IN VITRO CULTURE ESTABLISHMENT FROM HYPOCOTYL AND COTYLEDON EXPLANTS OF TWO BANGLADESHI SUNFLOWER (*Helianthus annuus* L.) VARIETIES

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

Afsana Tasnim Oshin

Student ID: 11136003

March, 2015

Department of Mathematics and Natural Sciences

Biotechnology Programme

BRAC University

Dhaka, Bangladesh

Dedicated
To
My Beloved Parents and
Lovely Sister

DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "***In vitro* culture establishment from hypocotyl and cotyledon explants of two Bangladeshi sunflower (*Helianthus annuus* L.) varieties"** submitted by the undersigned has been carried out under the supervision of Dr. Aparna Islam, Associate Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree of diploma.

(Afsana Tasnim Oshin)
Candidate

Certified

(Dr. Aparna Islam)
Supervisor

Associate Professor
Biotechnology Programme
Department of Mathematics and Natural Sciences
BRAC University, Dhaka.

Acknowledgement

Firstly I would like to thank Allah Almighty for His immense blessings that gave me the strength and patience to accomplish my work successfully.

I would like to express my sincere gratitude and esteem to Professor Naiyyum Chowdhury, Coordinator, Biotechnology programme, Department of Mathematics and Natural Sciences, BRAC University and Professor A. A. Ziauddin Ahmad, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for their valuable suggestions, inspiration, cooperation and encouragement throughout my four year study period in BRAC University. It had been a great privilege for me to be their student.

I express my earnest gratitude and respect to my supervisor Dr. Aparna Islam, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University for her helpful advices, affectionate guidance and constant support throughout this research study.

I am glad and extremely grateful to my parents and my sister for their immense love, continuous support and encouragement in every stage of my life.

I want to thank all the members of Plant Tissue culture and Biotechnology lab of BRAC University, namely Shahana Chowdhury, Noor Jahan, Rabab Mahdi, Anamika Datta, Sreoshee Rafiq and Iftekhar Mahmud Chowdhury for their care, affection, helpful advices, good ideas, guidance and support during my research work. I am also grateful to the lab assistants, Forkan Mia and Arifuzzaman, who had been always there to help me in the lab works.

I am also thankful to my friends, especially Maisha Mosharrat Chowdhury, Sheikh Ziauddin Ahmed, Tasmin Naila, Mashiat Nawar and Rhedia Tehrin for their good company, ideas, various helps and support throughout my degree program.

March, 2015 **Afsana Tasnim Oshin**

List of Abbreviations

The following abbreviations have been used throughout the text.

BARI - Bangladesh Agricultural Research Institute

BRAC - Bangladesh Rural Advancement Committee

BAP - 6-Benzylaminopurine

NAA - Napthalene acetic Acid

IAA - Indole-3 Acetic Acid

IBA - Indole-3 Butyric Acid

GA_3 - Gibberellic Acid

Kn - 6- Furfurylaminopurine

2,4-D - 2, 4-Dichlorophenoxy acetic acid

MS - Murashige and Skoog (1962) medium

LS - Linsmaier and Skoog (1965) medium

NPK- Nitrogen Phosphorus Potassium

NaOCl - Sodium hypochlorite

$HgCl_2$ - Mercuric chloride

Fe-EDTA - Ferric ethylenediaminetetraacetic acid

NaOH - Sodium hydroxide

pH - Power of hydrogen

cm - Centimeter

gm - Gram

mg - Milli gram

ml - Milliliter

L - Liter

Fig - Figure

psi - Pounds per square inch

SD - Standard deviation

rpm - Rotations per minute

Abstract

Sunflower (*Helianthus annuus*.L) is the world's second most important oilseed crop. Plant tissue culture and gene transformation are the important tools for improvement of this crop. The purposes of this study are to optimize seed sterilization procedure, determine the most suitable and efficient explants along with their suitable age and size, and to optimize the hormonal concentration for shoot regeneration. Seeds being treated with 70% ethanol for 3 minutes and 14% Clorox for 20 minutes, with their testa being removed before being inoculated into the germination medium, were found to be optimum for the tissue culture technique. In this study, two varieties, BARI surjomukhi 2 and BRAC Hysun 33 were used as plant materials. Hypocotyls and cotyledons were excised from 5, 7 and 9 days and cultured on MS medium supplemented with eight different combinations of BAP and NAA hormones. The experiments were kept in 18/6 hour light/dark photoperiod at 26±2ºC. It was determined that MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA is the best combination for 7 day old hypocotyl explants of both BARI surjomukhi-2 and BRAC Hysun 33 varieties. From 7 day old hypocotyl explants, the best shoot regeneration response of BARI surjomukhi-2 and BRAC Hysun 33 variety was 60% and 53.3%, with the best grown shoot that grew up to 2.08 cm and a of length 1.61 cm within 30 days of regeneration respectively. This clearly showed that MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA is the best hormonal media combination for hypocotyl explants. Shoot regeneration efficiencies of the cotyledon explants on the same medium were lower in comparison with the hypocotyl explants. Cotyledon explants only grew large in size with time, but did not show any positive response like shoot regeneration. So, hypocotyls were proved to be better explants than cotyledons. In future, rooting at the base of the regenerated shoots will be tried to generate whole plantlet.

Table of Contents

1. Introduction

The scientific name of sunflower is *Helianthus annuus*, which belongs to the family Compositae. The name sunflower is derived from the flower head's shape, which resembles the Sun. It is an annual plant grown as a crop for its edible oil and edible fruits. It is the world's second most important oil seed crop next to soybean in the world. Sunflower is one of the most important sources of edible vegetable oil throughout the world due to its high oil content (40% - 52%), no cholesterol and high non saturated fatty acids content (Leland, 1996; Khalifa and Awad, 1997). To grow best, sunflowers need full sun. They grow best in fertile, moist, and well-soaked soil. Biotechnological approaches like tissue culture techniques are required for the improvement of sunflower because it increases the yield by selecting desired characteristics. Tissue culture is a pre-requisite for transformation. To develop protocols for an efficient regeneration, it is important to determine the roles and connections of different genotypes, explant sources, and hormonal effects (Bayraktaroglu and Dağüstü, 2011).

1.1. Morphology of sunflower

The sunflower plant has straight and hairy stem, which has a height of 125-140 cm. It has a strong taproot system and lateral spread of surface roots. Sunflower leaves are phototropic in nature, and structurally they are broad, petiolate, coarsely toothed, rough and mostly alternate.

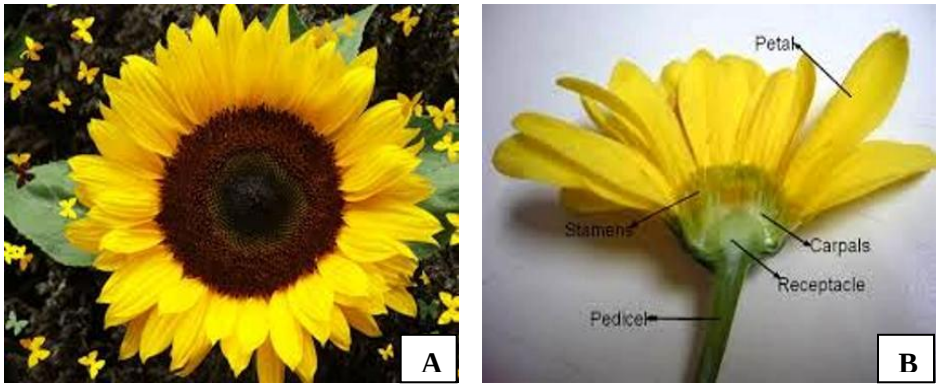

Figure**: A.** A sunflower. **B.** Sunflower labeled longitudinally.

The stems are typically unbranched and along with most other parts of the plant, it varies from being glabrous or very smooth to very densely pubescent. Stem length is determined by the number of internodes. The first leaves are always opposite but in some varieties, become alternate. The color intensity of the leaves could vary from light to dark green (Dedio, 2005; Heiser, 1978).

Sunflower contains a single large inflorescence (flower head) at the top of the unbranched stem. The sunflower head is not a single flower but is made up of 1,000 to 2,000 individual flowers joined at a common receptacle. The flowers around the circumference are called ligulate ray flowers without stamens or pistils. They are usually large and yellow but the color can range from lemon-yellow, orange to reddish. The remaining flowers are perfect flowers (with stamens and pistils). The flowers in the center of the head are called disk flowers which mature into fruits, known as sunflower seeds. The disk flowers are arranged spirally (Dedio, 2005). Anthesis (pollen shedding) begins at the periphery and proceeds to the center of the head (Putnam, *et al*., 1990).

The achene, or fruit of the sunflower consists of a seed, also called the kernel, and adhering pericarp, usually called the hull. In the absence of fertilization, the achenes will be empty, with no kernel. Achenes vary from 7 to 25 mm in length and 4 to 13 mm in width. They may be linear, oval or almost round (Dedio, 2005).

1.2. History

The wild sunflower is one of the few crops that are native to North America but commercialization of the plant took place in Russia, which recently returned to North America to become a cultivated crop. Selection for high oil in Russia began in 1860 and was largely responsible for increasing oil content from 28% to almost 50%. The American Indian first cultivated the plant into a single headed plant with a variety of seed colors including black, white, red, and black-white striped. Argentina, the combined European Union, China, India, Turkey and South Africa are all significant producers of sunflower (National Sunflower Association 2014).

