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KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE

THE EFFECTS OF ACTIVATED CHARCOAL AND PHYSICAL STATE OF CULTURE MEDIUM ON MICROPROPAGATION OF *MORUS NIGRA* L.

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THE EFFECTS OF ACTIVATED CHARCOAL AND PHYSICAL STATE OF CULTURE MEDIUM ON MICROPROPAGATION OF *MORUS NIGRA* L.
(YÜKSEK LİSANS TEZİ)

AVIN KHORSHEED KHALID

ÖZET

Bu çalışmada, urmu dutu olarak bilinen *Morus nigra*'nın üç genotipi agarla jelleştirilmiş veya sıvı halde olan ve 0.0 veya 0.5 g L^{-1} aktif kömür içeren Nas ve Read (2004) ortamı üzerinde kültüre alınmıştır. Kültür ortamında kömürün varlığı ve ortamın fiziksel hali (sıvı veya jel) bir eksplantten elde edilen ortalama sürgün sayısını önemli derecede etkilemiştir. Bir eksplantten elde edilen ortalama sürgün sayısı 1,1 ile 5.8 arasında olmuştur. Aktif kömür içermeyen ortam üzerinde daha fazla sürgün elde edilmiş ve sıvı ortama kıyasla jelleştirilmiş ortam üzerinde elde edilen sürgün sayısı daha yüksek olmuştur. Sürgün uzunluğu bakımından ise sıvı ortamın jelleştirilmiş ortama kıyasla daha iyi olduğu belirlenmiştir. Mikrosürgünlerin köklenme oranı düşük kalmış fakat köklendirilen mikrosürgünlerin %90'dan fazlası başarılı bir şekilde dış ortama aktarılmıştır.

Anahtar Kelimeler: Aktif kömür, kültür ortamı fiziksel hali, jelleştirilmiş ortam, sıvı ortam, *Morus nigra.*

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THE EFFECTS OF ACTIVATED CHARCOAL AND PHYSICAL STATE OF CULTURE MEDIUM ON MICROPROPAGATION OF *MORUS NIGRA* L.
(M.Sc. THESIS)

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ABSTRACT

Three genotypes of *Morus nigra* were cultured on agar – gelled or liquid Nas and Read (2004) medium containing 0.0 or 0.5 g L^{-1} activated charcoal. Medium physical state and presence of activated charcoal in the medium greatly affected mean shoot number per cultured explants. Mean shoot numbers obtained per explants varied between 1.1 and 5.8. Medium containing no charcoal produced more shoots compared to medium containing charcoal and agar - gelled medium produced more shoots compared to liquid medium. However, mean shoot length on liquid medium was higher than that on agar –gelled medium. Rooting of microshoots was low, however, over 90% of rooted microshoots were successfully acclimatized and transplanted ex vitro.

Key Words: Activated charcoal, culture medium physical state, gelled medium, liquid medium, *Morus nigra*

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LIST OF SYMBOLS AND ABBREVIATIONS

µM	:Micro mole
BA	:Benzyl adenine
IBA	:Indole -3-butyric acid
NRM	:Nas and Read medium
LM	:Liquid medium
GM	:Gelled medium
AC	:Activated charcoal
EtOH	:Ethanol
SAB	:Shoot axillary but
RIM	:Root indication medium

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1. INTRODUCTION

Black mulberry (*Moru nigra* L.) belongs to the genus *Morus* and of the Moraceae family. Approximately 10-16 species of mulberry are cultivated worldwide. *Morus nigra* L. is also known as ‘‘amoreira’’, ‘‘amorapreta’’ or ‘‘negra’’ (Ercisli and Orhan, 2007; Wasano *et al*., 2009). It is a woody perennial tree or shrub. It is an economically important plant used for its fruit and is vital to sericulture industry.

Mulberries are good sources of vitamins and minerals, especially contain a high amount of anthocyanin (Gerasopoulos and Stavorulakis, 1997). Berry fruits are economically important crops in many countries. Interest in berry fruits has recently increased because they are excellent sources of health-promoting vitamins, anti-oxidants and other valuable nutrients (Song and Sing, 2004). Fruit are used as worming agent, for dysentery and as a laxative, odontalgic, anthelmintic, expectorant, hypoglycemic and emetic (Baytop, 1996). The fruits of mulberry activate kidney energy and thus are used as antichloristic. Mulberry is rich in phenolic, which possess a wide spectrum of antimutagenic and anticarcinogenic properties. *Morus nigra* fruit contain high amounts of total phenolic and several phytonutrients, ascorbic acid. *Morus nigra* is also used in folk medicine and considered as a medicinal plant. Mulberry is well adapted to different climatic conditions ranging from tropical and sub-tropical areas to temperate regions. It is widely distributed in varied geographical and ecological zones.

Morus nigra are commercially propagated by stem cuttings, seeds and grafting. Often cuttings are difficult to root. It is a difficult to propagate species, as it tends to bleed heavily when cut for grafting. Successful rooting ability of stem cuttings depends on the propitious genotype and environmental conditions. Also, the success of grafting depends on cambium activity and compatibility. It also can be propagated by seeds but seed propagation is not practiced due to the varied nature of the seedlings become of its cross pollination and difficulty in seed germination. Moreover, dependence on season and slow multiplication rates are some of other major limiting factors in conventional propagation (Pati *et al*., 2006). These difficulties hinder propagation and establishment of new commercial orchards. In vitro production of micro cuttings and their subsequent rooting can be employed as an alternative to other conventional clonal propagation methods (Debergh and Maene, 1981).

The demand for black mulberry plants is increasing from year to year. Therefore, it is necessary to increase acreage of certified plantations of *morus nigra* mother plants. A stock plant orchard has to be established from healthy, virus-free plants. Such plants can be obtained and multiplied by using micropropagation (tissue culture) techniques.

Plant micropropagation is an integrated process in which cells, tissues, or organs of selected plants are isolated, surface sterilized, and incubated in a growth promoting aseptic environment to produce many clonal plantlets (Altman, 2000). Plant tissue culture (micropropagation) procedures have been employed in few *Morus* species and micropropagation techniques have rarely been utilized in mulberry. Micropropagation of mulberry through tissue culture provides a valuable tool for a more efficient and rapid multiplication of plants. Large number of uniform (genotypically and phenotypically identical) plants can be produced in a relatively narrow space and short time. Plant cell and tissue culture offers means not only for rapid mass multiplication of existing stocks but also for the conservation of important, elite and rare plants. The advantages of micropropagation are that large numbers of uniform, genetically stable plants are produced under sterile conditions with a minimum of stress imposed on the growing plants. It is used extensively for mass propagation of cultivated trees and forest plants (Bajaj, 1986).

The success of micropropagation largely depends on genotype and the chemical composition and physical state of the culture medium used. Morphological and physiological disorders may become serious problems and the cultures may not be maintained everlastingly if a suboptimal culture medium is used (Nas and Read, 2004). Chemical composition of the culture medium has been reported to affect all types of morphogenic responses (Morard and Henry, 1998; Schnapp and Preece, 1986; Sahrawat *et al*., 1999; Gyulai *et al*., 1992). A liquid or gelled medium with activated charcoal may affect shoot proliferation.

