in China (about 24 species) and the Japan (about 19), though America is also rich in *Morus* species. However, the genus is poorly represented in Africa, Europe and the Near East. In Vietnam, about 100 mulberry cultivars of the species *M. alba, M. nigra* and *M. laevigata* are available. In China, about 15 species exists, out of which, *M. alba,M. multicaulis, M. atropurpurea* and *M. mizuho* are cultivated for sericulture. The mulberry species *M. insignis, M. rubra* and *M. celtidifolia* exist in Latin America also. In India, generally *M. indica, M. alba,M. serrata* and *M. laevigata* are grown naturally in northern India. But,

meanwhile, several cultivars of *M. multicaulis, M. nigra, M. sinensis* and *M. philippinensis* have also been introduced (Ravindran *et al.*, 1997).

Bangladesh is also one of the fertile lands along with good suited climatic condition for cultivation of mulberry. Main distribution of the plant is confined to greater Rajshahi region and south western part of the country. In Chuadanda, Meherpur, Kustia and Jhenaidah most number of plants are noticed. In some districts of this region, mulberry is used as roadside plantation besides commercial cultivation for silk industry.

Mulberry leaves are important for silkworm due to its rich content of different nutrients. It contain various minerals and extracts, such as beta carotene, GABA-1, amino acids, carotenoids, flavonoids, chlorophyll, vitamin C, B_1, B_2, B_6, A and they are rich in fiber. The leaves contain six times more calcium than green tea, 25 times more than milk and 40 times more than cabbage. With respect to iron it contains 2.5 times more than green tea and 10 times more than spinach (www.health.wikinut.com).

Mulberry leaf production is the agricultural part of sericulture which is important in the rearing of silkworms. Silk is a fibrous protein of animal origin. About four to five hundred species of animals are known to produce silk but only few are known to be commercially exploited. Nearly, 95% of the silk comes from the mulberry silk worm, *Bombyx mori*. Commercial silk from all other sources is collectively called as non-mulberry silk. A mulberry silk constitutes nearly 95 per cent of the total silk production; silk in popular terms may refer only to mulberry silk and sericulture only to the rearing of mulberry silk worm. The conscious cultivation of mulberry plants for harvesting leaves to be used as food for silkworms is referred to as moriculture (Singhal *et al.*, 2001).

Though, industrially leaf has the most important use, but other parts of mulberry plants are not useless. Mulberry fruits are an ingredient of a particularly seductive drink known as mulberry wine and mulberry leaves are often eaten as a vegetable and are useful as a cattle feed. A new UK fruit juice company Fairjuice has launched a super fruit drink prepared from pure fresh pressed mulberry fruits which is full of antioxidants. It is also a source of resveratrol which is considered to be beneficial for

heart health. It also suppresses the appetite, which is why it has been reported as a useful drink against obesity (http://www.foodbev.com/news/fairjuice-launches-superfruit-mulberry-juice#.VIlR1WfMeQA, viewed on 11 Dec., 2014).The fruit is also used in modern medicine for the preparation of syrup; to add flavors and natural color in medicines (Singhal *et al.*, 2003).

Integration of fish, livestock, and crop production is famous in case of agri-farming in many countries. The system recycles resources, reduces organic pollution (livestock and poultry manure are good organic fertilizers for fish farming), and combines fish farming with mulberry cultivation for raising silkworms. The silkworm pupae are used as fish feed, and the worm faeces and wastewater from silk processing as pond fertilizers. Pond silt is used as fertilizer for fodder crops, which can in turn be used to feed livestock, poultry, and fish (Singhal *et al.*, 2003).

South-western region of Bangladesh is well known for its poor economy in comparison with other parts of Bangladesh. Natural calamities as well as man-made global warming are causing serious problems in the ecology of this region. Soil and water salinity are increasing day by day that delimits agricultural activities. Main agricultural activities are limited into rain fed Aman rice cultivation followed by fish culture. Efforts are being carried out to introduce crops which are resistant to salinity. But it is time consuming and limited to only few crops or plants. Silk worm pupae are excellent source of protein and it can be a good feed item for fish. As rearing of silk worm is completely dependent on leaves of mulberry, there remains a great opportunity of large scale plantation of mulberry in this area.

Under the above circumstances the present research work was undertaken with the following objectives:

- To select the most suitable substrate for mass propagation of mulberry using stem cuttings
- To develop a protocol for *in vitro* regeneration of mulberry through nodal segments

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Experiment 1

STANDARDIZATION OF MACROPROPAGATION TECHNIQUE OF MULBERRY (*Morus alba*)

1.1 Introduction

1.1.1 Macropropagation of mulberry plant

Propagation is a common practice employed in all plants in order to obtain healthy and resistant plants. Different modes of propagation of trees have a lot of importance in order to grow trees on large scale.

Mulberry is propagated either through seeds or by vegetative means. Vegetative propagation is the most common method of propagation because of various advantages like maintenance of particular characters of the plant. Propagation through seeds has got certain limitations and is used only for breeding new varieties.

Asexual propagation has been one answer because of the shortened time requirement for cuttings of superior trees to root and grow; this method of reproduction is fast becoming a very important nursery management tool (Khan *et al.*, 2007).

Clonal plants produced true to type plants (Afzal *et al.*, 2006). Use of cuttings is one of the possible techniques for vegetative propagation of trees and it was observed that cuttings play important role in the rooting of important timber species (Awang *et al.*, 2009). Cuttings are probably the cheapest and effective method for propagating new plants. A cutting is any detached plant part that, under favorable conditions will produce a new plant identical to parent plant (Jain *et al.*, 1990).

1.1.2 Rooting media for propagation of mulberry plant

Rooting media has an important role in initiation and development of root and root growth followed by shoot proliferation and growth. Along with different media rooting hormone and supporting artificially supplied nutrient can promote the whole process.

Lot of research works have been carried out to check different aspects of propagation of mulberry plants while using cutting. But very few works have been conducted in case of effect of rooting media combined with rooting enhancer or nutrient on

producing successful saplings. *Morus alba* is an important tree for sericulture and other purposes like timber, fruit, shade etc.

Generally it's possible to produce saplings by cuttings of mulberry directly in the soil. But it does not ensure more number of saplings from comparatively less space as in nursery, where propagation can be carried out with prepared media in poly-bag or other container. On the other hand, number and quality of produced saplings in direct soil might be different from place to place depending on soil condition. But prepared media enriched with rooting hormone or growth substances which are previously incorporated with media or treated on cuttings could help to increase success than direct soil.

So keeping in view the importance of mulberry tree a research work was conducted to compare the effect of different rooting media on development of successful saplings using mulberry stem cuttings under shade house. The objective of the present experiment was:

- To select a suitable media for mass propagation of mulberry using stem cutting

1.2 Review of Literature

A number of researchers have worked on macropropagation of mulberry in different regions of the world on different variety and on propagating media.