1.3. The Reproductive Biology of *H. annuus*

The sunflower is an annual crop that is propagated by seed only and can hybridize spontaneously with several wild/weedy relatives (Burke *et al.,* 2002). Until the 1960's the cultivars grown were open-pollinated and cross-pollinated mostly by insects. They were highly self-incompatible along with the wild species. Current commercial sunflower varieties are self-compatible, however environmental conditions can influence the level of self-fertility expressed (Snow *et al.,* 1998). Pollen transfer is through insect pollinators, mainly bees. The pollen is spiny and adapted to be transported by insects. Little is pollinated by wind as the pollen is very heavy weight (Fick, 1978). It may be viable for several days. Although the anthers containing the pollen and the stigma are on the same floret, the two lobes of the stigma are initially not exposed to their own pollen. However, they are susceptible to pollination from other florets of the same head by insects, wind or gravity (Dedio, 2005).

1.4. Uses

As edible oil: Sunflower oil is considered as a premium oil because of its high level of unsaturated fatty acids and lack of linolenic acid, bland flavor and high smoke points. It supplies more Vitamin E than any other vegetable oil. The oil also contains variable amounts of vitamins A, D and K. It is a combination of monounsaturated and polyunsaturated fats with low saturated fat levels. The primary fatty acids in the oil are oleic and linoleic (typically 90% unsaturated fatty acids), with the remainder consisting of palmitic and stearic saturated fatty acids (National Sunflower Association 2014). Sunflower oil is valued for its light taste, frying performance and health benefits. The primary use of it is in salad dressings, cooking oil, margarine cooking, baby formula, lubrication, bio-fuel, hydrolic fluids, soaps, and illumination. There are three types of sunflower oil available, namely linoleic and high oleic sunflower oil (Putnam, *et al*., 1990). Commercially available sunflower varieties contain a range from 39% to 49% oil in the seed (Putnam, *et al*., 1990).

As meal: Sunflower meal is rich in fiber, has a lower energy value and is lower in lysine but higher in methionine than soybean meal. Protein percentage of sunflower meal ranges

from 28% for non-dehulled seeds to 42% for completely dehulled seeds (National Sunflower Association 2014). Sunflower oil is a valued and healthy vegetable oil and sunflower seeds are considered as a healthy, tasty snack and nutritious ingredient in many foods. The seeds can be squeezed to extract oil from it. These seeds are used for human consumption and can also be used as birdfeed and as a high protein meal for livestock (Dedio, 2005). The by-products of the seed (seed cake) is a rich source of proteins (35%) and carbohydrates (18- 20%) for animals and poultry feed (Ibrahim, 2012; Aminifar and Galavi, 2014).

Industrial Applications: Sunflower can be used as purple dye for textiles, body painting, varnishes, plastics and other decorations because of its good semidrying properties without color modification associated with oils high in linolenic acid (Putnam, *et al*., 1990). Parts of the plant are used medicinally ranging from snakebite to other body ointments. The oil of the seed can be used on the skin and hair, while the dried stalk can be used as a building material (National Sunflower Association 2014). The use of sunflower oil (and other vegetable oils) as a pesticide carrier, and in the production of agrichemicals, surfactants, adhesives, plastics, fabric softeners, lubricants and coatings has been explored. The utility of these applications is usually contingent upon petrochemical feedstock prices (Putnam, *et al*., 1990). The flowers are used as a yellow dye, and the plant itself can be used for fodder, silage and as a green-manure crop (Dedio, 2005).

1.5. Cultivation of sunflower

Sunflower is grown in many semi-arid regions of the world. It is tolerant to both low and high temperatures but more tolerant to low temperatures. Sunflower seeds will germinate at 3.9°C, but temperatures of at least 7.8 to 10°C are required for satisfactory germination. Seeds are not affected by vernalization (cold) in the early germination stages. Seedlings in the cotyledon stage have survived temperatures down to -5°C. At later stages freezing temperatures may injure the crop. Optimum temperatures for growth are 21 to 26°C, but a wider range of temperatures (18 to 33°C) show little effect on productivity. Extremely high temperatures have been shown to lower oil percentage, seed fill and germination

(Putnam, *et al*., 1990). In Bangladesh, sunflower is normally seeded in May, as it requires a long season to mature (Sunflower Production Guide 2014). It is essential that the seed is placed deep enough to reach sufficient moisture levels because sufficient moisture results in more rapid germination. Sunflower threshes easily and harvesting is done in October when the moisture content is below 12% (Dedio *et al.*, 1980; Dedio, 2005).

The sunflower is considered to be somewhat of a drought tolerant plant and will grow in a variety of soil types from sands to clay and a wide range of soil pH's from 5.7 to over 8. Sunflowers do however possess a low salt tolerance and require well drained soil. Application of fertilizers having nutrients like nitrogen, phosphorous and potash can increase sunflower growth and yield substantially (Kho, 2000; Prasad *et al*., 2002; De Varennes *et al*., 2002; Cechin and Fumis, 2004; Sadras, 2006).

1.6. Constraints of sunflower cultivation and production

1.6.1. Diseases

A four-year rotation is recommended for sunflower mostly because of its high susceptibility to sclerotinia. In case of downey mildew, it can be controlled chemically by seed coating with a fungicide (Dedio, 2005). The most serious diseases of sunflower are caused by fungi. The major diseases include rust, downy mildew, verticillium wilt, sclerotinia stalk and head rot, phoma black stem and leaf spot. The severity of these disease effects on total crop yield might be ranked as: (i) sclerotinia, (ii) verticillium, (iii) rust (recently more severe), (iv) phoma, and (v) downy mildew. Resistance to rust, downy mildew, and verticillium wilt has been incorporated into improved sunflower germplasm (Putnam, *et al*., 1990). Various insects like cutworms and wireworms can also attack the crop. The sunflower beetle, banded sunflower moth and the seed-eating insects can cause extensive defoliation shortly after emergence and later on at bud stage (Burke and Rieseberg, 2003).

1.6.2. Precocious flowering and poor rooting

Sunflower is disgracefully recalcitrant to genetic transformation and regeneration when subjected to tissue culture (Pearson *et al.,* 2007). It has been found that the plantlets which are usually obtained from shoot tip and cotyledon culture resulted in precocious (mature) flowering *in vitro* within 20-25 days of culturing. On the other hand, premature flowering has been obtained in regenerants from immature embryo, especially on a medium containing high sucrose (6%) along with 0.5mg/l BAP and 0.5mg/l IAA. But *in vitro* flowered plantlets could not be established in soil very effectively due to its poor rooting. This is why *in vitro* flowering is considered difficult in sunflower tissue culture. For root induction, 0.5mg/l of activated charcoal to 1/3 MS media and 1% sucrose resulted in increase in rooting to the extent of 68-88%. However, this is important to consider that transferring to rooting media should be done before or when flowering is just initiating for better results (Patil *et al*., 1993).

1.6.3. Hyperhydricity

The special conditions during *in vitro* culture, such as low level of light, high relative humidity, ample sugar and nutrients results in the formation of plantlets of abnormal morphology, anatomy and physiology. Hyperhydricity is a physiological malformation affecting clonally propagated plants generated under tissue culture conditions. This condition is associated with chlorophyll deficiency, poor lignification, and excessive hydration of tissues, which result in malformed plantlets that cannot survive *ex vitro* conditions after transplanting because the affected shoots fail to root. These plants also have reduced or retarded growth and thickened, malformed stem and leaves. Efforts are being made in this species to control this undesirable phenomenon, hence reducing its effects if not eliminated (Fauguel, *et al.,* 2008).

1.7. Improvements of sunflower

Because of the importance of sunflower, many studies on yield improvement have been done. The results of some research have shown that genetic gain in oil content has been relatively low and is slowing down, which means that the increase in oil yield during the

past decade was mainly due to the increased seed yield per ha (Aminifar and Galavi, 2014).

Early breeding was by mass selection, which involved selecting heads for some specific trait. Because sunflower is a highly cross-pollinating crop, there was no control of pollination. In this way, varieties for characters, such as, disease resistance, oil content and seed characteristics were developed. Later on, Pustovoit (1964) in USSR in the 1920's developed a much more successful technique called the method of reserves. It involves testing of seed from individual heads for various characters in an evaluation nursery for two years, followed by controlled pollination of selected heads. In this way, a dramatic increase in oil content was achieved along with improvement in yield (Burke, *et al.,* 2002). Genetic improvement for seed yield and oil-content in sunflower cultivars was initiated in the early 1970s (Chigeza *et al.,* 2012). Periodic assessment of the benefits of continued investment in plant breeding allows prioritization of traits especially those linked to quality, quantity and a reduction in the impact of crop production on the environment (Chigeza, *et al.,* 2012; Aminifar and Galavi, 2014).

Primary step of plant tissue culture is establishment of aseptical culture. For this reason, Taški-Ajdukoviã and Vasiã (2005) have established different sterilization methods for overcoming internal bacterial infection in sunflower seeds. The obtained result showed that the combination of 5% commercial bleach and dry heating gives the best result of sterilization of the sunflower seeds, and hence overcome the problem with internal bacterial infection. This represents an excellent method to obtain plants free of microbial contamination for tissue culture.

However, many other different ways of sterilization had been followed by other scientists. Abdoli *et al*. (2007) removed the pericarps and washed the seeds under running tap water before culturing the seeds. Seeds were surface sterilized with 70% ethanol for 3 minutes and Sodium hypochlorite (NaOCl) solution containing 2-3 drops of Tween-20 detergent for 20 minutes. On the other hand, Rao and Rohini (1999) had undergone a completely

different way of seed sterilization. The seeds were surface sterilized for 10 minutes in 0.1% Mercuric chloride ($HgCl_2$).