Micropropagation from axillary buds is often the most appropriate propagation technique because axillary buds have entire rudimentary vegetative shoot apical meristem and can be induced to develop in to plants easily which are similar to the parental type. The growth of *in vitro* cultured tissues and morphogenesis is very influenced by the genotype even more than by any other factors (George, 1993).

Propagation of *Morus nigra* via conventional propagation techniques is not satisfactory. Micropropagation seems to be an attractive alternative propagation but there are only a few studies that report *in vitro* propagation of different *Morus* species. Therefore, the objective of this study was to develop a micropropagation method that allows rapid proliferation of *Morus nigra* plants and overcomes the limitations associated with the traditional techniques used for propagation of black mulberry. To reach this objective the possible effects of charcoal and culture physical state (culture medium) on culture growth of *Morus nigra* were investigated.

2. LITERTURE REVIW

2.1. General Characteristics of Mulberry

It is believed that mulberry first originated in the foothills of Himalayas and later dispersed into Asia, Europe, Africa and America (Sanchez, 2000). Mulberry thought to have originated from the lower ranges of the Himalayas in either India or China. In most European countries, including Turkey and Greece, mulberries are grown for fruit production rather than foliage (Ercisli, 2004; Gerasopoulos and Stavroulakis, 1997). Mulberries are widely spread throughout all regions from the tropics to the sub-arctic and from sea level to altitudes as high as 4000 m (Machii *et al*., 2000; Tutin *et al*., 1996). They are found from temperate to subtropical regions of the Northern hemisphere to the tropics of the Southern hemisphere and they can grow in a wide range of climatic, topographical and soil conditions (Tutin, 1996; Vijayan *et al*., 1997). Black mulberry (*Morus nigra* L.), a tree native to Western Iran, is widely cultivated in Southern Europe, Southwest Asia and in Mediterranean countries for its juicy fruit as well as for its valuable industrial, medicinal products and as an ornamental plant. However, this species is somewhat difficult to propagate as it tends to produce exudates heavily when cut (Ďurkovič *et al*., 2009). Mulberry could be classified into foliage, fruit, timber or ornamental mulberries (Hotta, 1958).

Morus nigra is a woody perennial tree, plays a very significant role in the sericulture industry as its foliage is used as an important feed crop for silkworms (*Bombyx mori* L.) (Lu, 2002). All the mulberry species have white sap that contains latex (Hora 1981). The different ploidy levels ranging from diploids with 28 chromosomes to docosaploid with 308 chromosomes are common among mulberry species (Basavaiah *et al*., 1989). There are three main mulberry species, namely black (*Morus nigra*), red (*Morus rubra*) and white (*Morus alba*) for fruit production (Yaltirik, 1982). The mulberry ovary is about 2 mm long (Radford, 1968). Mulberry fruit contain 89.3% water, 2.2% protein, 0.2% fat, 2% glucose, 2.3% fructose, 2.2% dietary fiber, 0.19% malic acid, 0.59% citric acid, 0.8% ash, 121 kJ energy and 100 g fruit contain 10 mg vitamin C, 0.01 mg thiamin, 0.01 mg riboflavin, 0.7 mg niacin, 0.01

mg p-carotene, 310 mg K, 6 mg Na, 20 mg Ca, 12 mg mg, 0.3 mg Fe and 0.2 mg Zn (Wills *et al*., 1987).

Mulberry is propagated through conventional methods such as grafting, air layering, cuttings and by seed sowing, depending on the cultivar (Lu *et al*., 2002). Seed propagation is not desirable due to the heterogeneous nature of the seedlings become of cross pollination (Das, 1983; Hossain *et al*., 1992). In some countries such as Bangladesh propagation of mulberry through cuttings is restricted to a single season (September–October) (Zaman *et al*., 1997). Developing inbred mulberry is not easy due to a longer juvenile period, high inbreeding depression and the dioecious nature of the plant (Vijayan, 2010).

Mulberry is one of the most important ornamental and aromatic crops, represents a major commodity in the commercial floriculture market and in essential oil industry. It is a valuable food for birds and animals. It is a good source of vitamins and minerals (Gerasopoulos and Stavorulakis, 1997). It is a good source of sugars, acids and anthocyanin content, which are also responsible for their color, taste and presumably also their antioxidant properties. The extracts of black mulberry have moderate antioxidant activity (Lin and Tang, 2007). Black mulberry juice contains a high potassium and citric acid and low level of sodium. It is recommended for young children and elderly people. Juice and fruit characteristics of mulberry almost remain constant and do not change from year to year (Darias-martin *et al*., 2003).

Morus plants are highly important for medicinal, economical, industrial and clinical uses (Andreoni, 2005; Sánchez, 2000). Besides its use as food, the fruits, leaves and barks are well known as traditional medicine in Turkey and have been used for many years as an anti-fever, as a laxative, anthelmetic, expectorant and facilitate discharge of urine and lower blood pressure (Baytop, 1999).

Owing to the popular use of leaves as a reliever of hot flashes and the symptoms of premenstrual tension (Miranda *et al*., 2010), it can be said that substances capable of exerting some estrogenic or progesteronic effect are present among the constituents of *Morus nigra* leaves. The hypoglycemic effect has been attributed to the *Morus nigra* leaves (Oryan *et al*., 2003). The fruits, leaves, barks and roots are used as laxative, sedative, expectorant, refresher, emollient, calmative, diuretic, hypoglycemic agent, antiseptic, anti-inflammatory, antioxidant, and in the treatment of eczema, and oral inflammation (Oryan *et al*., 2003; Naderi *et al*., 2004; Ercisli and Orhan, 2007; Padilha *et al*., 2010). Mulberry leaves have long been used in Chinese folk medicine to alleviate fever, suppress atherosclerosis, alleviate hypertension, and also have diuretic and hyperglycemic effects (Harauma *et al*., 2003; Jia *et al*., 1999). Mulberry leaves are also used as fodder for livestock (Sánchez, 2000). The nutritional and physiological aspects of mulberry leaves and mulberry leaf hydrocolloid have been reported extensively (Gai *et al*., 2005; Jia *et al*., 1999; Wu, 1994). The hot-water-extracted crude polysaccharide of mulberry leaves is composed of three fractions the first fraction constituted mainly of rhamnose, arabinose, and galactose, in a ratio of 21:16:20. The second fraction constituted mainly of rhamnose and glucose in a ratio of 3:1, and the third fraction constituted mainly of rhamnose (Ouyang *et al*., 2005).

Mulberry leaves have hypoglycemic and hypolipidemic effects in type 2 diabetic patients (Andallu *et al*., 2001). Mulberry leaf extracts have been reported to significantly reduce blood glucose in alloxan-induced diabetic mice (Hosseinzadeh and Sadeghi, 1999).

The fruit of black mulberry is usually 1 to 4 cm long, oval in shape with bright red color before full ripeness and it is purple-black when fully ripe (Darias-Martin *et al*., 2003). Fruit contains essential fatty acids that humans cannot synthesis, and must be obtained through diet. Essential fatty acids are long chain polyunsaturated fatty acids derived from linoleic, and oleic. These chemicals regulate numerous body functions including blood pressure, blood viscosity, immune and inflammatory responses (Pawlosky *et al*., 1996; Simopoulos and Salem, 1996). The variation of phenolic compounds in the fruit depends on many factors, such as degree of maturity at harvest, genetic differences, and environmental conditions during fruit development (Zadernowski *et al*., 2005). The red-coloured fruit of *Morus nigra* which taste very pleasant when eaten fresh also have other uses as marmalades, juices, liquors, natural dyes and in the cosmetics industry (Ercisli and Orhan, 2007).