Langawit and Faustino (2008) used different rooting media to produce saplings from mulberry cuttings of different varieties. Different rooting media used in this experiment were: Garden Soil as a Control (T_0), Compost + Poultry Liter + Garden Soil (T_1), Silkworm Rearing Waste + Garden Soil (T_2), Poultry Liter + Cow Dung Manure + Garden Soil (T_3), Sand + Sphagnum Moss + Garden Soil (T_4), and Silkworm Rearing Waste (T_5). All the different manures and silkworm rearing waste were fully decomposed before they were used. The results of this study found that the Batac mulberry variety cuttings planted in the rooting media mixture of Compost + Poultry Liter + Garden Soil (T_1), Poultry Liter + Cow Dung Manure + Garden Soil (T_3) and Sand + Sphagnum Moss + Garden Soil (T_4) gave the best results.

Propagation of mulberry through cuttings is still one of the most cost effective methods of clonal multiplication. Koyuncu and Senel (2003) conducted an experiment to determine the influence of cuttings collection time and planting methods for hard wood cuttings on the rooting percentage of black mulberry (*Morus nigra* L.). The dormant cuttings were prepared from one-year-old shoots during January, February and March, 2001. Hardwood cuttings treated with 5 gl^{-1} IBA rooted in perlite using bunch (BP) and rows (RP) as well as in hydroponics (HP). Rooting percentage, callusing percentage, number of roots per rooted cutting and average root length per (cm) were evaluated. The average rooting of black mulberry cuttings varied from 3.3 to 60.0%. The best rooting percentage was obtained from bunch planting and from January cuttings. Among the three methods they examined, bunch planting seemed the best method of black mulberry hardwood cuttings.

An effort was carried out by Ahmad *et al.* (2011) to find out the smaller size of cuttings for propagation of *Morus alba* cuttings of 5 cm, 10 cm, 15 cm and 20 cm were planted in polythene tubes of 9 cm x 18 cm, after filling the bags with soil/sand mixture (1:2 ratio), and were placed in the experimental area. Half out of total were placed in outdoor and remaining under polythene sheet. Root growth was found maximum in outdoor as compared to under polythene sheet.

Qadri *et al.* (2011) tried to propagate temperate region mulberry plants through cuttings under polyhouse condition. They reported that more than 50% saplings were survived. They found that uniform distribution of rainfall during the growing period helps in the growth, crop stand and timely sprouting.

Kako (2012) tried to identify the effect of IBA and Kinetin on budding success percentage of black mulberry cutting. In his research, the effect of cuttings diameter and ındole-3-butyric acid (IBA) doses on hard wood stem cuttings of black mulberry were studied. Cuttings (18-20 cm length) were taken from early February and treated with different IBA doses (0, 1000, 2000, 3000 and 4000 mg/l). Cuttings were planted in sandy loam soil under air condition in order to root. The results of the study revealed that cuttings diameter and IBA had significant effects on rooting percentage, length and diameter of transplants.

Baqual *et al.* (2007) conducted an experiment with six varieties of mulberry and under two environmental conditions- open and polyhouse. Among them polyhouse condition resulted vigorous rooting.

Kalyoncu *et al.* (2009) studied the effects of relative humidity and ındol-3-butyric acid (IBA) on softwood top cuttings of two black mulberry (Types 1 and 2) and one white mulberry (Type 3). In their experiment, cuttings were taken from early June (14 Haziran) and applied to the different IBA doses (0, 1000, 2000, 3000 and 4000 ppm). Cuttings were planted in pumice medium under misting system in the greenhouse for 48 days in order to root. The highest rooting percentage they observed from Type 1 (black mulberry) in 2000 and 3000 ppm IBA doses application (100%). The lowest one was control group from Type 2 (black mulberry) which was not rooted. With respect to root numbers, the highest value was found from Type 3 (21.73 number/cutting) and Type1 (16.42 number/cutting); the lowest one was control group of Type 2 (0.00 number/cutting). The longest root was determined from 3000 ppm IBA dose of Type 1 (11.23 cm); the highest root branching value was found from Type 3 in 3000 ppm IBA dose (16.20 number/cutting) application.

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1.3 MATERIALS AND METHODS

1.3.1 Materials

In the present research hard wood stem cuttings of 10 -12 cm of 8 – 10 mm diameter with 3 active buds were taken from two years old tree from Khulna University campus from March to May, 2014. The initial hard wood cuttings of mulberry (variety BM-1) were collected from Sericulture Research Institute, Rajshahi and plants were established in Khulna University campus.

Various substrates were used as rooting media to raise saplings from mulberry stem cuttings:

1. Garden soil (Collected from Khulna University campus)
2. Garden soil + Cutting aid paste (CAP- a rooting hormone containing IBA, NAA, PHB, H_3BO_3, vitamins, surfactant, talc. etc. produced and marketed by Liebigs Agro Chem. Pvt. Ltd., Kolkata)
3. Garden soil + Agronourish
4. Coarse sand
5. Coarse sand + Cutting aid paste (CAP)
6. Coarse sand + Agronourish
7. Coconut coir
8. Coconut coir + Cutting aid paste (CAP)
9. Coconut coir + Agronourish
10. Garden soil (50%) + Cow dung (50%)
11. Garden soil (50%) + Cow dung (50%) + Cutting aid paste (CAP)
12. Garden soil (50%) + Cow dung (50%) + Agronourish
13. Garden soil (50%) + Vermicompost (25%) + Cow dung (25%)
14. Garden soil (50%) + Vermicompost (25%) + Cow dung (25%) + Cutting aid paste (CAP)
15. Garden soil (50%) + Vermicompost (25%) + Cow dung (25%) + Agronourish

Agronourish is a commercial powdery fertilizer product made from humic acid, enriched with micronutrients.

1.3.2 Methods

1.3.2.1 Preparation of media

The experimental substrates (rooting media) mentioned above were prepared manually with care and treated with fungicide and filled in polybag (4" X 5").

1.3.2.2 Planting of cuttings into media

Before planting of stem cuttings, each poly bag was gently soaked with water. Then a small hole was prepared in every poly bag. Cuttings were planted into those holes in 45° angle while the tips were directed to north. After planting, medium around the bases were pressed gently to remove air from holes prepared before planting. For each treatment similar cutting size were used. Cuttings were watered immediately after planting.

1.3.2.3 After planting care

Cuttings after planting into media were regularly inspected to detect any abnormal change, water deficit or excessiveness, leaf dropping, pest attack or leaf drying.

1.3.2.4 Data collection

Data were collected for three times at 10 days interval on the following parameters.