Bayraktaroglu and Dağüstü (2011) carried out their seed sterilization procedure by placing the seeds in 96% alcohol for 2 minutes, then in 50% commercial bleach solution containing a drop of detergent, agitated/ stirred for 20 minutes, followed by 5-6 times rinsing in sterile distilled water. The pericarps and achenes were removed after that, before inoculating the seeds into the germination medium containing 3% sucrose and 0.8% agar (pH 5.7). Whereas, Faugel *et al.* (2008) firstly removed pericarps of the seeds, then surface sterilized the seeds in 98% ethanol for 30 seconds, followed by soaking them in 5% NaOCl for 15 minutes.

T. Elavazhagan *et al*. (2009) reported to have produced an efficient protocol for plant regeneration using cotyledon and meristem explants of sunflower, where cotyledon showed highest percentage of response of callus induction on MS medium supplemented with 2mg/l BAP and 0.5mg/l GA_3. Highest frequency of multiple shoot bud induction (49.4%) was observed on MS medium with 5mg/l BAP and 0.5mg/l IBA. In a study conducted by Abdoli *et al*. (2007), *in vitro* shoot regeneration efficiency was checked without inducing hyperhydricity in sunflower, where cotyledons were regenerated on MS medium containing 4.4µM BAP, 5.4µM NAA and various concentrations of agar-agar. The addition of agar concentration showed to be useful in improving the quality of sunflower regenerated shoots by reducing hyperhydricity. Ozyigit *et al.* (2014) established plant tissue culture protocol on MS media supplemented with various plant growth regulators using hypocotyl and cotyledon explants. The highest shoot regeneration was observed using hypocotyl explants in MS media supplemented with 1 mg/1 BAP and 0.5 mg/l NAA. Shoot regeneration efficiencies with the cotyledon explants on the same medium were lower in comparison with hypocotyl explants. They proved that direct shoot regeneration of the cotyledons and young hypocotyls are advantageous explants. *In vitro* regeneration was performed by Bayraktaroglu and Dağüstü (2011) with the aim of developing efficient callus and shoot regeneration from different explants of sunflower. Roots, hypocotyls and cotyledons were excised from 4

day-old seedlings and cultured on embryo induction medium supplemented with 1mg/l BAP, 1mg/l NAA and 0.1mg/l GA_3. This was observed after 4 weeks, where it was found that root explants produced statistically high callus formation compared to cotyledon and hypocotyl explants. They showed that the effect of genotype, explant age, hormone type and concentration, basal medium and explant source are very important on callus induction and plant development.

In another paper, Ozyigit *et al.* (2005) reported their establishment of plant tissue culture systems on Murashige and Skoog (MS) media supplemented with various plant growth regulators using mature embryos of sunflower. For callus induction MS medium and 1 mg/l 2,4-D were used and for shoot regeneration MS medium and 1 mg/l BAP and 0.5 mg/l NAA were used. Callus induction ratios were around 80–92%. The latest study of Ozyigit *et al.* (2007) aims to observe the effect of genotype, hormone concentrations and culture conditions on sunflower callus induction and indirect plant regeneration. Calli were obtained from hypocotyl and cotyledon explants. Seeds germinated on MS media contained no hormones. Hypocotyl and cotyledon explants were cultured on MS media supplemented with 1 mg/l 2, 4 -D and different percentage of callus inductions were obtained. Calli were cultured on MS medium, 1 mg/l BAP and 0.5 mg/l NAA. Some genotypes showed high regeneration response while others showed lower on the same media with hypocotyl and cotyledon derived calli. This study showed that genotypic differences affect callus induction and plant regeneration in sunflower tissue culture studies.

1.7.1. Activities regarding sunflower in Bangladesh

In Satkhira of Bangladesh, sunflower was cultivated on 10 hectares of land in 2013 and about 30 to 35 mounds of sunflower seeds were produced per acre. Sunflowers have been cultivated in 150 hectares of land in Dhanshagor, Rayenda, Khontaka, Southkhali unions of Sharonkhola upazila of Bagerhat district in the year 2013 and it is expected that over 2000 tonnes of oil would be produced from these. BRAC started cultivation of this crop initially since 1998 and recently, has taken a fresh initiative to expand the cultivation of

sunflower in this area after Sidr disaster (http://businessnews24bd.com/satkhira-farmers-getting-inclined-to-sunflower-cultivation/November 16, 2014).

CSISA-CIMMYT Khulna hub has taken initiative for Sunflower production in saline and water limited condition in southern Bangladesh, in collaboration with Bangladesh Agricultural Research Institute (BARI) and Khulna University. They distributed seeds of BARI surjomukhi-2 among farmers of Titukhali village of Batiaghata Upazila under Khulna district in a block of 8 acres of land and gave necessary training to the farmers collaboration with BARI. The crop condition is very good. Local farmers are expecting more yield and income from sunflower (http://www.khulnanews24.com/index.php/local-news/255-initiatives-for-sunflower-production-in-saline-environment-of-southern-bangladesh.html/ November 16, 2014).

1.8. Objective of this paper

In this present study, the varieties BARI surjomukhi-2 and BRAC Hysun 33 have been used to establish a tissue culture protocol for sunflower plants. These varieties have not been previously reported in being involved to any such protocol establishment.

Therefore, aim of the present study is:

- to optimize seed sterilization procedure (according to highest aseptic germination rate)
- to determine the most suitable and efficient explants
- to determine the suitable age and size of explants
- to optimize hormonal concentration for shoot regeneration.

2. Materials and methods

2.1. Materials

2.1.1. Plant Material: BARI surjomukhi-2 and BRAC Hysun 33

In the present investigation, sunflower (*Helianthus annuus*) seeds of the varieties BARI surjomukhi-2 and BRAC Hysun 33 were used. BARI surjomukhi-2 seeds were collected from Bangladesh Agriculture Research Institute (BARI), which is located at Joydebpur, Gazipur. BRAC Hysun 33 seeds were collected from BRAC Agricultural Research and Development Centre (ARDC). Seeds were preserved at 4°C temperature in Plant Biotechnology Laboratory, BRAC University, Mohakhali, Dhaka, Bangladesh.

BARI surjomukhi-2 seeds are black colored, and have the ability to produce plants of height 125-140 cm, which has unicum broad leaves. This variety was released by BARI in the year 1982. The variety takes 90-100 days to mature and can produce a successful seed yield of 1.4-2.0 ton/ha. These seeds are sown in the Rabi season (between mid December and mid January) and its harvesting time is within March-April. The major disease by which it suffers most is the leaf blight disease, which can be managed by three spray with Rovral 50WP (0.2%) at 10 days interval (KIB Information service).

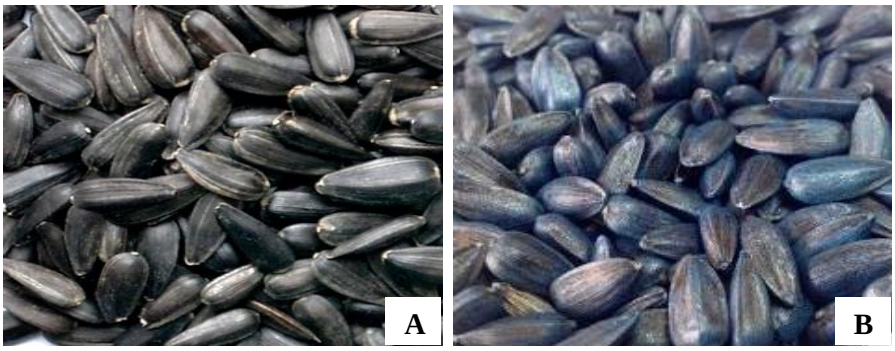

Figure: **A.** BARI surjomukhi-2 seeds. **B.** BRAC Hysun 33 seeds.

BRAC Hysun 33 seeds are blue colored, which can produce plants of height 90-110 cm. Sunflowers can grow in all types of soil. They grow best in moist soil. The lifespan of these plants ranges from 120-140 days. The variety produces a successful seed yield of

1.4-1.5 ton/acre and its germination rate is 100%. The most suitable time for sowing these seeds is from October to December. Slightly high moist condition leads the *Fusarium* species to attack the plants, resulting the plants to suffer from rotten diseases.

2.2. Methods

2.2.1. Media preparation

In the present study, Murashige and Skoog (MS) medium (1962) in full or half strength were used for *in-vitro* regenerations. MS medium were supplemented with hormones to produce regeneration medium.

2.2.1.1. Preparation of stock solutions

Preparation of different stock solutions was the first step for the preparation of the medium. As different constituents were required in different concentrations, separate stock solutions for macro-nutrients, micro-nutrients, organic solution and Iron-EDTA solutions were prepared.

2.2.1.2. Macro-nutrients (Stock solution-I) stock preparation for MS media

The stock solution of macro-nutrient was made in such a way that its strength was 10 times (10X) more than the full strength of the medium. For this purpose, the weight of different salts required for 1 liter of stock were weighed accurately and were sequentially dissolved one after another in a beaker. The final volume of the solution was made up to 1000 ml by further addition of distilled water into the 1000ml measuring cylinder. The solution was finally poured into a Scott bottle (clear glass container). After proper labeling, the solution was stored in a refrigerator at 4°C temperature for several weeks.

Table 2.1: The components and amount of each component needed to make 1 liter (10X) of Stock solution-I:

Macro-nutrients components	Amount (mg/l)	Amount for 10X stock solution (mg/l)
KNO_3	1900	19
NH_4NO_3	1650	16.5
$MgSO_4.2H_2O$	370	3.70
$CaCl_2.2H_2O$	440	4.40
KH_2PO_4	170	1.70

2.2.1.3. Micro-nutrients (Stock solution-II) stock preparation for MS media

This part of the stock solution was made with all the micronutrients. This was made 100 times (100X) the full strength of components and volume up to 1000 ml of distilled water. This solution was autoclaved and stored at 4°C in refrigerator.