2.2. Tissue Culture

Plant tissue culture is the technology of growing plant cells, tissues or organs cut off from the mother plant, on artificial media under aseptic conditions. It comprises techniques and methods used for research into many botanical disciplines and has several practical applications (George *et al*., 2008). Plant tissue culture was first used almost 100 years ago to explain some of the most basic questions in developmental biology (Smith, 2000). *In vitro* culture is one of the important tools of plant biotechnology that exploits the totipotency nature of plant cells, a concept by Haberlandt (1902) and unequivocally demonstrated for the first time by (Steward *et al*., 1958). The in vitro techniques were developed initially to demonstrate the totipotency of plant cells predicted by Haberlandt in 1902 (Maheswaran *et al*., 2000). Totipotency is the ability of a plant cell to perform all the function of development, which are characteristics of zygote, the ability to develop into a complete plant (Gupta and Ibarki, 2006). Micropropagation appears to be a promising tool to overcome limitations for both large-scale propagation and enhancement of woody species.

There has been a rapid increase in the number of researchers involved in the plant tissue culture within the last century, because:

1. A large number of clonal plants can be obtained. It is very useful where plants are difficult to propagate vegetatively by other methods.
2. It is often much quicker than conventional means.
3. With the use of meristem culture it is likely to produce certified virus free plants.
4. New cultivars can be developed from cultures due to mutations of rapidly dividing cells.
5. Micropropagation can be used for rejuvenation of plant materials.
6. Micropropagation can result in production of new desirable characteristics.
7. Production is independent of the season and can continue throughout the year.

8. Vegetative produced material can be stored for long periods.

9. Plant material needs little consideration between subcultures and no labor is required for weeding, spraying or watering (Thiart, 2003).

A successful micropropagation protocol involves a series of step, each with a specific set of requests. These are:

1. Initiation of aseptic culture.

2. Shoot multiplication.

3. Rooting of micro shoots.

4. Hardening and field transfer of tissue culture raised plants (Murashige *et al*., 1974).

The first three stages are carried out under aseptic conditions, while the last stage is carried out in a greenhouse environment.

2.3. Initiation of Aseptic Culture

There are only a few studies that report *in vitro* propagation of different *Morus* species. Thus the problems faced at the initiation stage of *Morus* culture are largely unknown.

2.4. Shoot Multiplication

The most widely used method of plant regeneration in mulberry is micropropagation through axillary bud culture (Jain *et al*., 1990; Sharma and Thorpe, 1990; Yadav *et al*., 1990; Pattanaik *et al*., 1996). *In vitro* clonal propagation through axillary bud propagation is a reliable approach for the multiplication of trees mostly within a reasonable time frame irrespective of seasonal considerations (Shirin *et al*., 2005). *In vitro* propagation of white mulberry has been achieved from numerous explants, such as axillary buds (Jain et al. 1990), shoot-tips (Hayashi and Oka 1995) and callus (Bhau and Wakhlu 2001). *In vitro* multiplication potential of apical buds of aseptic and field grown plants of mulberry (*Morus alba*) var. Berhampur supplemented with various concentrations of BAP has been reported. Rapid *in* *vitro*-propagation procedure, in which very young axillary buds can be cultured, established, multiplied, rooted, and transplanted to soil within three months was achieved (Paul *et al*, 2001). A high sprouting percentage (80%) of axillary buds and shoot tips of *Morus alba* L. was achieved under *in vitro* micropropagation conditions. The *in vitro* proliferated shoots were multiplied rapidly on MS medium supplemented with BAP (2.0 mg/l) and NAA (0.2mg/l). Multiplication was also achieved by culture of explants on MS medium fortified with BAP (2.5 mg/l) + glutamine (1mg/l). This medium facilitated the elongation of shoots and sprouting of axillary buds of *in vitro* grown shoots (Anis *et al*., 2003). Anuradha and Pullaiah (1992) and Oka and Ohyama (1975) reported that nodal explants of mulberry containing axillary buds showed the best response for sprouting and shoot elongation. Axillary buds developed into shoots as well as inflorescences irrespective of the hormones used in the MS medium. Inflorescences were excised at an early stage of induction to accelerate the development of the shoot cultures. Low concentration of 2,4-D (0.5 mg/l) stimulated sprouting whereas higher concentration (2.0 mg/l) resulted in rapid callus proliferation through axillary bud cultures of mulberry (*Morus alba* L.) (Chitra and Padmaja, 1999). With a slight increase or decrease in hormonal concentrations a decline in shoot as well in root number was observed (Mei-Chun Lu, 2002). Plant propagation has been achieved in mulberry *in vitro* using meristem/shoot tip cultures, axillary buds, intermodal segments, hypocotyl regions, cotyledons, and leaves (Mandal, 1999; Vijayan *et al*., 2011).

In vitro plant regeneration from apical/ axillary shoot buds and nodal explants methods has been reported in a number of mulberry species (Mhatre *et al*., 1985; Ohyama and Oka, 1987; Hossain *et al*., 1992; Pattnaik and Chand, 1997; Chitra and Padmaja, 1999). *In vitro* micropropagation of *morus india* was achieved by apical/axillary shoot culture (Sahoo *et al*., 1997). *In vitro* micropropagation of *Moru sindica* from axillary buds was better in the presence of IBA and kinetin (Prabhakar and Basavaraju, 2000).

Shoot multiplication is an important factor determining the appropriateness of the tissue culture method for the mass propagation of tree species (Quraishi *et al*., 1996). The increase in shoot proliferation rates during series of subcultures may possibly be due to *in vitro* adaptation. An increase in the shoot number in each passage along with increase in callus at the base of cultures was observed when explants were transferred to a fresh medium in the V-1 and S-34 cultivars of *Morus* (Tewary *et al*., 1995). Several cytokines especially BA is routinely used for shoot propagation from nodal cuttings and axillary buds (Oka and Ohyama, 1974; Hossain *et al*., 1990; Jain *et al*., 1990; Yadav *et al*., 1990). Zaman *et al*. (1992b) evaluated the role of agar type and pH on shoot proliferation in *Morus alba*, and noted that the percentage of shoot-proliferating explants as well as the number of shoots per explant increased when the level of agar was raised up to 0.6%. Further increases decreased both parameters. The optimum pH for bud break ranged from 4.0 to 5.5; less acidic levels, in fact, decreased the rate. For producing multiple shoots, pH 5.5 was the best, and variations either up or down caused this frequency to decrease (Zaman *et al*. (1992b).

Shoot multiplication is an important stage in any propagation program since it determines the number of produced plants. In this stage, the number of propagules is multiplied by repeated subculture until the desired (or planned) number of plants is attained. The shoot multiplication rate also varies in different species and is affected by the culture medium (Davies, 1980; Khosh-Khui and Sink, 1982a; Rout *et al*., 1990 and Pati *et al*., 2006).

In some micropropagation methods, multiplication stage will include the prior induction of meristematic centers from which adventitious organs may develop. Some of the propagules produced at Stage II (especially shoots) can also be used as the basis for further cycles of multiplication in that they can usually be cultured again (subcultured) to increase their number (Trigiano and Gray, 1996 and Salman, 1988).