- Number of bud/shoot emergence cutting^{-1}
- Average length of shoot (cm)
- Number of leaves cutting^{-1}

During the measurement for collecting information, centimeter scale and digital slide calipers were used.

After 45 day of planting, the following data were collected through destructive method.

- Number of roots cutting^{-1}
- Average length of roots (cm),
- Average diameter of roots (mm),
- Fresh weight of roots(g) cutting^{-1},

- Dry weight of roots(g) cutting^{-1},
- Fresh weight of shoots(g) cutting^{-1},
- Dry weight of shoots(g) cutting^{-1} and
- Leaf area index

For the measurement of above parameters, five plants were gently uprooted at the end of the experiment. Rest five plants were planted into the soil and observed for next 30 days.

1.3.3 Preparation and measurement of parameters

Simple meter scale was used to measure the length of the shoot. The numerical values for this parameter were recorded using the scale in centimeter value.

For measuring the number of roots, they were gently cleaned up by water; the water was removed with tissue paper and then detached from plants for measuring length by measuring scale of length in centimeter. All root length from each plant were measured separately and made the sum. In this way length of root was taken for every plant from every treatment.

The same roots used in length measurement were used to measure diameter. Measuring diameter of every single root, average was calculated.

The roots after measuring diameter were gathered plant wise to measure the fresh weight using electrical balance.

Roots were packed in brown paper separately and labeled and then kept in to oven to remove water. After drying properly, the samples were kept in oven and adjusted the temperature in normal environment while care was taken to retain them from absorbing water from air. When the root samples came in normal environment temperature the weight again measured using digital balance. Average weight was calculated for every plant. Then they were analyzed statistically.

Fresh weight of root was also measured at the same time of measuring that of shoot. Just after detaching shoots from the cuttings were preserved in polythene packet. Digital balance was used in measurement.

The fresh shoots used for fresh weight were packed in brown paper and labeled and taken to oven at 70°C temperature for 48 hours in oven to remove water. After complete drying the samples were kept in oven to adjust with normal environment temperature. Then they were taken out side and weighed separately using digital balance in gram.

1.3.4 Data transformation

Values obtained as zero after collection of data were processed by transforming into non-zero figures with the help of ARCSIGN method for the convenience of data analysis in MSTAT C.

1.3.5 Experimental design

The experiment was laid out in Completely Randomized Design (CRD) with 15 treatments and 10 replications for each.

1.3.6 Statistical analysis

Analysis of variance (ANOVA) was done for the collected data with MSTAT-C program, and means were compared with new Duncan Multiple Range Test (DMRT) program in computer.

1.4 RESULTS

The findings of the experiment regarding effect of different substrates on rooting and on other growth parameters to produce mulberry saplings are presented in this chapter.

1.4.1 Bud/shoot emergence

During propagation of a plant through cuttings it needs to plant cutting with active buds. The propagation starts with the emergence of the buds. Quick and vigorous emergence of bud is very important in this case. In the present study, the maximum number of bud (2.0) was emerged on 10^{th} day after planting from sand + CAP, coconut coir, coconut coir + Agronourish and 25% VC + 25% CD + 50% soil + Agronourish. On 20^{th} day, the highest number of bud was found from sand (1.9), sand + CAP (2.0), sand + Agronourish (1.9), coconut coir (2.0), coconut coir + CAP (1.9), coconut coir + Agronourish (2.0) and 25% VC+ 25% CD + 50% soil + CAP (1.9). On 30^{th} day the maximum number of emergence was from sand + CAP (2.0), coconut coir (2.0), coconut coir + Agronourish (2.0) and 25% VC + 25% CD + 50% soil + Agronourish (2.0). The analyzed data indicated that there were significant differences among different media used (Table 1).

1.4.2 Average shoots length (cm)

The quickness of development of shoot in length after emergence is the first sign for the effectiveness of a rooting media for multiplication of plants through cuttings. In a good rooting media, the shoots will develop faster before or at the same time with the development of roots. In the present experiment, the highest average length of shoot on 10^{th} day was recorded from the cuttings planted in sand media (5.45 cm), on 20^{th} day from sand + Agronourish media (11.16 cm) and coconut coir media (11.41 cm) (Table 1). On 30^{th} day the highest average length of shoot was found from sand (13.98 cm). Statistical analysis of the values indicated that there was significant difference among different treatments for length of shoot on all three dates.

1.4.3 Average leaf number

Number of the leaf emerged determines the rate of success of propagation of plant through cuttings. As more as possible the proper size and number of leaves will be

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Table 1. Effect of rooting media on shoot growth of mulberry saplings

Treatment	10th day			20th day			30th day		
	No. of shoots	Length of shoots(cm)	No. of leaves	No. of shoots	Length of shoots(cm)	No. of leaves	No. of shoots	Length of shoots(cm)	No. of leaves
Soil	1.8^{ab}	0.91^{g}	$1.3^{d}(1.2)$	1.5^{ab}	1.84^{g}	1.9^{de}	1.4^{b}	2.95^{gh}	1.6^{efg}
Soil + CAP	1.5^{b}	0.93^{g}	$1.3^{d}(1.2)$	1.3^{b}	2.25^{g}	1.7^{de}	1.4^{b}	2.75^{h}	2.1^{efg}
Soil + Agronourish	1.7^{ab}	0.92^{g}	$1.28^{d}(1.15)$	1.7^{ab}	1.92^{g}	1.3^{e}	1.7^{ab}	1.87^{i}	1.4^{fg}
Sand	1.9^{ab}	5.45^{a}	$1.89^{ab}(3.1)$	1.9^{a}	10.94^{a}	7.3^{a}	1.8^{ab}	13.98^{a}	8.4^{a}
Sand + CAP	2.0^{a}	3.72^{c}	$1.88^{ab}(3.1)$	2.0^{a}	9.63^{b}	6.0^{b}	2.0^{a}	9.83^{d}	5.7^{c}
Sand + Agronourish	1.9^{ab}	4.53^{b}	$1.83^{ab}(2.9)$	1.9^{a}	11.16^{a}	7.0^{a}	1.9^{ab}	12.57^{b}	6.5^{bc}
Coconut coir	2.0^{a}	4.88^{b}	$1.97^{a}(3.4)$	2.0^{a}	11.41^{a}	7.6^{a}	2.0^{a}	12.61^{b}	6.7^{bc}
Coconut coir + CAP	1.8^{ab}	4.9^{d}	$1.8^{ab}(2.8)$	1.9^{a}	8.08^{c}	5.4^{b}	1.9^{ab}	9.71^{d}	7.0^{b}
Coconut coir + Agronourish	2.0^{a}	4.78^{b}	$2.05^{a}(3.7)$	2.0^{a}	9.81^{b}	7.6^{a}	2.0^{a}	11.93^{c}	5.65^{c}
Soil + 50% CD	1.6^{ab}	0.83^{g}	$1.3^{d}(1.2)$	1.7^{ab}	2.82^{f}	1.3^{e}	1.7^{ab}	2.65^{h}	1.1^{g}
Soil + 50% CD + CAP	1.6^{ab}	3.44^{c}	$1.64^{bc}(2.25)$	1.7^{ab}	3.57^{e}	2.3^{d}	1.8^{ab}	3.57^{f}	2.2^{efg}
Soil + 50% CD + Agronourish	1.6^{ab}	0.55^{g}	$0.71^{e}(0.0)$	1.6^{ab}	1.9^{g}	1.3^{e}	1.9^{ab}	3.25^{eg}	2.3^{ef}
25%VC + 25%CD + 50% Soil	1.6^{ab}	1.94^{f}	$1.35^{d}(1.35)$	1.6^{ab}	3.25^{ef}	2.4^{d}	1.6^{ab}	3.73^{f}	2.5^{ef}
25%VC + 25%CD + 50% Soil + CAP	1.7^{ab}	2.37^{e}	$1.47^{cd}(1.7)$	1.9^{a}	6.15^{d}	3.5^{c}	1.9^{ab}	4.73^{e}	4.3^{d}
25%VC + 25%CD + 50% Soil + Agronourish	2.0^{a}	1.78^{f}	$1.4^{cd}(1.5)$	1.9^{a}	3.22^{ef}	2.3^{d}	2.0^{a}	4.84^{e}	2.7^{e}
CV (%)	22.32	11.25	12.06(30.73)	21.81	5.87	16.36	20.40	5.37	18.97
Level of significance	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

