In vitro Culture Establishment from Cotyledon and Embryonic Axis Explants of Two Bangladeshi Sunflower (*Helianthus annus* L.) Varieties

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

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Dedicated to

My Parents

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled **"*In vitro* Culture Establishment from Cotyledon and Embryonic Axis Explants of Two Bangladeshi Sunflower (*Helianthus annus* L.) Varieties"** submitted by the undersigned has been carried out under 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 or diploma.

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List of Abbreviation

The following abbreviations have been used throughout the text.

2,4-D	2,4-Dinitrophenylhydrazine
BAP	6-Benzylaminopurine
BARI	Bangladesh Agricultural Research Institute
EDTA	Ethylenediaminetetraacetic acid
HCL	Hydrochloric Acid
Kn	Kinetin
MS	Murashige and Skoog (1962) medium
NAA	Napthalene acetic Acid
NAOH	Sodium Hydroxide
TDZ	Thidiazuron

Abstract

Sunflower is considered as source of cholesterol free edible oil. Because of cross pollinating nature of this plant, it is difficult to identify true breeding homozygous line, thus, breeding becomes intricate. Besides, due to biotic and abiotic stresses, development of this crop is urgent. This study was aimed to develop a potential alternative to improve qualitative and quantitative production of this crop through basic tissue culture regeneration. Several attempts with various explants have already discovered in past but no initiative was reported for Bangladeshi sunflower varieties. In present study, optimum seed sterilization method; the efficiency of different explants; effect of different growth hormones in different combinations and concentrations and rooting efficiency of initiated shoots were demonstrated for two farmer popular Bangladeshi sunflower varieties, namely BARI Surjomukhi 2 and BRAC Hysun 33. The seeds were washed with 70% ethanol for 3 minutes followed by vigorous hand shaking with 14% Clorox for 20 minutes. To collect explant, named embryonic axis, testas were removed and 3 mm piece from proximal portion of the seed was excised and inoculated in different combinations and concentrations of hormone supplemented media. For explant cotyledon, sterilized seeds were inoculated in germination media to obtain different ages seedlings, thus five, seven and nine days cotyledon explant was collected. In case of explant embryonic axis, BRAC Hysun 33 variety showed maximum (58.33%) regeneration in 1.0 mg/l BAP with 0.1 mg/l NAA hormone supplemented media while in BARI Surjomukhi 2 variety, 2.0 mg/l BAP gave highest (46.67%) regeneration. Elongated shoots were inoculated in 0.2 mg/l IBA containing media for root induction but further studies are needed to optimize rooting media concentration. The second explant, cotyledon remained non-regenerative in both varieties. Further studies need to be done to develop regeneration system from this explant. In future reproducibility of this protocol using embryonic axis needs to be evaluated.

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Chapter 1: Introduction

1. Introduction

Sunflower (*Helianthus annus* L.) is a member of Asteraceae family. It is an annual, short duration (125-130 days) plant. The botanical name of sunflower is *Helianthus*, derived from Latin words Helios (Sun) and Anthos (Flower). The name itself resembles the structure of flower; round yellow head signifies sun with rays. It is believed that the flower head rotates with the rotation of sun. According to National Sunflower Association, this important agricultural crop was originated in North America (present day Arizona and Mexico) among American Indian tribes around 3000 BC. According to United States Department of Agriculture, Ukraine, Russia, European Union and Argentina are the top four producers of sunflower which account for 70% of global production (National Sunflower Association, 2014).

1.2 Description of sunflower plant

The average height of sunflower plant is 3 to 12 feet in length. The flower of sunflower plant blooms heliotropically, sometimes droopily. It is a composite flower.