2.5. The Effect of Physical Status of Nutrient Medium on Shoot Multiplication

Generally, tissue culture media are solidified with any of the gelling agents. Agar is widely used for the solidification of the medium. If the concentration of agar is increased, the medium become hard and does not allow the diffusion of nutrient into the tissue. Sometimes, the solid media will accumulate the toxic substances namely, oxidized, phenolic compounds released from tissue and hamper further growth of tissue (Maheswaran *et al*., 2000).

Water potential is determined by osmotic potential of the solutes in the medium (Beruto *et al*., 1995; Fujiwara and Kozai, 1995; Owens and Wozniak, 1991). The water potential of media solidified with gels is more negative than that of a liquid medium, due to their matric potential (Amador and Stewart, 1987). Thus agar-solidified medium has lower water availability and uptake by the plants than liquid medium (Debergh, 1983 and George, 1993). This lower uptake of nutrients could explain the lower rate of development of plantlets in solid media compared to liquid media (Kuria *et al*., 2008). A ten-fold difference in the regeneration rate of sugar cane was observed by Anders *et al*. (1988) on media gelled with the best and least effective seven brands of agar.

The nutritional requirements of plant cultures can be supplied by liquid media but growth in liquid medium may be retarded and development affected by oxygen deprivation and hyperhydration. The oxygen concentration of liquid media is often insufficient to meet the respiratory requirements of submerged cells and tissues. It can be increased either by raising the oxygen concentration of the medium or placing cells or tissues in direct contact with air.

In liquid medium, cultures may grow better than on agar- solidified medium. Culture may be fully or only partially immersed in the medium (George *et al*., 2008). Plantlets can be cultured by incomplete submersion in liquid medium. One method of aerating tissues is by the mechanical flooding and evacuation of tissues by liquid medium. Narayan *et al*. (1989) standardized a technique for this. Using intermodal segments of *Morus alba*, they first pretreated the tissue on an MS liquid medium supplemented with 2.2 pM BAP in 250 mL Erlenmeyer flasks. An alternative approach to aeration is to apply the liquid medium over the plant tissues as a nutrient mist.

2.6. Rooting Stage

This stage is characterized by preparing shoots or shoot clusters for rooting and then their successful transfer to soil. In rooting stage, auxins are used for adventitious root formation. Auxin plays a role in many developmental processes, one of them is rooting. Generally, when the concentration of auxin is low, root initiation is favored, and when the concentration is high, callus formation occurs (Smith, 2000). *In vitro* rooting capacity depends on the interaction of internal and external factors (Hyndman *et al*., 1982). Several factors such as the species, cultivar; age and size of microshoots; media; inorganic salts; carbohydrate; activated charcoal; growth regulator and physical factors may affect rooting (Pati *et al*., 2005). For rooting of microshoots of *Morus nigra*, the most common auxins that have been used are NAA and IBA. IBA is the best auxin for induction of rooting for *Morus nigra* (Yadav *et al*., 1990). The average number of roots induced per shoot after 30 days of culture was highest with 0.25mg/l IBA for *Morus nigra* (Yadav *et al*., 1990).

2.7. Effect of Charcoal on Rooting of Microshoots

Use of activated charcoal in vitro culture may affect growth mainly shoot elongation but also rooting (Fridborg and Eriksson, 1975; Damiano, 1978; Dumas and Monteuuis, 1995; Bousselmame *et al*., 2001). A 0.2% concentration of charcoal in the rooting medium decreased the root induction hesitation in *Morus indica* (Vijaya *et al*., 1999). Rooting was also achieved by direct insertion of *in vitro*-grown mulberry shoots into a vermiculite moistened plant bed (Katase, 1993). Rooting was good in half strength MS medium supplemented with IBA (4μM) along with activated charcoal (AC) as compared to AC-deficient medium (Owen *et al*., 1995; Wann *et al*., 1997). Addition of activated charcoal to the shoot proliferation medium reduced the time needed for adventitious root formation by at least 2 weeks and inhibited the formation of callus at the basal end of the shoots in *Morus alba* (Sharma and Thorpe, 1990).

Incorporation of activated charcoal in the tissue culture media may either have a beneficial or adverse effect on growth and development, depending on the species and tissue used. Previous studies indicated that the effect of activated charcoal can be attributed to establishing a darkened environment, adsorption of undesirable substances or growth regulators, or both (Pan and Staden, 1998).

2.8. Acclimatization and Field Establishment

The ultimate success of shoot or node culture depends on the ability to transfer shoots to ex vitro and reestablish growing plants in greenhouse or field conditions. This comprises acclimatizing or hardening-off plantlets to conditions of significantly lower relative humidity and higher light intensity. Micropropagated plants are difficult to transplant for two primary reasons: a heterotrophic mode of nutrition and poor control of water loss (Trigiano and Gray, 2000).

Plants are cultured in vitro under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients and in an atmosphere with high level of humidity. This contributes a culture induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of migropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field (Hazarika, 2003). Plants developed through tissue culture are heterotrophic, lack cuticle on their epidermis (Adelberg *et al*., 2000), and have non-functional stomata (Czynczyk and Takubowski, 2007). Such plants cannot survive the outside unfavorable conditions. Thus, they need hardening and acclimatization, where they receive a special treatment before they can be transferred to the soil in order to stimulate photosynthesis, cuticle development and their stomata start functioning.

3. MATERIALS AND METHODS

The present study was carried out in the ÜSKİM plant tissue culture laboratory, Kahramanmaras Sutcu Imam University, Turkey, between October 2013 and August 2014.

3.1. Plant Materials

Mulberry (*Morus nigra* L.) axillary buds were used as the experimental materials. Culture of mature *Morus nigra* genotypes A5, T6 and T8 grown *in vitro* were used as the explant source. These genotypes were in culture on Nas and Read (2004) medium containing 0.25 mg BA (6-benzyladenine) L^{-1} and 0.01 mg IBA (indole-3-butyric acid) L^{-1} (Table 1). The cultures were maintained before and during the experiment at 24±1°C with a 16/8 h (light/dark) photoperiod under cool-white fluorescent light at 80 µmol $m^{-2}s^{-1}$.

Table 3.1. Composition of Nas and Read (2004) medium used in this study

Macrominerals	mg L^{-1}
NH_4NO_3	530
$Ca(NO_3)_2 \cdot 4H_2O$	700
$CaCl_2 \cdot 2H_2O$	90
$MgSO_4 \cdot 7H_2O$	1600
KNO_3	550
KH_2PO_4	1300
Microminerals	mgL^{-1}
H_3BO_3	6.2
$CuSO_4 \cdot 5H_2O$	2.5
$MnSO_4 \cdot H_2O$	20
$Na_2MoO_4 \cdot 2H_2O$	2.5
$ZnSO_4 \cdot 7H_2O$	8.8
Sequestrene 138 Fe	100
Vitamins	mgL^{-1}
Thiamine (B1)	0.60
Riboflavin (B2)	0.21
Nicotinic acid (B3)	1.15
Pyrodoxine (B6)	0.60
α - Tocopherol (E)	20.0
Vitamin C (ascorbic acid)	1.0
Glycine	0.85
Myo-inositol	200
Sucrose (gL^{-1})	30

3.2. Culture Medium and Culture Conditions

The medium used for culture growth was Nas and Read (NRM) (2004) medium. The cultures were maintained in a growth chamber at 24±1^{o}C with a 16/8 h (light/dark) photoperiod under cool-white fluorescent light at 80 µmol $m^{-2}s^{-1}$.