ARC SIGN data transformation method was used for no. of leaves during data analysis in MSTAT C. Original data are given in parenthesis.

CAP = Cutting Aid Paste
CD = Cow dung
VC = Vermicompost

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developed from a cutting, it will strengthen the demand of producing a good quality sapling. Here, in the present study, the statistically analyzed data indicated that there was significant difference among the different rooting media for number of leaf bearing after emergence of shoot. Maximum average number of leaf on 10^{th} day was found from cuttings in coconut coir media (1.97) and coconut coir + Agronourish (2.05). On 20^{th} day, the highest average number was recorded from media, sand (7.3), sand + Agronourish (7.0), coconut coir (7.6) and coconut coir + Agronourish (7.6) (Table 1). On 30^{th} day the highest number of leaf was recorded from sand (8.4).

1.4.4 Average root number

The number of root determines the rate of success of propagation. Generally cuttings producing more number of roots are regarded as to produce more successful plantlets which will put support to further growth and development. Average number of root was count at 45^{th} day of planting after unbiasly picked up 5 plants from each treatment. From the analysis of data for the average number of roots from the cuttings there was also found a statistically significant difference among treatments. In the present experiment, the maximum average number of roots was counted from sand + CAP media (3.48) (Table 2).

1.4.5 Average root length (cm)

Length of root determines how many media portion it will anchorage. It is directly linked to the amount of nutrient the plant will absorb. So, increase in the length of root is an important factor to justify the effectiveness of the rooting media. By this, it indicates the quickness of producing new plants from cuttings. If the length of initiated root increases quickly, it indicates that the media is good enough to propagate the type of the plant easily and quickly using that media. The highest value (3.59 cm) was identified from sand + Agronourish (Table 2). For the average length of root, there was also significant difference among the treatments used.

1.4.6 Average root diameter (mm)

To prove any media good enough for propagation of any specific plant, it is obvious that it will ensure the smooth and proper development of root and increase in diameter is a part of this. In analysis of variance, statistically significant difference was proved among the treatments. Maximum average diameter of roots was observed from the

cuttings grown on sand (2.43 mm), sand + CAP (2.46 mm), sand + Agronourish (2.44 mm), coconut coir (2.49 mm) and coconut coir + CAP (2.47 mm) (Table 2).

1.4.7 Root fresh weight (g)

The total amount of root from a newly produced plant is measured by fresh weight and it is linked to the length and diameter of roots of that plantlet. Generally the sapling producing more amount of root is considered as better quality plantlet. In the present study, statistically significant difference was also found for the fresh weight in analysis of variance among the treatments. Maximum value of fresh weight (1.43 g) was found from sand + Agronourish medium (Table 2).

1.4.8 Root dry weight (g)

Dry weight of root is a part which contributes in total biomass. It is determined by excluding the liquid portion. So, it indicates the total amount of carbohydrates and other solid materials in root which constitutes main body of plant. There was significant difference among different treatments in respect of dry weight of roots. Among the treatments, sand + CAP medium was found the best which produced the highest dry weight of root (0.82 g) (Table 2).

1.4.9 Shoot fresh weight (g)

This weight is taken for the over ground part of the plant body just after seperating the roots. This is important in measurement of plant growth as more as canopy produced by a plant is supposed to be a successful plantlet especially during propagation. Rooting media has a strong influence on this as it supplies sufficient support for the development of the plant. Statistically significant variation was found among the treatments for fresh weight of shoot. The highest fresh weight of shoot was recorded in the present experiment from five media viz. sand (2.13 g), sand + CAP (2.23 g), sand + Agronourish (2.83 g), coconut coir (2.65 g) and coconut coir + CAP (2.52 g) (Table 2).

1.4.10 Shoot dry weight (g)

Shoot dry weight indicates the total over ground biomass of a plant excluding water. Statistically significant difference was found for shoot dry weight. The maximum