Table 2.2: The components and amount of each component needed to make 1 liter of Stock solution-II:

Micro-nutrients components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
KI	0.83	0.0083
H_3BO_3	6.20	0.0620
$MnSO_4.4H_2O$	22.30	0.2230
$ZnSO_4.7H_2O$	8.60	0.0860
$Na_2MoO_4.2H_2O$	0.25	0.0025
$CuSO_4.5H_2O$	0.025	0.00025
$CoCl_2.6H_2O$	0.025	0.00025

2.2.1.4. Iron-EDTA (Stock solution-III) stock preparation for MS media

Solution III was made 100 times (100X) the final strength of Iron chelate ($FeSO_4.7H_20$) and $Na_2EDTA.2H_2O$ and volume up to 1000 ml of distilled water as described for the stock solution III. Heat was applied to dissolve the salts. The solution was autoclaved and

stored at 4°C for several weeks in an amber bottle to prevent penetration of sunlight into the solution as the solution is sunlight sensitive.

Table 2.3: The components and amount of each component needed to make 1 liter (100X) of Stock solution-III:

Iron-EDTA solution components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
$FeSO_4.7H_2O$	27.8	0.278
$Na_2EDTA.2H_2O$	37.3	0.373

2.2.1.5. Organic solution (Stock solution-IV) stock preparation for MS media

It was also made 100 times (100X) the final strength of medium, and volume was brought up to 1000 ml of distilled water. This solution was autoclaved and stored at 4°C for future uses.

Table 2.4: The components and amount of each component needed to make 1 liter of Stock solution-IV:

Organic solution components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
Nicotinic acid	0.5	0.005
Pyridoxin HCl	0.5	0.005
Thiamin HCl	0.1	0.001
Glycine	2.0	0.02

2.2.2. Preparation of stock solution for growth regulators (hormones)

The effects of the following hormones were examined in the present study.

1) Auxin: Napthalene acetic-acid (NAA)
2) Cytokine: 6- Benzyl amino purine (BAP)

Preparation of all the growth regulators is almost similar**.** To prepare any of the above mentioned hormonal stock solutions, 20 mg of hormone powder has to be taken in a clean beaker, where required amount of appropriate solvent (1N NaOH) was added to dissolve

the hormone powder. The dissolved mixture was then made up to 200 ml in volume with the addition of distilled water. This will give the stock solution a concentration of 10X. This solution was sterilized and preserved in a refrigerator at 4°C for several weeks. The growth regulators along with their solvents are listed below.

Table 2.6: Growth regulators and their solvent.

Growth regulator	Solvent
BAP	1 N NaOH
NAA	1 N NaOH
IBA	1 N NaOH

2.2.3. Preparation of 1 liter of MS medium

MS medium was prepared from these stock solutions. The components and amount of each component are mentioned below in the following table.

Table 2.5: Components and their amount in making of 1 liter MS medium

Components	Amount (for 1000ml)
Macro-nutrients (10X)	100 ml
Micro-nutrients (100X)	10 ml
Organic (100X)	10 ml
Iron - EDTA (100X)	10 ml
Sucrose	30gm
Myo-inositol	0.10 gm

The following procedure shows the general steps that should be maintained to produce MS media supplemented with the desired hormones in order to produce the regeneration media:

1) For the preparation of MS medium, 250 ml distilled water was taken in a 1 liter volumetric flask.

2) 100 ml of Stock solution I (Macro-nutrients), 10 ml of Stock solution II (Micro-nutrients), Stock solution III (iron-chelate) and Stock solution IV was added sequentially and mixed thoroughly.
3) 100 mg of Myo-inositol was added to this solution and was dissolved completely.
4) Then 30 gm of sucrose was dissolved as carbon source.
5) To obtain different required concentrations of various hormone(s) the stock solutions were added individually or in case of multiple supplementations added sequentially to this solution and were mixed thoroughly. Since each of the hormonal stock solution contained 10 mg in 100 ml of solution, the addition of 10 ml of any hormonal stock solution to make 1 liter of medium resulted in 1.0 mg/l concentration of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of the stock solution of the medium following this procedure.
6) Required amount of hormonal stock was also added in the medium.
7) The whole mixture was then made up to 1 liter with further addition of distilled water.
8) The pH of the medium was adjusted to 5.8 using a digital pH meter with the help of 1N NaOH or 1N HCl, whichever was required.
9) To solidify either 8.0 g (at 0.8%) of plant-agar was added to the desired medium. To dissolve the solidifying agent, the whole mixture was heated in a microwave oven.
10) Finally, the molten medium was dispensed in the conical flasks and sealed properly using aluminum foil. The vessels were marked with the help of a permanent marker to indicate specific hormonal supplement.

2.2.4. Precaution for aseptic culture

Almost all the steps of tissue culture, including inoculations and aseptic manipulations were carried out in the laminar air flow cabinet. The cabinet has to be switched ‘ON’ along with the Ultra-Violet (UV) light for at least 30 minutes before use. The inner surface of the clean bench is sterilized by UV rays and thoroughly cleansed by 70% ethanol after 30 minutes UV treatment to overcome the contaminants. All the necessary

instruments, like, scalpels, forceps, and flasks were sterilized by autoclave machine. At the time of inoculation and explant cutting, the required instruments were carefully sterilized by flaming method of sterilization inside the cabinet. Both hands are needed to be rinsed and sterilized with hexisol hand rub. All measures were taken to obtain maximum contamination-free condition during the work. Any contaminated culture and older non-regenerative plant parts should be autoclaved before disposal according to the bio-safety rules.

2.2.5. Media sterilization

All the media were prepared and poured into conical flasks and then autoclaved for 15 minutes at 120 psi for sterilization.

2.2.6. Seed sterilization

The seeds sterilization protocol has been optimized in this study. Seeds were sterilized in both ways, with and without testa (seed coat/pericarp). Seeds were initially washed under running tap water, along with a few drops of Trix detergent for a time period of 30 minutes, 2 hours and 24 hours individually. These seeds were then taken in a conical flask and sterilized by shaking in autoclaved distilled water for 2 minutes inside the laminar air flow cabinet. Following a further wash of the seeds in 70% ethanol for 3 minutes, they were again washed by stirring in autoclaved distilled water for three times with 1 minute duration of each wash. After that, the seeds were completely immersed in 14% Clorox and placed in a shaker at 120 rotations per minute (r.p.m.) for a range of period of time: 10 minutes to 20 minutes. Clorox solution was poured out and the seeds were again washed 3 times (1 minute for each wash) in autoclaved distilled water until all the bubbles disappear. The testa of the seeds were removed after the sterilization procedure, before being transferred to germination media. The seeds were kept soaked in autoclaved distilled water for 24 hours in dark chamber to obtain explants of age 5 days, 7 days and 9 days. The flasks were placed in dark chamber to create proper environment for germination.

2.2.7. Seed germination

The surface sterilized seeds were then given two more autoclaved distilled water wash to remove their surface waxy film and then inoculated into the conical flask containing MS (Murashige and Skoog 1962) media, also considered as germination media, for allowing growth of the seedling, from which explants can be collected. Seeds in the germination medium were kept in the dark chamber to resemble the dark environment under soil as it provides suitable environment for germination. Then after 24 hours, the cultures were shifted to 20-25ºC day-light temperature in a 16 hour photoperiod under white fluorescent light to allow shoot growth after germination.

2.2.8. Collection of explants

Explants were collected from aseptically germinated seeds. Explants are hypocotyls and cotyledonary leaves. These two explants were collected from the shoot parts on the basis of their age: 5, 7 and 9 days.

2.2.9. Regeneration media for shoot regeneration

Explants were inoculated into MS media, which was supplemented with hormones, like, BAP and NAA of various concentrations and combinations to produce the regeneration media in order to achieve direct and indirect regeneration. Cultures were maintained on the same media which eventually results into a well developed shoot production. Hormonal concentration of BAP and NAA varied in a wide range of eight combinations.

Table 2.7: The combination of the eight different concentrations of BAP and NAA.

BAP (mg/l)	NAA (mg/l)
1	0
	0.1
	0.5
	1
2	0
	0.1
	0.5
	1

2.2.10. Subculture

The *in-vitro* cultures grown shoots are required to be transferred into fresh media almost at an interval of 3 weeks. This is done to provide sufficient nutrients to the developed shoots, so that morphogenic changes may be achieved.

2.2.11. Root induction

For induction of sufficient roots from the *in vitro* grown shoots, half strength MS medium supplemented with 1mg/l IBA was used. Media was solidified using phytagel (12g/l). Shoots were separated by a sharp cut at the internode before placing into the above rooting media.

3. Results

The aim of this present study was to establish an efficient regeneration protocol for micropropagation of the sunflower varieties BARI surjomukhi-2 and BRAC Hysun 33. In this experiment, two explants were chosen: hypocotyl and cotyledon. Suitability of these explants for regeneration and optimization of different hormonal concentrations of BAP and NAA were accomplished through repeated experimental trials.

3.1. Seed sterilization and aseptic seed germination

BARI surjomukhi-2 seeds were sterilized in two ways, with and without testa (seed coat/pericarp). When the seeds with testa were inoculated into germination media, contamination was observed indicating insufficient sterilization. It has been found that 20 minutes of Clorox treatment and removal of the testa of the seeds after the sterilization procedure, before being transferred to germination media was effective for sterilization. So this step was followed in further experiments. On the other hand, the seeds which were sterilized after initial removal of their testa, gave no contamination but their germination rate was not as good and efficient as the seeds with testa. Hence, seeds with testa being removed after the sterilization procedures, treated with 70% ethanol for 3 minutes and 14% Clorox for 20 minutes was considered to be optimum for being used in the tissue culture technique (Table 3.1).