3.3. Determining the Effects of Charcoal and Medium Physical State on Culture Growth

To find out the possible effects of charcoal and culture physical state (culture medium) on culture growth the following culture media were used:

1. Liquid Nas and Read Medium (NRM) + 0.5 gL^{-1} activated charcoal.
2. Liquid NRM containing no charcoal.
3. Gelled NRM + 0.5 gL^{-1} activated charcoal.
4. Gelled NRM containing no charcoal.

Nas and Read (2004) medium containing 0.01 mgL^{-1} Indol-3-Butryric Acid (IBA), 0.25 mgL^{-1} Benzyl adenine (BA), and 30 gL^{-1} sucrose was used as the culture medium. If charcoal was to be added to a medium, first 0.5 gL^{-1} charcoal was added to the medium, and then the PH was adjusted to 5.5. To gelled (solidify) medium, 5.5 gL^{-1} Merck microbiological agar was added. The media were autoclaved at 121^{o}C and 1.2 kgf cm^{-2} for 15min. After autoclaving, the medium was distributed to 15 x 250 mm glass–test tubes.

3.4. Experimental Design

At least 10 replications (test tubes containing 1 axillary bud) of each genotype were randomly applied to each treatment, and then culture vessels were randomly placed on the growth shelves. Cultures (test tubes containing 1 axillary bud) were first maintained in the dark for 1 week at 24±1^{o}C. Then, they were subjected to a 16/8 h (light/dark) photoperiod under cool-white fluorescent light at 80 µmol $m^{-2}s^{-1}$ in the same growth chamber for 4 weeks. At the end of a-5 week culture period, the experiment was repeated twice by using explants randomly taken from shoots grown on the same fresh media.

3.5. Rooting of Microshoots

3.5.1. The Effect of IBA on *In Vitro* Rooting of Microshoots

Shoots obtained on multiplication medium and ≥ 2cm long were used for rooting experiments. To induce rooting microshoots obtained on multiplication medium (NRM containing 0.01 mgL^{-1} IBA, 0.25 mgL^{-1} BA) were cultured on gelled (NRM containing 0, 0.5, 1.0 or 1.5 mgL^{-1} IBA). Four replications (Magenta vessels containing 5 microshoots) of each genotype were applied to each IBA level. Shoots were first maintained in the dark for 1 week at 24°C, and then were subjected to a 16/8 h (light/dark) photoperiod under cool-white fluorescent light at 80μmol $m^{-2}s^{-1}$ for three weeks. Data of rooting percentage (%), average number of roots and average root length were recorded after 4 weeks.

3.5.2. Acclimatization of Rooted Microshoots

Four weeks after root induction, rooted shoots were thoroughly washed under tap water to remove agar from roots. Then shoots were transferred into plastic pots containing a mixture of 60% peat and 40% perlite. Rooted shoots that transplanted to pots were covered with clear plastic cups for 1 week, then, the cups were perforated (about 1 cm^{-2}), and plants were kept covered for another week. At the end of the second week the cups were removed, and plants were left to grow under 16/8 h (light/dark) photoperiod under cool-white fluorescent light at 80μmol $m^{-2}s^{-1}$ for two weeks. At the end of the fourth week, the acclimatized plants were transplanted into bigger pots containing the same mixture described above. Then, after one month, the plants were shifted to the greenhouse (shaded with a mesh blocking 50% of the light) and left to grow under uncontrolled temperature (min. 18^{o}C–max. 27^{o}C) and relative humidity conditions (Nas *et al*., 2012).

3.6. Statistical Analysis

All statistical analysis were performed using Statistical Analysis System (SPSS 16). Comparison of treatment means was done using LSD at $P \leq 0.05$ used. Data for explants in a culture vessel were divided by the number of explants, and the mean shoot number and number shoot length were used for statistical analysis.

4. RESULTS AND DISCUSSION

4.1. The Effect of Charcoal and Medium Physical State (medium) on Shoot Multiplication

Chemical composition of culture medium (with or without charcoal) and medium physical state (liquid or gelled) had a great influence on the number of shoots obtained per cultured explants. The effects of genotype and medium on shoot multiplication, however, were insignificant (Table 4.1.).

Table 4.1. Analysis of variance of shoot number of *Morus nigra* genotypes on different culture media (with or without charcoal and liquid or gelled)

Source of variations	Sum of Squares	DF	Mean square	F
Genotype	0,94	2	0,47	1,00
Medium	80,46	3	16,09	34,14***
Genotype x Medium	2,66	6	0,66	0,28

***: Significant at $P \leq 0.001$ level

Mean number of shoots obtained per explants are shown in Table 4.2. and Figure 4.1. Clearly medium containing no charcoal was superior to medium containing charcoal as it produced more shoots. Moreover, gelled medium containing no charcoal was better than liquid medium containing no charcoal (Figure 4.1.).

Table 4.2. The effect of charcoal and medium physical state on mean numbers of shoots of *Morus nigra* genotypes

Medium	Mean Number of Shoots (± std. dev)/Explant		
	Genotype A5	Genotype T6	Genotype T8
Agar + charcoal	1.0 ± 00	1.1 ± 0.1	1.2 ± 0.2
Agar + no charcoal	5.5 ± 1.5	5.8 ± 1.3	5.2 ± 0.4
Liquid + charcoal	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.1
Liquid + no charcoal	4.3 ± 0.3	3.9 ± 1.2	2.5 ± 0.1