Table 2. Effect of rooting media on root and shoot growth of mulberry saplings

Treatment	No. of roots	Average length of roots (cm)	Average diameter of roots (mm)	Fresh weight of roots (g)	Dry weight of roots (g)	Fresh weight of shoots (g)	Dry weight of shoots (g)	Leaf Area Index
Soil	0.71^{g}(0.0)	0.71^{g}(0.0)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.38^{bc}	0.14^{c}	3.06^{e}(8.86)
Soil + CAP	1.76^{e}(2.6)	1.99^{d}(3.46)	2.24^{c}(4.5)	0.72^{g}(0.02)	0.71^{b}(0.003)	0.25^{bc}	0.11^{c}	2.78^{f}(7.25)
Soil + Agronourish	0.71^{g}(0.0)	0.71^{g}(0.0)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.18^{c}	0.05^{c}	071^{h}(0.0)
Sand	2.95^{b}(8.2)	3.26^{b}(10.15)	2.43^{a}(5.39)	1.2^{c}(.95)	0.74^{b}(0.044)	2.13^{a}	0.45^{b}	5.11^{b}(25.65)
Sand + CAP	3.48^{a}(11.6)	2.85^{c}(7.6)	2.46^{a}(5.53)	1.39^{b}(1.43)	0.82^{a}(0.175)	2.23^{a}	0.58^{a}	5.73^{a}(32.34)
Sand + Agronourish	2.98^{b}(8.4)	3.59^{a}(12.38)	2.44^{a}(5.46)	1.43^{a}(1.54)	0.76^{b}(0.076)	2.83^{a}	0.6^{a}	5.74^{a}(32.41)
Coconut coir	1.92^{de}(3.2)	2.86^{c}(7.65)	2.49^{a}(5.7)	0.84^{e}(.21)	0.71^{b}(0.008)	2.65^{a}	0.41^{b}	4.91^{c}(23.58)
Coconut coir + CAP	2.1^{cd}(4.0)	2.93^{c}(8.35)	2.47^{a}(5.6)	0.94^{d}(0.38)	0.73^{b}(0.039)	2.52^{a}	0.4^{b}	5.58^{a}(30.59)
Coconut coir +Agronourish	1.37^{f}(1.4)	1.46^{f}(1.67)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.62^{bc}	0.15^{c}	3.47^{d}(11.54)
Soil + 50% CD	0.71^{g}(0.0)	0.71^{g}(0.0)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.13^{c}	0.05^{c}	0.71^{h}(0.0)
Soil + 50% CD + CAP	0.71^{g}(0.0)	0.71^{g}(0.0)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.33^{bc}	0.03^{c}	0.71^{h}(0.0)
Soil + 50% CD + Agronourish	0.71^{g}(0.0)	0.71^{g}(0.0)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.11^{c}	0.06^{c}	0.71^{h}(0.0)
25% VC+25%CD +50% Soil	2.21^{c}(4.4)	1.63^{ef}(2.16)	2.28^{bc}(4.7)	0.76^{f}(0.07)	0.71^{b}(0.009)	1.05^{b}	0.32^{b}	2.6^{g}(6.24)
25%VC+25%CD+50% Soil + CAP	0.71^{g}(0.0)	0.71^{g}(0.0)	0.71^{d}(0.0)	0.71^{i}(0.0)	0.71^{b}(0.0)	0.48^{bc}	0.31^{b}	0.71^{h}(0.0)
25%VC+25%CD+50% Soil + Agronourish	1.51^{f}(1.8)	1.82^{de}(2.82)	2.34^{b}(5.0)	0.72^{h}(0.01)	0.71^{b}(0.002)	0.56^{bc}	0.06^{c}	0.71^{h}(0.0)
CV (%)	7.64(16.12)	9.47(22.33)	2.31(6.36)	1.31(9.59)	3.40(171.08)	35.9	27.34	3.07(6.85)
Level of significance	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Data were collected after 45 days of planting

ARC SIGN data transformation method was used for no. of roots, length of roots (cm), diameter of roots (mm), fresh weight of roots (g), dry weight of roots (g) and leaf area index during data analysis in MSTAT C. Original data are given in parenthesis.

CAP = Cutting Aid Paste

CD = Cow dung

VC = Vermicompost

shoot dry weight was found from sand + CAP (0.58 g) and sand + Agronourish (0.6 g) (Table 2).

1.4.11 Leaf area index

Leaf area index is a dimensionless quantity that characterizes plant canopies. It is defined as the one sided green leaf area per unit ground surface area in broadleaf canopies. It is used to predict the photosynthesis primary production, evapotranspriration and it also determines the crop growth. In the present study, the maximum leaf area index was recorded from sand + CAP (5.73), sand + Agronourish (5.74) and coconut coir + CAP (5.58) (Table 2).

1.5 DISCUSSION

1.5.1 Bud/shoot emergence

For the appearance of the bud from the cuttings, using sand and coconut coir media with their associations to produce highest number of buds, the reason may be the high porosity of the rooting media which can satisfy a good air-water relationship. A good air-water relationship is essential for proper rooting from the cuttings. As this air-water relationship was higher in to the media of sand and coconut coir and their associations, it might be the reason for the maximum and quick bud emergence from those media.

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1.5.2 Average shoots length (cm)

As first shoot emergence occurred from the cuttings planted in the sand and coconut coir media with their associations where possibility of air circulation was higher due to good air-water relationship into those media, rate of growth of shoot might be higher.

1.5.3 Average leaf number

Generally as much as any plant or shoot grows, it produces more leaves accordingly. In the present experiment, leaves counted from the cuttings planted in to the media coconut coir and sand supplemented with Agronourish which has followed the normal rules.

1.5.4 Average root number and length

The media which provide facility to grow root easily when the media is loose enough, have sufficient water holding capacity, can supply nutrients sufficiently, the development of root might be higher there. Here maximum average number of root was from sand + CAP. CAP is rooting hormone which accelerates the initiation of roots. The maximum average length of roots resulted from sand + Agronourish media, where Agronourish is multi-nutrient supplying agent. After the initiation of roots their length increases to the highest dimension as there was nutrient supply from Agronourish.

1.6 SUMMARY

To develop a successful propagation method it needs to emphasize on some parameters by measuring in different stages of development during the study period. These parameters determine the effectiveness of propagation by the method used in the experiment. Some of such type parameters were selected and data were collected under the present study.

For emergence of buds from stem cuttings using different rooting media in polybag, sand + CAP, coconut coir + Agronourish and 25% VC + 25% CD + 50 % soil Agronoursh media worked the best, by producing quick and maximum number of buds. On the other hand, shoot grew quickly to reach a highest length comparing other media used in the experiment found on sand. But coconut coir media has also the potentiality to make the faster growth of shoot. Because, on the 20th day a highest data was recorded from sand for the same parameter. For the production of leaves after emergence, sand media also proved here the best to produce maximum number of leaves. Besides, some other media like coconut coir + CAP and coconut coir + Agronourish also has the possibility to produce better results. For production and easily spreading of root, comparatively loose media could produce the better result. Here also sand + CAP media produced the maximum number of roots from the cuttings. CAP may accelerate the cuttings to produce early roots. But the length of roots was higher in sand + Agronourish media as Agronourish supplied some extra nutrients after initiation of roots. For diameter of root several media was proved the best, viz. sand, sand + CAP, sand + Agronourish, coconut coir and coconut coir + CAP. There was no significant difference among the above mentioned media to produce the maximum diameter. Fresh weight of root was found the highest amount from using sand + Agronourish media. But the dry weight of root was highest from a different media, sand + CAP. Fresh weight of shoot again found the highest from five media as like as diameter of root, viz. sand, sand + CAP, sand + Agronourish, coconut coir and coconut coir + CAP. But the dry weight of shoot was the highest from two media- sand + CAP and sand + agronourish. Maximum leaf area index was resulted from three media with statistically no significant difference among them. Here sand + CAP, sand + Agonourish and coconut coir + CAP were the best to produce highest leaf area index.