Similarly, seeds of BRAC Hysun 33 variety were sterilized both including their testa (seed coat) and without testa. The best response was given by those seeds that were sterilized with their testa. The testa of the seeds were removed before the seeds was being transferred to germination medium. Here also, a constant 70% ethanol wash was given to all types of seeds for 3 minutes duration along with a Clorox treatment of both 14% and 30% concentration for 20 minutes time period. Seeds with testa in germination media gave contamination, so in order to avoid contamination, testa were removed before transferring the seeds to germination media (Table 3.1).

Table 3.1: Optimization of seed sterilization of BARI surjomukhi 2 and BRAC Hysun 33

Variety	Treatments	Testa/ Seed coat (+)or Seed coatless(-)	Contamination	Percentage of seeds germinated (%)	Average time required for germination (days)
BARI surjomukhi 2	70% ethanol wash for 3 minutes + 14% Clorox treatment for 20 minutes	+ SC	Yes	90 (1)	2.67
		- SC	No	66.67 (0.58)	7.33
	70% ethanol wash for 3 minutes + 14% Clorox treatment for 15 minutes	+ SC	Yes	76.67 (1.53)	5.67
		- SC	No	56.67 (0.58)	8.33
	70% ethanol wash for 3 minutes + 14% Clorox treatment for 10 minutes	+ SC	No	36.67 (0.58)	9.67
		- SC	No	0 (0)	--
	70% ethanol wash for 3 minutes + 14% Clorox treatment for 20 minutes. Testa removed after seed sterilization	- SC	No	96.67 (0.58)	3.33
BRAC Hysun 33	70% ethanol wash for 3 minutes + 14% Clorox treatment for 20 minutes	+ SC	Yes	93.33 (0.58)	1.67
		- SC	No	33.33 (1.53)	7.33
	70% ethanol wash for 3 minutes + 30% Clorox treatment for 20 minutes	+ SC	Yes	83.33 (0.58)	2.33
		- SC	No	46.67 (1.53)	8.67
	70% ethanol wash for 3 minutes + 14% Clorox treatment for 20 minutes. Testa removed after seed sterilization.	- SC	No	96.67 (0.58)	2.67

+ SC = with seed coat; - SC = without seed coat; 0 = died, no shoot regeneration.
All experiments were performed three times with 30 seeds in every experiment.
SD value is mentioned within parenthesis.

3.2. Analysis of overall responses of two different explants of BARI surjomukhi-2 and BRAC Hysun 33

3.2.1. Effect of age of the explants on regeneration response

In the present study, hypocotyl explants were collected and cut into 1 cm pieces and the cotyledon explants were cut transversely at the edges. Excised hypocotyls and cotyledons were then inoculated into eight different hormone supplemented media for shoot induction and development**.**

3.2.1.1. Explant: Hypocotyl

Hypocotyl explants of three different ages (5, 7 and 9 days) were examined to determine the optimum age for regeneration response. When 5 days old explants of BARI surjomukhi-2 and BRAC Hysun 33 were inoculated, they were found to be dead within 2-3 days. On the other hand, it was difficult to collect 9 days old explants because the hypocotyls mature into hard stems by then, thus the explants were no longer young and soft. Still, hypocotyls explants of both the varieties had been collected on 9 days, which did not give any expected good result as they remained unchanged for few days before death within 3-4 days. In both BARI surjomukhi-2 and BRAC Hysun 33, 7 days old hypocotyls showed the highest rate of response, which is 97.9%, within 3.67 and 4.33 days, for regeneration initiation, respectively (Table 3.3).

If the hypocotyl explants are compared between the two varieties (BARI surjomukhi-2 and BRAC Hysun 33), it can be said that the BARI surjomukhi-2 variety is a better one as it produces a more healthier and longer shoot in lesser days (2.08 cm in 3.67 days) than BRAC Hysun 33 variety (1.61 cm in 4.33 days) (Table 3.3).

3.2.1.2. Explant: Cotyledon

Cotyledon explants of three different ages (5, 7 and 9 days) were examined to determine the optimum age for regeneration. 5 day old explants died immediately after some days

following inoculation. It was difficult to collect 9 days old explants because most of the cotyledons got matured and formed primordial leaves by 9 days (Table 3.4).

However, it has been found that the cotyledons only grew gigantically in size with time, but did not show any positive response like shoot regeneration. Callus was seen to have formed, along with numerous adventitious rooting, but no shoot or bud formation was evitable from the cotyledons. This confirms that cotyledons are not good explants for sunflower tissue culture. Since both the varieties gave no shoot regeneration in the cotyledon explant, it has been proved that this explant is not suitable for developing tissue culture protocol in the Bangladeshi sunflower varieties (Table 3.4).

Therefore, for further experiments, hypocotyls are better and more suitable explants compared to cotyledon explants.

3.2.2 Regeneration response of the two chosen explants under various hormonal supplementations

During this experiment, both direct and indirect regeneration were tried from the selected explants. Eight different hormonal concentrations and combinations were employed at this stage.

Table 3.3: Determination of the suitable age of hypocotyl explants of BARI surjomukhi-2 and BRAC Hysun 33 variety for regeneration

Varieties	Age of explant (days)	Days required for response (callus formation)	Percentage (%) of responsive explants	Average shoot length (after 30 days) in cm (SD)
BARI surjomukhi 2	5	--	0	--
	7	3.67	97.9 (0.58)	2.08 (0.77)
	9	9	50 (1)	--
BRAC Hysun 33	5	--	0	--
	7	4.33	97.9 (0.58)	1.61 (0.77)
	9	10.67	60.4 (1.55)	--

SD value is mentioned within parenthesis.

Table 3.4: Determination of the suitable age of cotyledon explants of BARI surjomukhi-2 and BRAC Hysun 33 variety for regeneration

Varieties	Age of explant (days)	Days required for response (callus formation)	Percentage (%) of responsive explants	Average Shoot length (after 30 days) in cm
BARI surjomukhi 2	5	--	--	--
	7	8.33	95.8 (0.58)	--
	9	12	52.08 (1.53)	--
BRAC Hysun 33	5	--	0	--
	7	8.5	93.75 (1)	--
	9	15.5	81.25 (1)	--

SD values are mentioned within parenthesis.

3.2.2.1. Effect of various hormonal supplementations on hypocotyl explants

3.2.2.1.1. Variety: BARI surjomukhi-2

Hypocotyl explants of BARI surjomukhi-2 showed indirect shoot regeneration in all the combinations of the hormonal concentrations. Callus formation occurred primarily in all, which successfully developed into a shoot in some, whereas remained as callus in the rest. Both types of callus were seen to have formed: translucent, jelly-like callus and also some friable callus. MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA gave the best shoot regeneration response (60%), with well-developed shoot that grew up to 2.08 cm within 30 days of regeneration. Second best result was given by MS media supplemented with 1mg/l BAP and 0.5mg/l NAA (40%), followed by 2mg/l BAP alone (26.67%). The results are shown in Table 3.5 (Fig 3.1).

3.2.2.1.2. Variety: BRAC Hysun 33

Good response was found in 7 days old hypocotyls of BRAC Hysun 33 variety, in MS media supplemented with combination of 1.0 mg/l BAP and with 0.1 mg/l NAA. The explant produced single shoot within 7 days, along with translucent callus formation, where the shoot grew up to a height of 1.61 cm with the highest shoot regeneration response (53.3%). In MS media supplemented with 1 mg/l BAP in combination with 0.5 mg/l NAA, shooting initiated around 25 days after inoculation without any callus formation. Some explants formed special structures within 10 days which ultimately did not form callus or shoot and these structures were obtained in media supplemented with 1 mg/l BAP in combination with 0.5 mg/l NAA and 2 mg/l BAP (Table 3.6, Fig. 3.2).

Table 3.5: Effect of different combinations of hormonal concentrations on shoot regeneration from 7 days old hypocotyls explants of BARI surjomukhi-2 variety

Hormones		Responsive explants (%)	Type of regeneration (callus type)	Shoot regeneration response (%)	Average shoot length in cm (after 30days)
BAP (mg/l)	NAA (mg/l)				
1	0	93.3 (0.58)	Indirect	0 (0)	--
	0.1	100 (0)	Indirect	60 (0)	2.08 (0.77)
	0.5	93.3 (0.58)	Indirect	40 (1)	1.12 (0.34)
	1	93.3 (0.58)	Indirect	0 (0)	--
2	0	100 (0)	Indirect	26.67 (0.58)	0.6 (0.34)
	0.1	93.3 (0.58)	Indirect	0 (0)	--
	0.5	93.3 (0.58)	Indirect	6.67 (0.58)	0.7 (0)
	1	86.6 (0.58)	Indirect	13.33 (0.58)	0.4 (0.14)

SD values are mentioned within parenthesis.

Table 3.6: Effect of different combinations of hormonal concentrations on shoot regeneration from 7 days old hypocotyl explants of BRAC Hysun 33 variety

Hormones		Responsive explants (%)	Type of regeneration (callus type)	Shoot regeneration response (%)	Average shoot length in cm (after 30 days)
BAP (mg/l)	NAA (mg/l)				
1	0	93.3 (0.58)	Indirect	0 (0)	--
	0.1	100 (0)	Indirect	53.3 (0.58)	1.61 (0.77)
	0.5	93.3 (0.58)	Direct	33.3 (0.58)	0.28 (0.15)
	1	100 (0)	Indirect	0 (0)	--
2	0	100 (0)	Direct	13.3 (0.58)	0.85 (0.21)
	0.1	93.3 (0.58)	Indirect	20 (0)	0.33 (0.15)
	0.5	86.6 (0.58)	Indirect	0 (0)	--
	1	93.3 (0.58)	Indirect	0 (0)	--

SD value is mentioned within parenthesis.