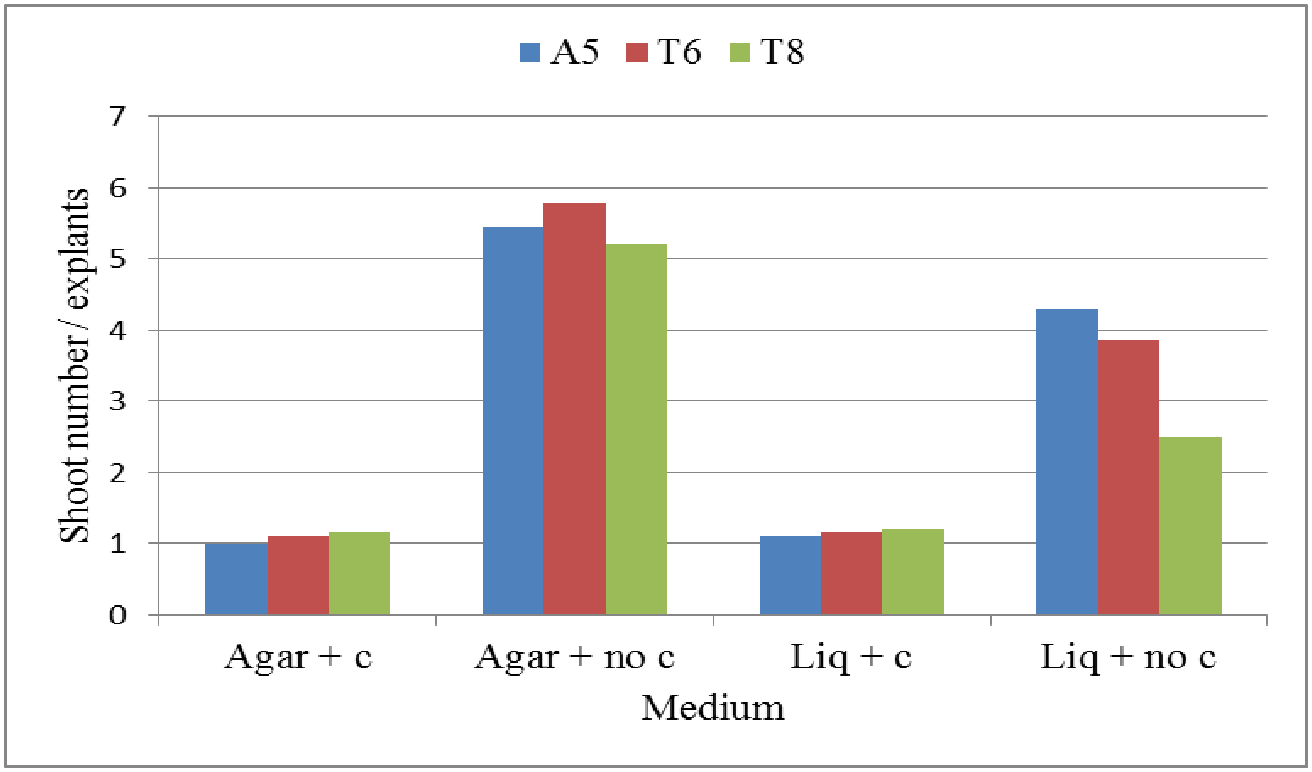

Figure 4.1. Mean number of shoots of *Morus nigra* genotypes obtained on liquid or gelled medium with or without charcoal

The highest numbers of shoots per cultured explant of genotype A5 (5.5 shoot/explant), genotype T6 (5.8 shoot/explant) and genotype T8 (5.2 shoot/explant) were obtained on agar-gelled medium containing no charcoal. On agar–gelled medium containing 0.5 gL^{-1} charcoal mean numbers of shoots obtained per cultured explants were 1.0, 1.1 and 1.2 for genotype A5, genotype T6 and genotype T8, respectively (Table 4.2).

Mean shoot number per explant obtained on liquid medium was significantly lower than that obtained on agar–gelled medium. Moreover, mean shot number obtained on liquid medium containing no charcoal was higher than that on liquid medium containing charcoal. Mean shoot numbers on liquid medium containing no charcoal were 4.3, 3.8 and 2.5 for genotype A5, genotype T6 and genotype T8, respectively. On liquid medium containing 0.5 gL^{-1} charcoal mean shoot numbers were 1.1, 1.1 and 1.2 for the same genotypes, respectively.

Our results indicate that agar-gelled medium containing no charcoal is better for shoot multiplication of *Morus nigra* compared to agar-gelled medium with charcoal and liquid medium with or without charcoal. Shoot numbers on gelled and liquid media containing charcoal were similar and were much lower than that on agar–gelled medium without charcoal. It is known that activated charcoal adsorb phenolic exudates secreted from explants and plant growth regulators in the culture medium as well. The inhibitory effect of charcoal on shoot number may be due to irreversible adsorption of plant growth regulators from the culture medium and that caused plant growth regulators to become less available for explants.

Similar shoot numbers per explant were obtained for all three genotypes of *Morus nigra* used in this study. This may indicate a close genetic relatedness of these genotypes. The explants of three genotypes were taken from individual trees. However, traditionally black mulberry is propagated by grafting. Thus, it is likely that the three genotypes might have been originated from a single tree.

4.2. The Effect of Charcoal and Medium Physical State (Medium) on Shoot Length

Similar to mean shoot number per cultured explant, chemical composition of culture medium (with or without charcoal) and medium physical state (liquid or gelled) influenced mean shoot length of all three *Morus nigra* genotypes used in this study (Table 4.3.).

Table 4.3. Analysis of variance of shoot length of *Morus nigra* genotypes on different culture media (with or without charcoal and liquid or gelled)

Source of variations	Sum of Squares	DF	Mean square	F
Genotype	0.342	2	0.17	0.64^{NS}
Medium	5.622	3	1.12	4.23**
Genotype x Medium	0.347	6	0.08	0.32^{NS}

^{NS}, **: Non-significant and significant at $P = 0.05$ level, respectively.

Mean shoot length of three genotypes are shown in (Table 4.4.). Contrary to mean shoot number, mean shoot lengths on liquid medium were higher than or similar to that on gelled medium. Gelled medium containing no charcoal and both liquid medium containing charcoal and containing no charcoal produced longer shoots compared to gelled medium containing 0.5 gL^{-1} charcoal. Shoot length on liquid medium with and without charcoal were similar (Table 4.4. and Figure 4.2.).

The longest shoots of genotype A5 (2.6 cm) and genotype T6 (2.5 cm), were obtained on liquid medium containing no charcoal. On agar–gelled medium containing no charcoal mean shoot lengths were 2.2 cm, 2.0 cm and 2.0 cm for genotype A5, T6 and T8, respectively (Table 4.4.).

Table 4.4. The effect of charcoal and medium physical on mean shoot length of *Morus nigra* genotypes

Medium	Mean Shoot Length ± std. dev. (cm)		
	Genotype A5	Genotype T6	Genotype T8
Agar + charcoal	1.1 ± 0.2	1.2 ± 0.4	1.0 ± 00
Agar + no charcoal	2.2 ± 1.8	2.1 ± 0.4	2.0 ± 00
Liquid + charcoal	2.1 ± 1.0	1.9 ± 0.1	2.2 ± 1.1
Liquid + no charcoal	2.6 ± 0.2	2.5 ± 0.8	1.9 ± 0.5