1.7 CONCLUSION

Silk industry is one of the promising agro-industry which is very much important to the civilization. Mulberry as because of a sole food of silkworm, the importance of mulberry cultivation is deeply related to this industry.

In the present study fifteen types of different rooting media were used to identify a best one which can help to produce good quality mulberry plantlets within short time, for further plantation. The study concluded that sand media can give the best result for multiplication of mulberry using stem cutting containing active buds. Addition of any rooting aid chemical may enhance the rate of success and make the process faster.

1.8 RECOMMENDATION

Further investigation can be undertaken to justify the multiplication process for validation. Number of treatments and replications could be increased to reduce the experimental errors. Moreover, the experiment could be undertaken in different environmental condition like in open sun, lathe house etc. There is scope of being more careful about water management into media.

Experiment 2

STANDARDIZATION OF MICROPROPAGATION TECHNIQUE OF MULBERRY (*Morus alba* L.)

2.2 REVIEW OF LITERATURE

Some techniques of microropagtion of mulberry, especially direct shoot regeneration *in vitro*, which are most resemble to the present study have been discussed below.

There are reports on micropropagation of mulberry (*Morus alba* L.) through *in vitro* culture of shoot tip and nodal explants by Anis *et al.* (2003). In the experiment, the researchers used Murashige and Skoog (MS) medium supplemented with different doses of both BAP and Kn. They achieved a high frequency of sprouting (80%) from nodal- and (70%) from shoot tip explants and shoot differentiation in the primary cultures of the explants. The combination of BAP (2 mgl^{-1}) and NAA (0.2 mgl^{-1}) is MS media proved best for multiple shoot formation. Slightly tender nodal explants of medium thickness (0.5 - 0.6 cm) with emerging greenish axillary buds responded more favorably in terms of bud sprouting and shoot differentiation. The survival percentage and their subsequent development into shoots varied from 35 to 80% in nodal and 20 - 70% in shoot tip explants on MS supplemented with various plant growth regulators. The frequency of sprouting was comparatively lower on Kn supplemented medium.

An efficient reproducible method was described by Attia *et al.* (2014), on rapid micropropagation of mulberry (*Morus alba* L. cv. Al-Taify) using axillary bud explants from mature plants. They induced shoots of mulberry plant on Murashige and Skoog (MS) medium supplemented with different concentrations of BAP. They found highest percentage of shoot initiation (90%) on MS medium supplemented with 2 mgl^{-1} BAP. In their experiment, they used 3-4 cm long shoots and observed a higher average number of elongated shoots (19) on MS medium contained 1 mgl^{-1} BAP and 1 mgl^{-1}Kn.

Khalafalla *et al.* (2007) reported the effects of 6-benzylaminopurine (BAP) alone or in combination with naphthalene acetic acid and Kinetin (Kn) alone or in combination with 2,4-dichlorophenoxy acetic acid (2,4-D) from their experiment, during induction on multiple shoot induction *in vitro* from the stem nodal segments on Murashige and Skoog (MS) medium. In their experiment maximum number of shoots (7.2 shoots per explants) was observed on the medium containing BAP (0.5 mgl^{-1}) in combination with NAA (0.5 mgl^{-1}).

Akram *et al.* (2012) studied axillary shoot proliferation and rooting of *Morus macroura* from nodal explants of mature trees. In their study the nodal explants (1.5 cm long) were cultured in MS medium supplemented with different levels of N6-benzyladenine (BA: 2, 4, 8, 10 or 12 µM) and 1-nepthaleneacetic acid (NAA: 2 or 3 µM for each). The rate of bud break was 100% at 10 µM BA in combination with either concentration of NAA (2 or 3 µM) after 6 days of culture. The length of axillary shoots was significantly improved by increasing the level of BA with Auxin. Most of the nodal explants (60.2%) developed surface calluses on the aerial portions of explants at comparatively lower BA levels (2-4 µM). They cut the belongating axillary shoots to prepare further nodal explants for multiple shoot induction. They found the MS medium supplemented with BA (8.8 µM) and 2µM indole-3-butyric acid (IBA)was quite effective for 65% shoot induction with 4.7 mean number of shoots and 8.4 mm shoot length after a week.

Balakrishnan *et al.* (2009) found the greatest multiple shoot formation with BA (0.5 and 1.0 mgl^{-1}) culturing mulberry shoot tip on MS media. They also observed a high frequency of regeneration (80%) and shoot differentiation in the primary culture of nodal explants of *Morus alba* L. on MS medium supplemented with 2, 4-D (0.3 mgl^{-1}) before proliferation of shoot in MS medium supplementation with BAP. GA3 (0.05 mgl^{-1}) facilitated the elongation of shoots followed by sprouting of axillary buds of in vitro grown shoots. They also observed a high frequency of rooting (86.7%) with development of healthy roots from shoots cultured on medium with 2, 4-D (1.0 mgl^{-1}).

Chattopadhyay *et al.* (2011) tested Micropropagation ability of two cytotypes (2n = 2x = 28 and 2n = 3x = 42) of mulberry (variety S1) in MS media fortified with N6-benzyl amino purine (BAP) than those of diploids. They observed maximum shoot initiation frequency (80%) with 4.4 µM of BAP on 4.2 days of culture in triploid; whereas, the axillary buds sprouted within 3.5 days with 100% shoot initiation frequency in diploid cultures. In their experiment, highest shoot length (4.8 and 5.6 cm per explant) was obtained at 8.8 and 4.4 µM BAP supplementations in diploid and triploid cytotypes, respectively.

Habib *et al.* (2003) undertook a study to develop a reproducible protocol for *in vitro* clonal propagation of white mulberry (*Morus alba* L.). They used different doses of BA and Kn in MS media primarily shoot regeneration and then used rooting hormone

to develop rooted plantlets. In their experiment axillary shoot proliferation was included in shoot tip and nodal segments of two sources on MS media fortified with 0.2, 0.5, 1.0 and 2.0 mgl^{-1} for selecting better explant type. Nodal and shoot tip explants were collected from mature field grown mulberry plants. The highest (60%) shoot tips showed proliferation on MS medium supplemented with 1.0 mgl^{-1} BA while 70% of the nodal segments produced shoots (proliferated) on the same media composition.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Mulberry, variety BM-1, released by Bangladesh Sericulture Research Institute (BSRI).

2.3.1.1 Explants

Nodal segments of 2.5 cm length containing one active bud collected from three years old mulberry tree from Khulna University campus.

2.3.1.2 Culture media:

For establishment of explants and further proliferation and shoot elongation half strength Murashige and Skoog (MS) medium was used (Murashige and Skoog, 1962).