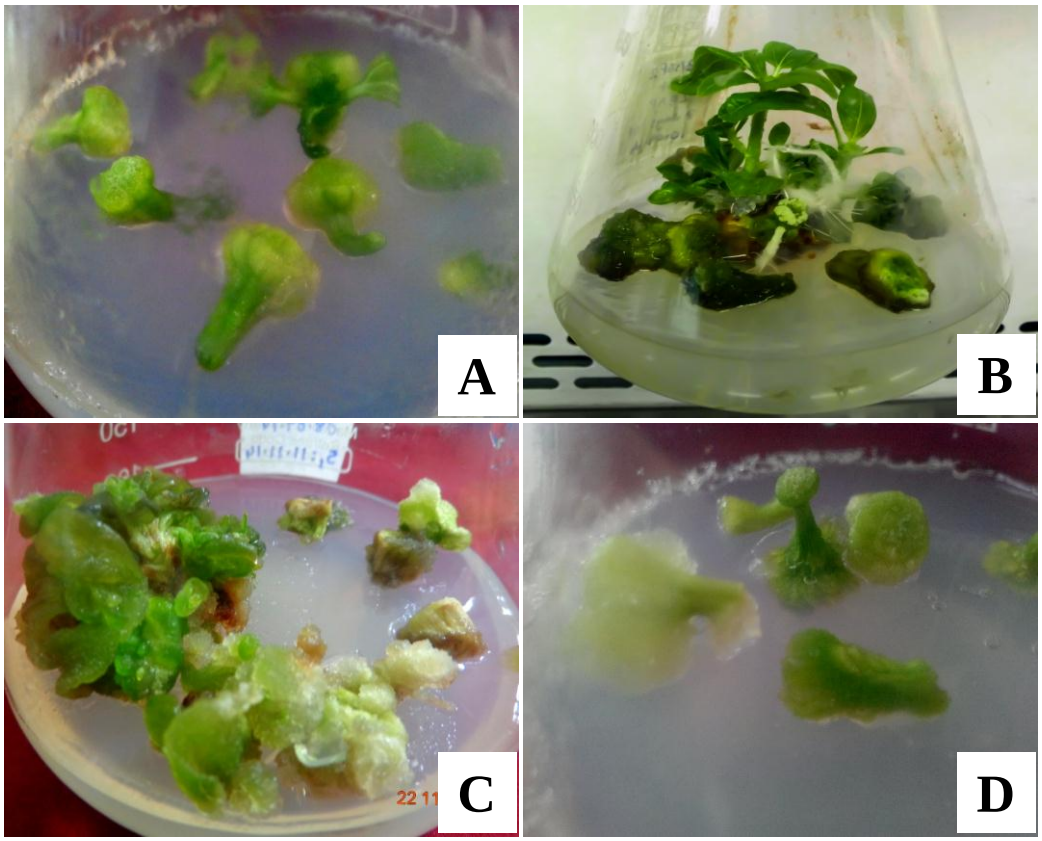

Fig. 3.1: Regeneration from hypocotyl explants of BARI surjomukhi 2 variety. **A**. Callus development in MS media supplemented with 1 mg/l BAP and 0.1 mg/l NAA within 7 days of inoculation. **B.** Successful shoot formation in MS media supplemented with 1 mg/l BAP and 0.1 mg/l NAA within 30 days of inoculation. **C.** Indirect regeneration and shoot formation in MS media supplemented with only 2 mg/l BAP (Photographs were taken 40 days after inoculation). **D.** Indirect regeneration in MS media supplemented with 1 mg/l BAP and 0.5 mg/l NAAwithin 7 days of inoculation.

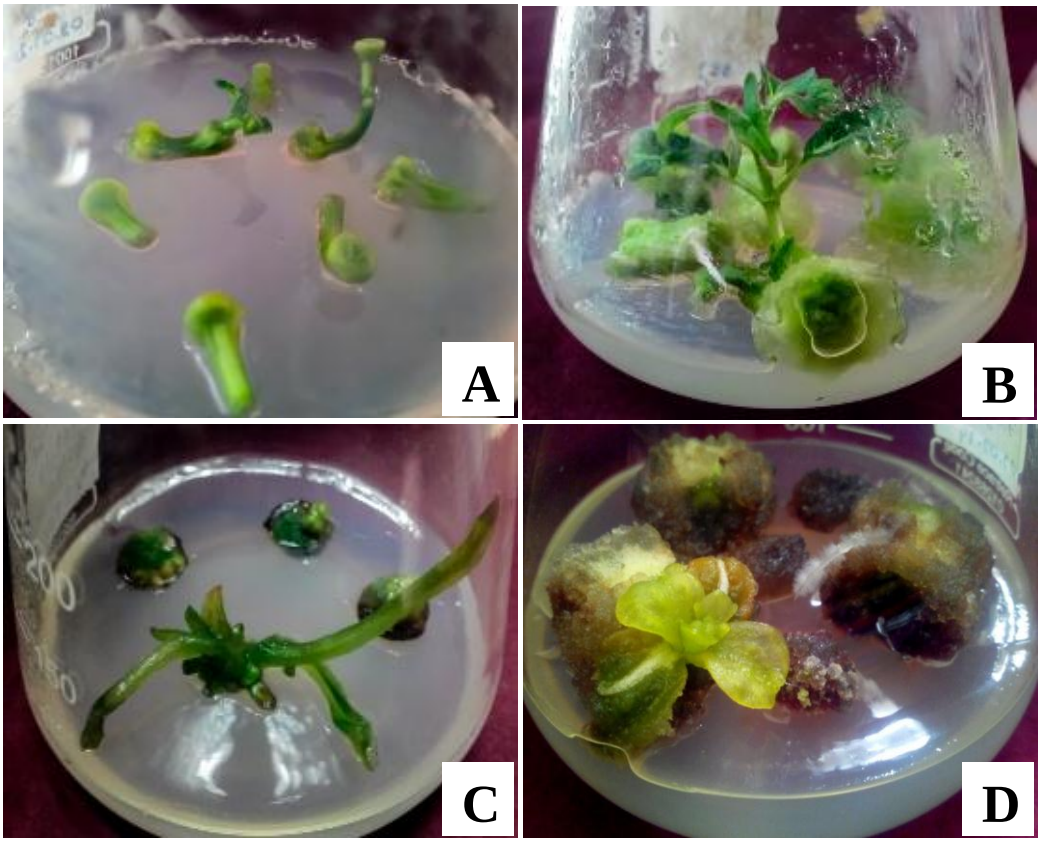

Fig. 3.2: Regeneration from hypocotyl explants of BRAC Hysun 33 variety. **A.** Direct regeneration in MS media supplemented with 1.00 mg/l BAP and 0.5 mg/l NAA within 7 days of inoculation. **B**. Indirect regeneration and successful shoot formation in MS media supplemented with 1.00 mg/l BAP and 0.1 mg/l NAA within 30 days of inoculation. **C.** Indirect regeneration and different structural formation of the explants in 2.00 mg/l BAP. **D.** Indirect regeneration in MS media supplemented with 2.00 mg/l BAP and 0.1 mg/l NAA (**C** and **D** photographs were taken 40 days after inoculation)

3.2.2.2. Effect of various hormonal supplementations on cotyledon explants

3.2.2.2.1. Variety: BARI surjomukhi-2

Cotyledon explants failed to show any regeneration response in any of the hormone supplemented media. They grew up gigantically upto a certain size, and then stopped their growth and any further regeneration response. As a result, no successful data could be collected from them as they did not regenerate any shoot. They developed both types of callus: translucent, jelly-like callus and also friable callus, along with numerous adventitious rooting and remained in that shape and form for a long time with no successive shoot initiation. In all the hormonal media concentrations, except 1mg/l BAP combined with 0.1mg/l NAA and 2mg/l BAP combined with 0.1mg/l NAA, explants gave indirect regeneration (Table 3.7, Fig. 3.3).

3.2.2.2.2. Variety: BRAC Hysun 33

Similar to BARI surjomukhi 2, cotyledon explants of BRAC Hysun 33 variety did not produce any shoot as well, but grew callus (Table 3.8, Fig. 3.4).

Hence, it can be concluded that the cotyledon explants are not suitable, whereas hypocotyls are excellent and efficient explants for sunflower tissue culture in Bangladesh.

Table 3.7: Effect of different combinations of hormonal concentrations on shoot regeneration from cotyledon explants of BARI surjomukhi-2 variety

Hormones		Responsive explants (%)	Type of regeneration (callus type)
BAP (mg/l)	NAA (mg/l)		
1	0	95.55 (0.58)	Indirect
	0.1	100 (0)	Direct
	0.5	100 (0)	Indirect
	1	91.11 (1.53)	Indirect
2	0	97.77 (0.58)	Indirect
	0.1	80 (2)	Direct
	0.5	91.11 (0.58)	Indirect
	1	84.44 (2.08)	Indirect

SD value is contained within parenthesis.

Table 3.8: Effect of different combinations of hormonal concentrations on shoot regeneration from cotyledon explants of BRAC Hysun 33 variety

Hormones		Responsive explants (%)	Type of regeneration (callus type)
BAP (mg/l)	NAA (mg/l)		
1	-	93.33 (0)	Indirect
	0.1	100 (0)	Indirect
	0.5	97.77 (0.58)	Indirect
	1	88.88 (1.53)	Indirect
2	-	97.77 (0.58)	Indirect
	0.1	77.77 (1.53)	Indirect
	0.5	82.22 (1.53)	Indirect
	1	80 (1)	Indirect

SD value is mentioned within parenthesis.