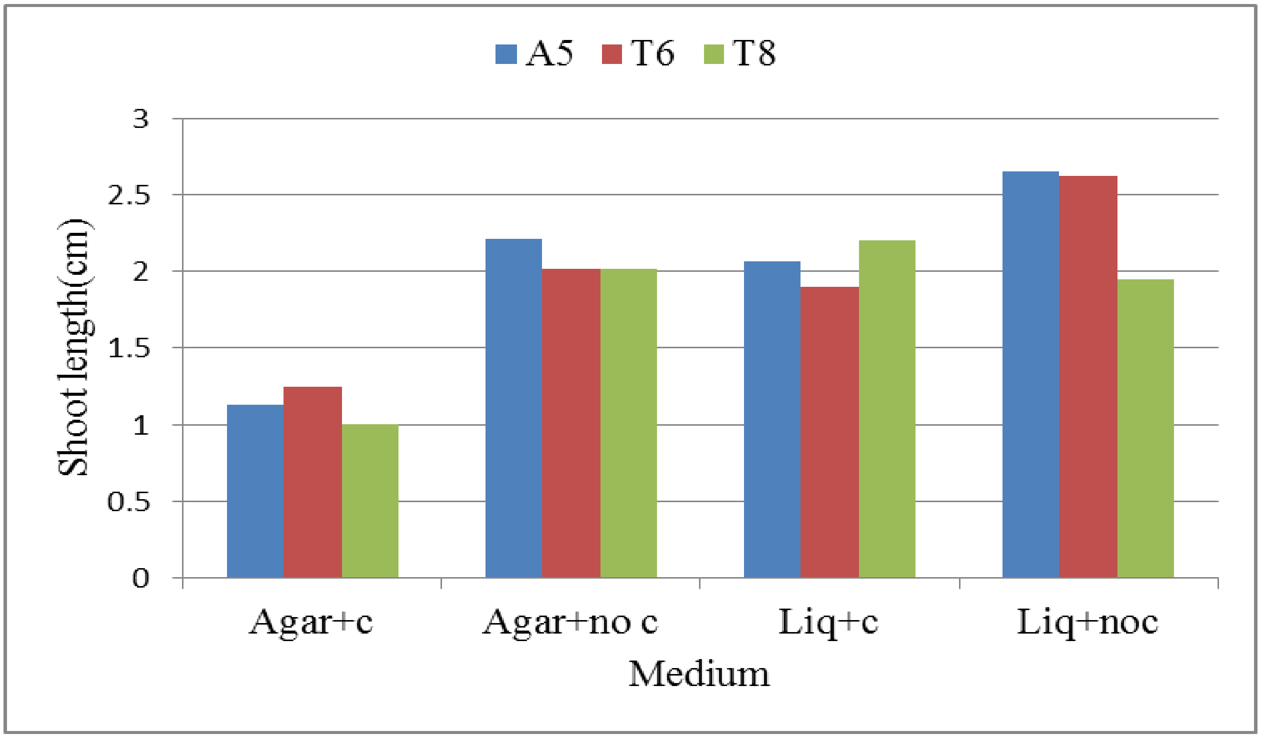

Figure 4.2. Mean shoot lengths of *Morus* n*igra* genotypes obtained on liquid or gelled medium with/without charcoal

A reverse correlation between the number of shoots per explant and shoot length was apparent. Shoot length obtained on liquid medium with or without charcoal was similar to or better than that obtained on agar–gelled medium. That the shoots obtained on liquid medium were longer than those on agar–gelled medium could be related to the low number of shoots obtained on liquid medium.

Similar shoot length were obtained for all three genotypes of *Morus nigra*. As explained above, this may indicate a close genetic relatedness of the genotypes used. The explants of three genotypes were taken from individual trees. However, traditionally black mulberry is propagated by grafting. Thus, it is likely that the three genotypes might have been originated from a single tree.

4.3. Rooting of Microshoots

4.3.1. The Effect of IBA on *In Vitro* Rooting of Microshoots

Four weeks after root indication rooting data were recorded. Regardless of IBA concentration and genotype rooting ratios were similar. Also the effect of IBA concentration and genotype interaction on rooting was insignificant (Table 4.5.).

Table 4.5. Analysis of variance of IBA on rooting percentage of *Morus nigra* genotypes

Source of variations	Sum of Squares	DF	Mean square	F
Genotype	22.23	2	11.1	1.205^{NS}
IBA	8.30	4	2.8	0.299^{NS}
Genotype x IBA	34.72	8	8.7	0.938^{NS}

^{NS}: Non-significant at $P \leq 0.05$ level. **: significant at $P \leq 0.05$ level.

Depending on IBA concentration and genotype, rooting percentage of microshoots varied between 10% and 17% (Table 4.5. and Figure 4.3.), however, the differences between means were statistically insignificant.

Table 4.6. Rooting percentages of *Morus nigra* microshoots in the presence of various IBA concentrations

IBA (mg L^{-1})	Rooting percentage of genotype (± std. dev.)			Mean
	Genotype A5	Genotype T6	Genotype T8	
0.0	13.0 ± 3	--	--	13.0 ± 3.0
0.5	17.0 ± 3	14.5 ± 4.2	14.0 ± 2.2	15.2 ± 1.4
1.0	15.5 ±2.2	16.5 ± 2.2	10.0 ± 2.2	14.0 ± 1.2
1.5	14.0 ± 2.2	14.0 ± 2.2	14.5 ± 2.2	14.2 ± 1.2
Mean	14.8 ± 1.3	15.0 ± 1.2	12.8 ± 1.2	

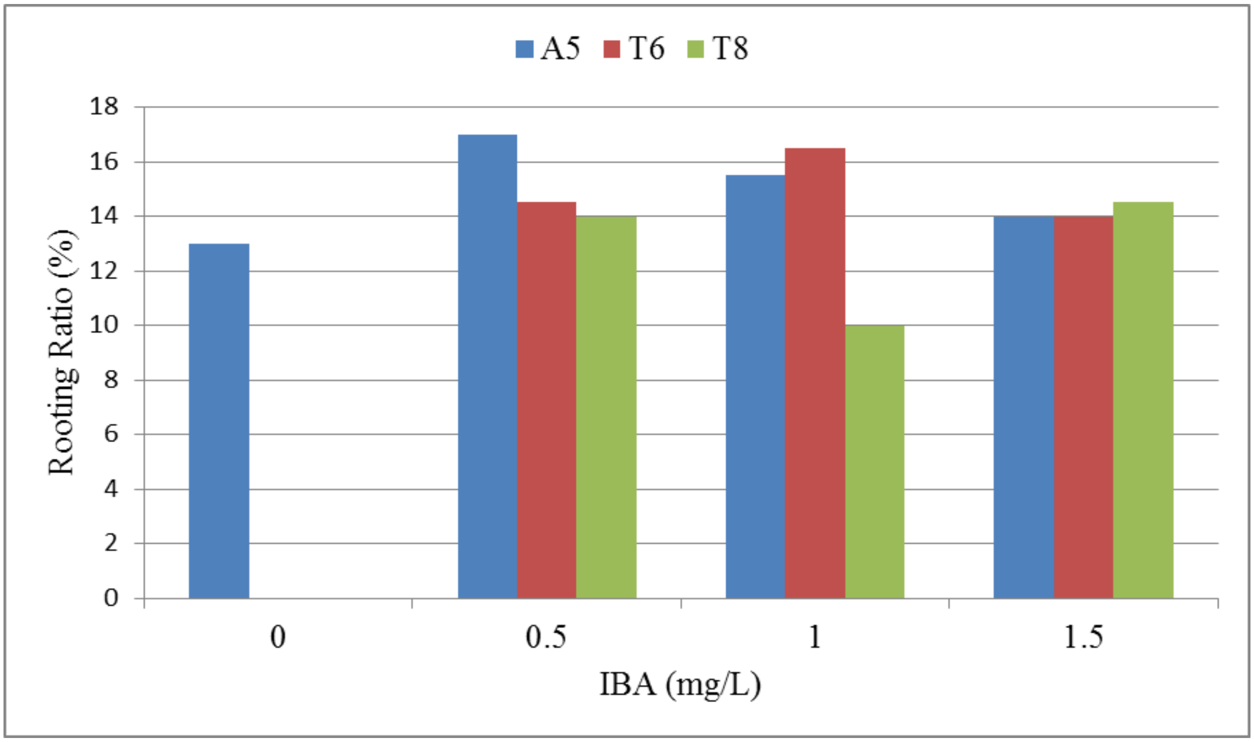

Figure 4.3. Mean rooting percentage of *Morus nigra* genotypes obtained on medium containing different concentrations of IBA (mg/l)

4.3.2. The Effect of IBA on *In Vitro* Root Number Per Microshoots

Similar to rooting percentage, the effects of IBA concentration, genotype and their interaction on root number per microshoot were insignificant (Table 4.7.).