2.3.1.3 Experimental treatments

1. No treatment (Control)
2. BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1})
3. BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1})
4. BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1})
5. BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1})
6. BAP(2.5 mgl^{-1}) + NAA (0.5 mgl^{-1})
7. BAP (3.0 mgl^{-1}) + NAA (0.5 mgl^{-1})
8. Kn (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1})
9. Kn (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1})
10. Kn (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1})
11. Kn (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1})
12. Kn (2.5 mgl^{-1}) + NAA (0.5 mgl^{-1})
13. Kn (3.0 mgl^{-1}) + NAA (0.5 mgl^{-1})

2.3.2 Methods

2.3.2.1 Experimental design

The experiment was set up in a Completely Randomized Design (CRD) with 13 treatments and 30 replications.

2.3.2.2 Preparation of stock solution

Before preparation of media used in experiment, stock solutions were prepared. Materials for macro stock, micro stock, iron stock, vitamin stock, inositol stock, glysine stock and EDTA stock solutions were collected from laboratory and after preparation, the stock solutions were stored in refrigerator. In the second phase BAP, Kn and NAA solutions were prepared in selected concentrations for the experiment and preserved in refrigerator.

2.3.2.3 Macro stock solution

1000 ml distilled water was taken in a beaker. 750 ml water was taken in to another beaker from that amount. All the salts and chemicals were weighed accurately using electric balance and dissolved in to that 750 ml water. Then the amount was made up to 1000 ml by addition from the previously taken water. The solution was well mixed up using stirrer. This was stored in refrigerator.

2.3.2.4 Micro stock solution

Measuring all prescribed chemicals they were dissolved in 100 ml distilled water. Finally they were well mixed up in the solution using stirrer. Thus the solution was preserved in refrigerator in 4°C temperature for further use.

2.3.2.5 Iron stock

The salt solution was prepared at 10 folds (10x) of the final strength of $FeSO_4$ and Na_2-EDTA in 100 ml of distilled water and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of distilled water. Finally, the stock solution was filtered, poured into clean amber glass bottle and preserved in refrigerator in 4°C temperature.

2.3.2.6 Vitamin stock

The prescribed amount of vitamins for the solution were measured separately and dissolved in distilled water at 100 folds (100x) of their final strength, mixed up properly and finally made up the volume upto 100 ml by addition of distilled water. The prepared solution was stored below 0°C in refrigerator.

2.3.2.7 Myoinositol

Myoinsitol was prepared individually as like as vitamins stock solution preparation and stored in the same way.

2.3.2.8 N6 - Benzyl Amino Purine (BAP) stock

To prepare the stock solution of BAP, 30 g of BAP was taken a 100 ml clean beaker in 50 ml distilled water and mixed up properly using magnetic stirrer. Then the volume was made up to 100 ml by adding distilled water and again mixed up to prepare well mixed solution. The solution was stored at 4 - 6°C temperature in refrigerator.

2.3.2.9 Kinetin (Kn) stock

30 g Kinetin was weighed and dissolved in distilled water in a 100 ml beaker with the help of a magnetic stirrer to prepare Kinetin (Kn) stock solution. Finally the solution was stored in refrigerated at 0°C temperature.

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2.3.2.10 Naphthalene Acetic Acid (NAA) stock

As like as Kinetin solution, 30 g Naphthalene Acetic Acid was dissolved in 100 ml distilled water in a 100 ml beaker and mixed well to prepare 100 ml NAA solution. The prepared solution was finally preserved in 0°C in refrigerator for further use.

2.3.2.11 MS basal media preparation

4,500 ml half strength MS media was prepared for multiple shoot regeneration. For convenience total amount was prepared in two steps; 3,000 ml and 1,500 ml. For 3,000 ml media, 21 g bacto agar was added to 1,500 ml double distilled water in a 2 L beaker and boiled on magnetic stirrer cum heater to dissolve the agar. Total amount of 90 g sucrose was dissolved in 500 ml double distilled water in a 1L beaker. 37.5 ml macro nutrients, 15 ml micronutrients, 15 ml vitamins, 30 ml inositol, 15 ml glysine and 15 ml EDTA solution were mixed with sucrose solution. Then the mixture was added to boiling agar solution stirred well using magnetic stirrer. The solution was taken to a volumetric flask and double distilled water was added to adjust the required amount. Using the same process, another 1,500 ml half strength MS media was prepared when the amount of agar was 10.5 g, sucrose 45 g, macro nutrients 18.75 g,

micronutrients 7.5 g, inositol 15 ml, vitamins 7.5 ml, glysine 7.5 ml and EDTA 7.5 ml.

4,200 ml solution was divided into 14 parts each containing 300 ml. From this total, two (600 ml in total) was kept as control after adjusting the pH range 5.6 – 5.8. In rest 12 parts, hormones were added to different doses.

For rest 12 parts each containing 300 ml media, hormones were mixed up.

In every case, pH was adjusted to 5.6 by adding 0.1 N HCl or 0.1 N NaOH before pouring of media into test tubes. Each test tube contained 10 ml media. Opening of test tubes were closed by immediately with autoclaved cotton plugs and covered with brown paper and autoclaved at 121°C maintaining 1.5 kg cm^{-2} pressure for 30 minutes.

2.3.2.12 Sterilization of explants

Fresh twigs were collected from mulberry plant. From them explants were taken by cutting them on both sides of node. The explants were then washed out thoroughly with running tap water for 30 minutes. Then the explants were washed out with a detergent solution, Tween 20 for 10 minutes. Immediately after washing with Tween 20, the explants were washed with distilled water for several times to remove the surface adhering contaminants. The explants were then taken into a Laminar Airflow Hood Chamber and kept in 70 % ethanol solution for 30 seconds. After that the explants were dipped into 0.1% $HgCl_2$ solution for 5 minutes. Finally the explants were washed in autoclaved double distilled water for 3 times. Excess water from the surface of the explants was removed using autoclaved tissue paper.

2.3.3 Inoculation and Incubation

The explants after sterilization were inoculated on culture medium inside Laminar Airflow Hood.

2.3.3.1 Inoculation

Surface sterilized explants were carefully inoculated into the medium with the help of forceps and fitted with cotton plugs. Before insertion into the medium excess amount

2.4 RESULTS

Results obtained in this experiment are sequentially presented and described below:

2.4.1 Effects of growth hormones

2.4.1.1 Frequency of proliferation of explants

Number of proliferated shoots was counted from every single culture in test tube under every replication. The statistical analysis of the recorded data from the experiment showed that there was no significant difference among the numbers of developed bud due to the effect of different hormone doses used in media (Table 3).