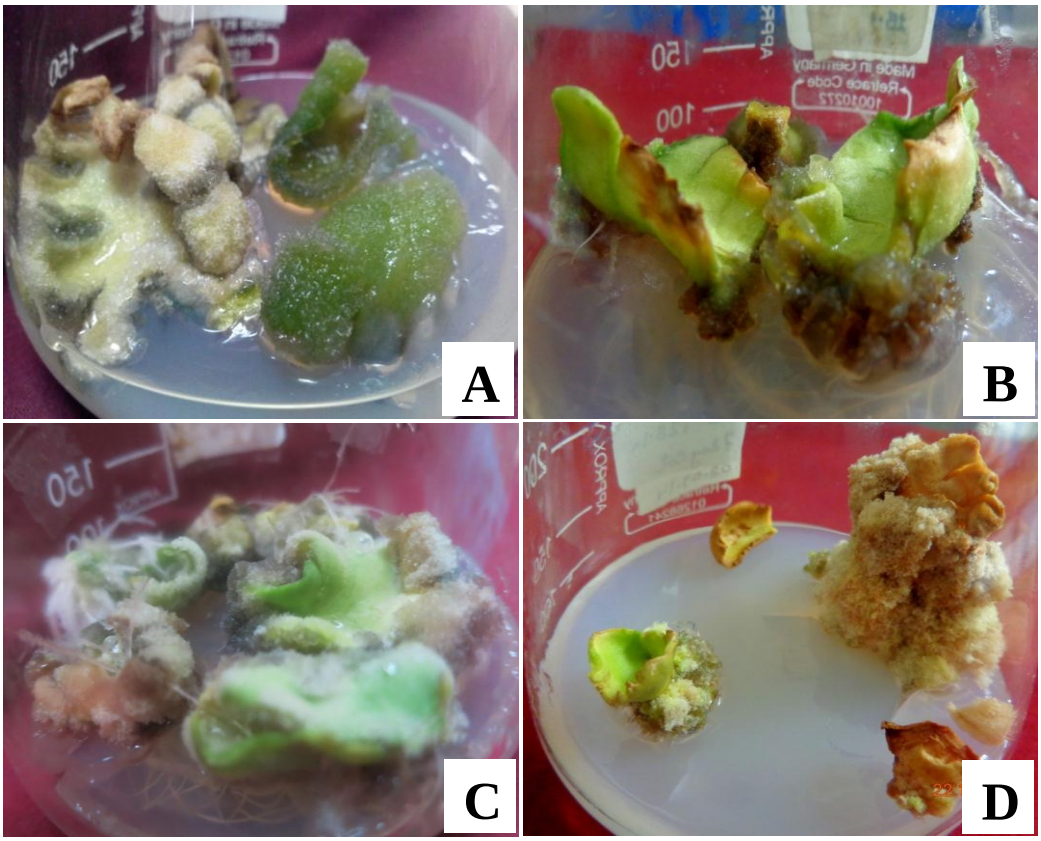

Fig. 3.3: Indirect regeneration from cotyledon explants of BARI surjomukhi 2 variety after 7 days of inoculation. **A.** Callus in MS media supplemented with 2.00 mg/l BAP and 1.00 mg/l NAA. **B-C.** Indirect regeneration in MS media supplemented with 1 mg/l BAP combined with 0.5 mg/l NAA and MS media supplemented with 1.00 mg/l BAP and 1.00 mg/l NAA, respectively. **D.** Friable callus formation in MS media supplemented with 2.00 mg/l BAP alone. (Photographs were taken 45 days after inoculation)

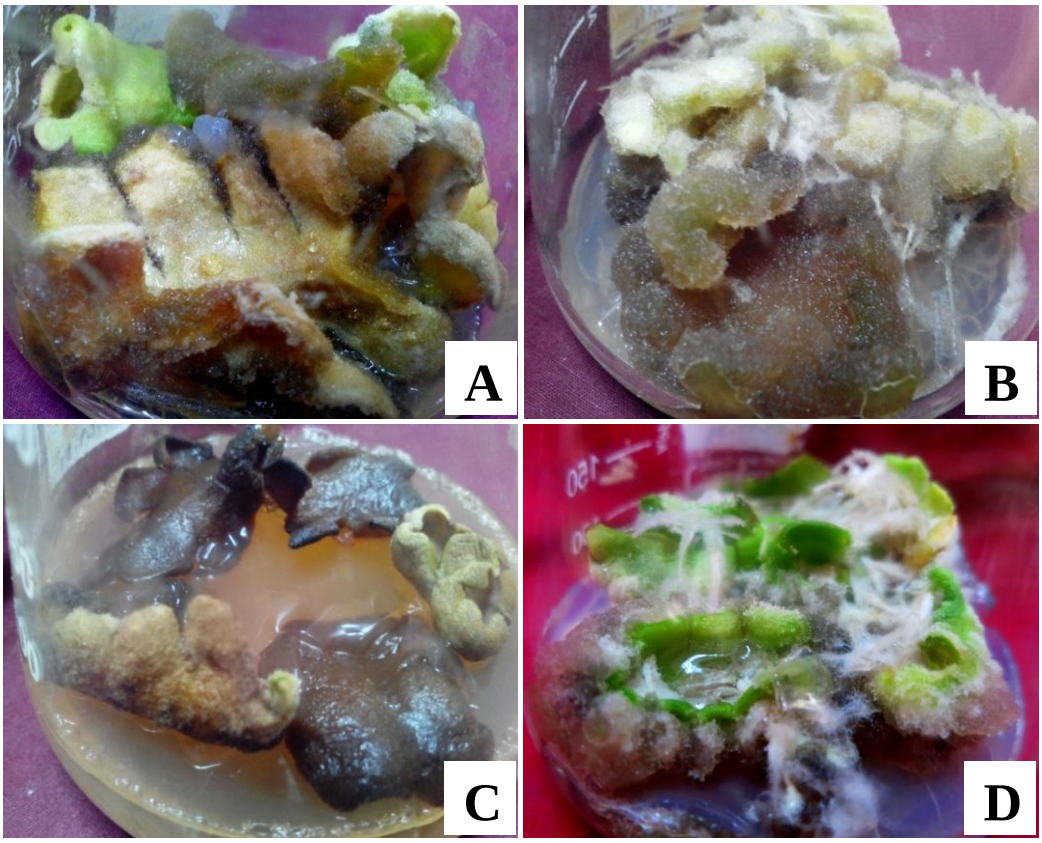

Fig. 3.4: Large callus formation from cotyledon explants of BRAC Hysun 33 variety. **A.** Callus in MS media supplemented with 1.00 mg/l BAP and 0.1 mg/l NAA. **B.** Callus in MS media supplemented with 2.00 mg/l BAP and 0.1 mg/l NAA. Callus eventually died becoming brownish black. **C.** Callus in MS media supplemented with 1.00 mg/l BAP and 0.5 mg/l NAA. **D.** Translucent callus formation and adventitious rooting in MS media supplemented with 1.00 mg/l BAP and 0.5 mg/l NAA. (Photographs were taken 45 days after inoculation)

4. Discussion

Several attempts had been taken to establish a tissue culture protocol for sunflower throughout the world. Since sunflower is recalcitrant to genetic transformation and regeneration when subjected to tissue culture (Pearson *et al*., 2007), and it has many other constrains while being cultivated including diseases (Putnam *et al.,* 1990), microbial infections (Taški-Ajdukoviã and Vasiã, 2005), precocious flowering, poor rooting (Patil *et al*., 1993), hyperhydricity (Fauguel *et al.,* 2008), sunflower is considered to be a challenging species in tissue culture (Pearson *et al*., 2007; Baker *et al.,* 1999; Kothari *et al*., 2002; Hewezi *et al.,* 2002; Radonic et al., 2006).

The present study was aimed to establish a regeneration protocol for two Bangladeshi sunflower (*Helianthus annuus*) varieties, BARI surjomukhi 2 and BRAC Hysun 33. Firstly, the seed sterilization procedures of both the varieties were established. Secondly, the most suitable and efficient explant was determined, along with the suitable age of the explant. Finally, the hormonal supplements and their concentrations in the regeneration media were optimized.

Many different explants, like hypocotyls, cotyledons, leaflets, mature and immature embryo, shoot-tip meristems and roots had been reported to be used for sunflower tissue culture (Elavazhagan *et al.,* 2009; Dagustu *et al.,* 2012; Abdoli *et al.,* 2007; Bayraktaroglu and Dagustu, 2011). Some reports discussed the influences of genotype and explants on callus induction and shoot regeneration, whereas some analyzed the shortening of the generation time in breeding programs using immature embryos (Dagustu *et al.,* 2012). They showed that the effect of genotype, type and age of explants, hormonal type and concentrations and basal medium are important on callus induction and plantlet development (Bayraktaroglu and Dagustu, 2011).

Among all the explants, hypocotyls and cotyledons had been reported to give the maximum response and produce statistically the highest percentage of callus (Elavazhagan *et al.,* 2009; Bayraktaroglu and Dagustu, 2011). So, in this study,

hypocotyls and cotyledon explants were examined to find out the most responsive explant for *in vitro* culture of sunflower. Hypocotyls and cotyledons explants increased their sizes after one week in the regeneration media, and then they gradually formed callus at second and third weeks.