Table 4.7. Analysis of variance of IBA on root numbers per microshoots of *Morus nigra* genotypes

Source of variations	Sum of Squares	DF	Mean square	F
Genotype	1.385	2	0.692	0.465^{NS}
IBA	3.247	3	1.082	0.726^{NS}
Genotype x IBA	2.316	4	0.579	0.388^{NS}

NS: Non-significant at $P \leq 0.05$ level.**: significant at $P \leq 0.05$ level.

The number of roots per microshoots varied between 1.2 and 3.7, and IBA concentrations and genotype 2.2 roots per microshoots were obtained (Table 4.8. and Figure 4.4.).

Table 4.8. The number of roots per microshoot of *Morus nigra* genotypes in the presence of various IBA concentrations

IBA (mg L^{-1})	Number of roots per mikroshoots (± std. err.)			Mean
	Genotype A5	Genotype T6	Genotype T8	
0.0	1.8 ± 1.2	--	--	1.8 ± 1.2
0.5	3.7 ± 1.2	2.5 ± 0.9	2.6 ± 0.9	2.9 ± 0.6
1.0	2.3 ± 0.9	2.7 ± 0.9	1.2 ± 0.9	2.1 ± 0.5
1.5	1.9 ± 0.9	1.9 ± 0.9	2.0 ± 0.9	2.0 ± 0.5
Mean	2.4 ± 0.5	2.4 ± 0.5	2.0 ± 0.3	

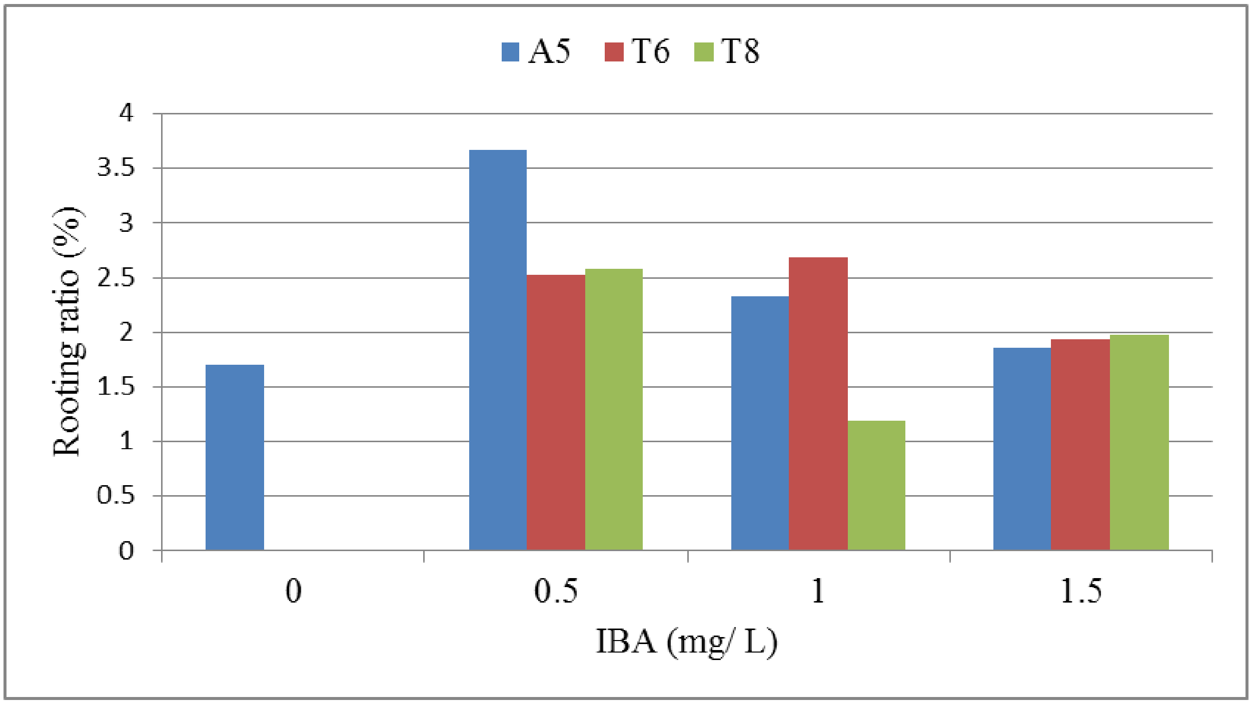

Figure: 4.4. Mean number of roots per microshoot of *Morus nigra* genotypes in the presence of various IBA(mg/l) concentrations

Morus nigra is a difficult to root species and low rooting ratios of woody cuttings is a major obstacle in traditional propagation. The low rooting ratio obtained in this study indicates that rooting of micropropagated shoots of *Morus nigra* may not be satisfactory as well. On the other hand, although statistically not significant, the highest rooting ratio and highest root number per shoot were obtained in the presence of 0.5 mgL^{-1} IBA concentration. Thus, providing other optimal conditions, a low level of auxin will be sufficient for rooting of microshoots. Rooting of micropropagated mulberry can also be achieved ex vitro by direct insertion microshoots into a vermiculite moistened plant bed (Katase, 1993).

Mei-Chun (2002) reported that 85% rooting of *Morus latifolia* was obtained at 1.0 mg L^{-1} IBA on half strength MS medium. Yadav *et al*. (1990) obtained the best rooting ratio and number of roots at 0.25 mgL^{-1} IBA in *Morus nigra*. Ohyama and Oka. (1987) and Jain *et al*. (1990) stated that the successful rooting of cuttings is dependent upon the auspicious environmental conditions and genotype.

4.4. Acclimatization of Rooted Microshoots

Rooted microshoots were removed from culture vessels and roots were washed with tap water to remove the agar. After removal of agar from roots, rooted microshoots were transferred to pots. Then the plantlets were subjected to the gradual acclimatization procedure explained in Materials and Methods (Figure 4.5). Over 90% of rooted microshoots survived acclimatization stage. Within the first weeks of acclimatization microshoots almost showed no sign of re-growth. However, about one month after acclimatization stage, fully hardened plantlets displayed new growth and they had normal looking phenotype (Figure 4.6.).

The acclimatization results indicate that once microshoots are rooted, they can successfully be transplanted ex vitro and a reasonable number of clone plants can be distributed to the growers in a relatively short period of time.

Figure 4.5. (A) Rooted microshoots of *Morus* n*igra.* (B) Microshoots transplanted to small pots and (C) covered with clear plastic cups to maintain high humidity

Figure 4.6. Fully hardened micropropagated plants of *Morus* n*igra* growing in the greenhouse

5. CONCLUSION AND RECOMMENDATIONS

Traditional propagation methods of black mulberry are not satisfactory for commercial propagation. Providing optimum culture medium and culture conditions, micropropagation can be an alternative propagation method.

Based on the results obtained from the present study the following conclusions can be drawn:

1. Agar-gelled medium gave better shoot multiplication compared to liquid medium and should be preferred for shoot proliferation.
2. If shoot length is concerned, first a desired shoot number should be obtained on agar – gelled medium then shoots should be cultured on a liquid medium to obtain longer shoots.
3. Charcoal in the culture medium was inhibitory for culture growth and its use in micropropagation of *Morus nigra* is not recommended.
4. Although the rooting of microshoots need to be improved, once microshoots are rooted, they can successfully be transplanted ex vitro and a reasonable number of clone plants can be distributed to the growers in a relatively short period of time.

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