2.4.1.2 Average length of shoot

Length of shoot might be another important parameter for success of shoot development in micropropagation using growth regulator in culture medium. In the present experiment, the maximum average length of shoot (19.77 mm) was achieved from BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1}) while the experimental data showed that there was significant differences among various concentration levels and combinations of hormones used in MS media (Table 3).

2.4.1.3 Average number of leaves

Number of leaf development could be an important plant growth character during multiplication of plants by tissue culture. In the present study, the number of leaves varied in different combinations of BAP and NAA and Kn and NAA and statistical analysis showed that there was significant differences among the treatments. Combination of Kn (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) produced the maximum number (4.8) of leaf which was statistically resemble to control, BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), BAP (3.0 mgl^{-1}) + NAA (0.5 mgl^{-1}) and Kn (2.5 mgl^{-1}) + NAA (0.5 mgl^{-1}) (Table 3).

Table 3. Effect of different levels of combinations of BAP and NAA and Kn and NAA on multiple shooting of mulberry after 30 days of inoculation

Treatment	No. of shoot tips inoculated	Frequency of proliferation of shoot buds (%)	Length of shoots (mm)	No. of leaves
Control	30	70.00	14.87^{b}	4.40^{abc}
BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	76.00	19.77^{a}	4.70^{ab}
BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	80.00	13.72^{c}	3.73^{cde}
BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	73.33	9.84^{f}	3.53^{cde}
BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	83.33	11.09^{d}	3.83^{bcde}
BAP(2.5 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	80.00	8.07^{h}	3.83^{bcde}
BAP (3.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	86.67	14.02^{c}	3.90^{abcd}
Kn (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	83.33	11.12^{d}	3.20^{e}
Kn (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	90.00	11.21^{d}	4.80^{a}
Kn (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	80.00	8.85^{g}	3.43^{de}
Kn (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	90.00	8.67^{g}	3.57^{cde}
Kn (2.5 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	76.67	10.34^{e}	4.33^{abce}
Kn (3.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	30	83.33	7.61^{i}	3.37^{e}
CV (%)		14.76	1.99	12.26
Level of significance		NS	0.01	0.05

BAP = Benzyl Amino Purine,
NAA = Naphthalene Acetic Acid,
Kn = Kinetin and NS = Not significant

2.5 DISCUSSION

In *in vitro* propagation, direct regeneration of plantlets is one of the fastest method of plant propagation. The first step and the most important one is regeneration of shoot.

The second experiment was designed and carried out to develop a protocol which can be a quick and efficient method of propagation of mulberry by regeneration of shoots directly using nodal segments of young mulberry stem.

2.5.1 Frequency of proliferation of explants

Different concentrations of BAP, Kn and NAA were used in half strength MS medium for optimizing multiple shoot proliferation. But no significant difference was noticed in the frequencies for shoot bud proliferation. Though, some other researchers' observation was different from the present study.

Khalafalla *et al.*(2007) reported about significant effect of the mentioned hormone on bud break and the maximum number of shoots (7.2 shoots per explants) from MS medium fortified with a combination of BAP (0.5 mgl^{-1}) and NAA (0.5 mgl^{-1}).

Anis *et al.*, 2003 observed highest shoot proliferation frequency (80%) from mulberry nodal segment and shoot tip culture in MS medium fortified with 2 mgl^{-1} BAP combined with 0.2 mgl^{-1} NAA.

The differences in case of obtaining the result may be due to the medium used, i.e., the experiment conducted by the above mentioned author were in full strength MS medium but the present study was in half strength MS medium.

2.5.2 Average length of shoot

Length of developed shoots was measured using digital slide calipers in mm scale. Here the different doses of two different hormones, each combined with NAA also bore statistically highly significant difference among them. The maximum length of shoots (19.78 mm) was recorded from MS medium fortified with BAP 0.5 mgl^{-1}.

Habib *et al.* (2003) observed maximum length of shoot (5.46±0.02 cm) from culture of nodal segment of mulberry using BA 1.0 mgl^{-1} as a supplement with MS media.

2.6 SUMMARY

Six different concentrations of N6 – benzylaminopurine (BAP) with one control and another six concentrations of Kinetin (Kn) with one control were used in the experiment. Medium sized test tubes were used with media content 10 ml per test tube. For each treatment there were 30 replications. Doses of BAP of 0.5 mgl^{-1}, 1.0 mgl^{-1}, 1.5 mgl^{-1}, 2.0 mgl^{-1}, 2.5 mgl^{-1} and 3.0 mgl^{-1} and Kinetin of 0.5 mgl^{-1}, 1.0 mgl^{-1}, 1.5 mgl^{-1}, 2.0 mgl^{-1}, 2.5 $mgl^{-1}$and 3.0 mgl^{-1} with control were taken as treatment. With each treatment of BAP and Kn doses, 0.5 mgl^{-1} NAA were added. The hormones were as a supplement of the half strength MS medium for direct regeneration of shoots from young mulberry stem cutting.

2.6.1 Frequency of proliferation of explants

For the emergence of bud from the explants almost all bud emerged from all treatment and there were no significant difference among the treatments.

2.6.3 Average length of shoot

There were some variation among different hormone concentrations and combinations in case of length of the sprouted shoots. The maximum shoot length (19.78 mm) observed in the hormone combination BAP (0.5 mgl^{-1}).

2.6.2 Average number of leaf

Good number of leaves emerged from almost all sprouted buds, though there was statistically significant difference among the treatments, where Kn (1.0 mgl^{-1}) resulted the best to produce maximum number of leaves (4.8).

2.6 CONCLUSION

Direct shoot regeneration is a prerequisite for quick multiplication technique in *in vitro* clonal propagation of plant. For proliferation of plants in a huge amount and to get disease free plantlets it's a very effective technique. Over the years different plant growth hormones are being used in media direct shoot regeneration. Sometimes combinations can provide a better result. In the present experiment, there was no significant difference for number of frequency of proliferation of explants (%) among the combination of the chemicals used. But for some other important parameters, like average length of the shoots (mm) and average number of leaf, the combination of the used chemicals played distinct role. In this case, the combination of N6 – benzylaminopurine (BAP) with concentration 0.5 mgl^{-1} and naphthalene acetic acid (NAA) in concentration 0.5 mgl^{-1} performed the best to achieve the highest length of shoot. For average leaf number, 0.5 mgl^{-1} Kn in association with NAA 0.5 mgl^{-1} proved the best over control and other concentrations among both BAP and Kn doses.

2.7 RECOMMENDATION

The present study paves the way for further attention and research on the propagation of mulberry. At the same time more attention could be given during preparation of shoot regeneration media and obviously during inoculation. More cautions during inoculation reduced the contamination in the present experiment. At the same time, more accurate measurement of the chemicals during preparation of the solutions had helped to produce better results also in the present study.

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