Ozyigit *et al.* (2014) established plant tissue culture systems on MS media supplemented with various plant growth regulators using hypocotyl and cotyledon explants. They observed highest shoot regeneration using hypocotyl explants in MS media supplemented with 1 mg/1 BAP and 0.5 mg/l NAA. Shoot regeneration efficiencies with the cotyledon explants on the same medium were lower in comparison with hypocotyl explants (Ozyigit *et al.*, 2007, 2014; Badigannavar and Kurkurvinnashetti, 1996). They proved that direct shoot regeneration of the cotyledons and young hypocotyls are advantageous explants. In another report, Ozyigit *et al.* (2005) reported that MS media supplemented with 1 mg/l BAP, 0.5 mg/l NAA and 1 mg/l 2,4-D were used for shoot regeneration. Callus was obtained from hypocotyl and cotyledon explants and the callus induction percentage was around 80–92%. MS media supplemented with hormones, like BAP, NAA, 2, 4-D and even GA_3 were also found to enhance plant regeneration (Elavazhagan *et al*., 2009; Ozyigit *et al.*, 2007; Badigannavar and Kurkurvinnashetti, 1996). Mohmand and Quraishi (1994) used LS basal medium supplemented with various concentrations of 2, 4-D, NAA and BAP in order to produce green and compact callus but no plant shoots were obtained.

The findings of Ozyigit and his colleagues (2014) partially resemble the data determined in the present study. It depicts that the highest shoot regeneration was observed using hypocotyl explants on MS media supplemented with 1 mg/1 BAP and 0.5 mg/l NAA. In this present study, it was determined that MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA is the best combination for 7 day old hypocotyls explants of both BARI surjomukhi-2 and BRAC Hysun 33 varieties. The *in vitro* regenerated shoots did not develop radicular system on the regeneration medium

If the two varieties (BARI surjomukhi-2 and BRAC Hysun 33) used in this present study are compared, it will be found that both the varieties give similar results in showing their shoot regeneration efficiency in specified hormonal media concentrations. Hypocotyl

explants of both the varieties developed healthy shoot in MS medium supplemented with 1mg/l BAP in combination with 0.1mg/l NAA, MS medium supplemented with 1 mg/l BAP in combination with 0.5mg/l NAA and MS medium supplemented with 2mg/l BAP alone. In BARI surjomukhi-2 variety, MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA gave the best shoot regeneration response (60%), with the best grown shoot that grew up to 2.08 cm within 30 days of regeneration. Similarly, in BRAC Hysun 33 variety, MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA gave the best shoot regeneration response (53.3%), with a shoot of length 1.61 cm within 30 days of regeneration. This clearly showed that MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA is the best combination for developing an efficient shoot from a 7 day old hypocotyl explant. When genotypes are compared, BARI surjomukhi-2 was found to respond better than BRAC Hysun 33 variety, as it gave better result in terms of both shoot length and percentage of shoot regeneration response. Therefore, there is no difference of hormonal requirement among these varieties. However, the response is genotype dependent.

This study has established the protocol of tissue culture of sunflower using hypocotyl explant. In future, rooting has to be established. This *in vitro* regeneration protocol of sunflower can be used then for the improvement of gene transfer systems in the sunflower varieties and hence, their genetic characteristics can be modified to enhance better productivity, which will be tolerant to the common constrains and provide nutritional quality of the oil extracted from sunflower.

5. References

Ahmad, R., Jabeen, N. (2009). Demonstration of growth improvement in sunflower (*Helianthus annuusL.*) by the use of organic fertilizers under saline conditions. *Pakistan Journal of Botany*, 41 (3): 1373-1384.

Aminifar, J., Galavi, M. (2014). Effective traits on yield improvement of sunflower (*Helianthus annuus*L.) during the last decades. *Sci. Agri.*1 (2): 73-75.

Aurori, A.C. (2011). Studies Regarding Some Factors Involved in Sunflower Protoplast and Tissue Explant Organogensis and Somatic Embryogenesis. Cluj,Napoca: Babeş-Bolyai University .

Badigannavar, A.M., Kururvinashetti, M.S. (1998). Callus induction and morphogenesis from somatic tissue cultures of sunflower - *Helianthus annuus L. Karnataka Journal of Agricultural Sciences*, 11, str. 63-66.

Bakht, J., Shafi, M., Yousaf, M. and Shah, H.U. (2010). Physiology, Phenology and yield of sunflower (autumn) as afected by NPK fertilzer and hybrids. *Pakistan J. Bot.*, 42(3): 1909-192.

Breccia, G., Vega, T., Felitti, S.A., Picardi, L., and Nestares, G. (2013). Differential expression of acetohydroxyacid synthase genes in sunflower plantlets and its response to imazapyr herbicide. *Plant Science* 208, 28-33.

Burke, J. M., K. A. Gardner and L. H. Rieseberg. 2002. The potential for gene flow between cultivated and wild sunflower (*Helianthus annuus*) in the United States. Am. J. Bot. 89(9): 1550-1552.

Burke, J.M. and L.H. Rieseberg. 2003. Fitness effects of transgenic disease resistance in sunflowers. Science 300:1250.

Dağüstü, M., Bayraktaroglu, N. (2011). Influences of Genotype and Explant on Callus Induction and Shoot Regeneration in Sunflower (*Helianthus annuus L.*). *Journal of Agricultural Science and Technology*, 5(2), 33.

Dagustu, N., Bayram, G., Sincik, M., & Bayraktaroglu, M. (2012). The Short Breeding Cycle Protocol Effective On Diverse Genotype Of Sunflower (*Helianthus annuus* L.). *Turkish Journal of Field Crops*, 17(2), 124-128.

Dedio, W. (2005). The Biology of *Helianthus annuus L.* (Sunflower). Plant Biosafety Office. Canadian Food Inspections Agency. Online at http://cera-gmc.org/docs/decdocs/05-209-009.pdf

Elavazhagan, T., Jayakumar, S., Chitravadivu, C. and Balakrishnan, V.(2009). In vitro culture and cytological studies on *Helianthus annus L. Bot. Res. Int.,* 2: 258-262. Fischer, C., Klethi, P., & Hahne, G. (1992). Protoplasts from cotyledon and hypocotyl of sunflower (*Helianthus annuus* L.): shoot regeneration and seed production. *Plant Cell Reports*, 11(12), 632-636.

Gurel, E. and Kazan, K. (1998). Development of an efficient plant regeneration system in sunflower (*Helianthus annuus L.*). *Turkish J. of Botany*, 22: 381-387.

Heiser, C. B., Jr. Taxonomy of Helianthus and Origin of Domesticated Sunflower. 1978 In: Sunflower Science and Technology. Agron. 19. pp. 31-53. Ed. Carter, J. F.

Jahangir, A. A., Mondal, R.K., Nada, K., Afroze, R.S., and Hakim, M.A. (2006). Response of Nitrogen and Phosphorus Fertilizer and Plant Spacing on Growth and Yield Contributing Characters of Sunflower. *Bangladesh J. Sci. Ind. Res.*, 41(1-2): 33-40.

Knittel, N., Gruber, V., Hahne, G., &Lenee, P. (1994). Transformation of sunflower (*Helianthus annuus* L.): a reliable protocol. *Plant Cell Reports,* 14(2-3), 81-86.

Liu, H., Xie, X., Sun, S., Zhu, W., Ji, J., & Wang, G. (2011). Optimization of'Agrobacterium'-mediated transformation of sunflower (*Helianthus annuus L.*) immature embryos.

Mohmand, A. S., & Quraishi, A. (1994). Tissue culture of sunflower. *Pakistan Journal of Agricultural Research*, 15(1), 153-160.
Online address: *http://www.sunflowernsa.com/all-about/*

Ozyigit, I. I., N. Gozukirmizi and B. D. Semiz (2007). Genotype dependent callus induction and shoot regeneration in sunflower (*Helianthus annuus L.*). *African Journal of Biotechnology*. 6(13):1498-1502.

Özyiğit, I.I., Bajrovic, K., Gözükırmızı, N. and Semiz, B.D. (2002). Direct Plant Regeneration from Hypocotyl and Cotyledon Explants of Five Different Sunflower Genotypes (*Helianthus Annuus L.*) from Turkey, *Biotechnology & Biotechnological Equipment*, 16:1, 8-11.

Ozyigit, I.I., Gozukirmizi, N., Semiz, B.D. (2006). Callus induction and plant regeneration from mature embryos of sunflower. *Russ. J. Plant Physiol*, 53 (4): 621-624.

Patil, M. S., Ramaswamy, N. M., and SreeRangasamy, S. R. (1993). In vitro flowing in sunflower (H.annuusL.). *Curr.Sc*., 65: 565 – 566.

Pearson, C.H., K. Cornish, C.M. McMahan, M. Whalen, D.F.J. Rath, N. Dong, and S. Wong. (2007). Using peat pellets in liquid media to root sunflower tissue culture plants. *Issues in new crops and new uses*, Eds J. Janick and A. Whipkey, 78–81. Alexandria, VA: ASHS Press.

Putnam, D.H., Oplinger, E.S., Hicks, D.R., Durgan, B.R., Noetzel, D.M., Meronuck, R.A., Doll, J.D., and Schulte, E.E. (1990). Sunflower. Alternative Field Crops Manual.

Sankara, Rao., K., Rohini, V.K. (1999) Agrobacterium-mediated transformation of sunflower (*Helianthus annuus L.*): a simple protocol. *Annals of Botany*. 83: 347–354.

Shin, D.H., Kim, J.S., Kim, I.J., Yang J., Oh, S.K., Chung, G.C., and Han, K.H. (2000). A Shoot Regeneration Protocol Effective on Diverse Genotypes of Sunflower *(Helianthus annuus L.). In Vitro Cellular and Developmental Biology-Plant*, vol. 36, pp. 273–278.

Sunflower Production Guide. NSA and Manitoba Agriculture and Food Publication.

Taski-Ajdukovic,K..J., Vasic, D.M. (2005). Different sterilization methods for overcoming internal bacterial infection in sunflower seeds. *Proc. Nat. Sci. MaticaSrpska*, Novi Sad. 109:59-64.