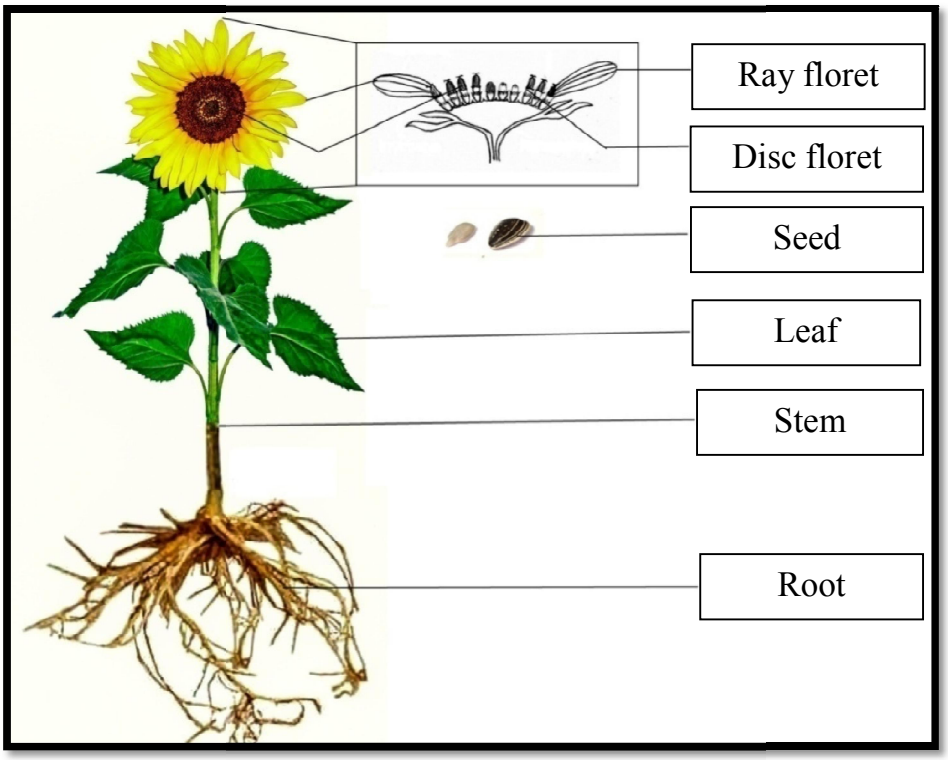

Fig. 1.1: A typical sunflower plant

The inflorescence is usually known as floral head or capitulum which can be of 6 to 15 inches in diameter depending on variety. It is a collection of 1000 to 2000 tiny flowers which are arranged in a spiraling pattern. Two types of floret named ray floret and disc floret typically form the whole flower. Ray florets are sterile as they lack both stamen and pistil. They mainly serve the purpose of alluring insects for pollination. Each disc floret possesses both male and female reproductive organ which provides reproductive opportunity for the plant. Disc florets are hermaphroditic and protandrous. These individual disc flowers produce their own seeds which are located in achene. They usually vary in size from 7 to 25 mm in length and 4 to 13 mm in width. Sunflower has thick, massive stem which can be up to 10 feet tall, depending on the number of internodes. The stem usually grows erectly and often unbranched. Sunflower plants have usually broad dark green color leaves. They are coarsely toothed. Each leaf has petiole and contains three main veins. Sunflower plant has long taproot system of around 4 feet long with abundant lateral surface roots.

1.3 Suitable growing condition

Sunflower is recognized all over the world for its immense beauty. It is a C3 plant. Its cultivation time is from December through April, around 125 to 130 days. According to the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (2010), it takes around 11 days from planting to emergence, 33 days from emergence to head visibility, 27 days from head visibility to first anther, 8 days from first to last anther, and 30 days from last anther to maturity. Though sunflower can grow in sandy or loamy soil, it cannot be grown in water-logged soil. The optimum temperature for growth is 23 to 28°C. As they have long taproots, it's better to plant sunflower in well dug, loose well draining soil. Sunflowers bloom in slightly acidic to somewhat alkaline pH 6.0 to 7.5. Once sunflower seeds are planted, they can tolerate heat and drought. Sunflower plants are vulnerable to summer winds and rain. They grow best in areas where direct sunlight is available (6 to 8 hours per day) (http://www.almanac.com/plant/sunflowers date: November 12, 2014).

1.4 The reproductive biology of sunflower

Sunflower is a monoecious plant and they reproduce sexually through cross pollination. Anther surrounds the style of the carpel and each anther contains pollens remain viable for several days. As the anther keeps growing, the corolla opens up distally to accommodate the emerging anther. Eventually all the pollens are pushed out of the anther. Primarily bees are considered as prime pollinator for transferring pollens from anther of other flowers. At the end of flowering, each flower in the head produces its own seed.

1.5 Importance of sunflower

The sunflower is cultivated globally as oil seed crop which yield one of the world's most important sources of cholesterol free edible oil. Recently, scientists are focusing on applying sunflower in cancer research as the plant contains histamine, a chemical found predominantly in leukemia patient.

1.5.1 Food value of sunflower

Sunflower seeds are consumed as food either by drying or roasting. The plant itself can be used as silage and green-manure crop. Moreover, after collecting oil from seed, the remainder is also used as fodder and poultry feed containing 50 to 60% protein (https://tjdemogarden.wordpress.com/2011/12/16/plant-profile-sunflower/ date: November 6, 2014). Sunflower seeds are considered as the best source of vitamin E (National Sunflower Association, 2014). The kernel of sunflower is the source of oil which contains around 45 to 55% oil. The content of saturated fatty acid in sunflower oil is lower than 10%. It is almost free from linolenic acid (omega 3). This oil is used as edible oil in form of margarine, salad dressing oil and cooking oil.

1.5.2 Medicinal value of sunflower

Sunflower flower is rich in carotenoids which help to prevent heart disease. Because of possessing diuretic and expectorant properties, sunflower seeds can be used to treat respiratory ailments like coughs, bronchitis malaria etc. Decoction prepared from sunflower root can be used as remedy from rheumatic aches and pains. Sunflower leaves can be used to made poultices which can be applied externally on swellings,

sores, snakebites and spider bites (https://tjdemogarden.wordpress.com/2011/12/16/plant-profile-sunflower/ date: November 6, 2014).

1.5.3 Industrial use of sunflower

Sunflower oil is used in cosmetics industry to produce soap, lotion etc. The ray florets of sunflower can be used for producing dye, especially yellow dye. Recent studies on sunflower discovered that the pith of sunflower stem is the lightest substance known with specific gravity of 0.028. This finding has essentially increased the commercial value of sunflower. This pith is presently used in manufacturing life saving appliances as well as slides for microscope (https://tjdemogarden.wordpress.com/2011/12/16/plant-profile-sunflower/ date: November 6, 2014).

1.6 Limitations of sunflower cultivation

Sunflower is considered as a high-risk crop because of probable losses from diseases caused by fungal, viral and bacterial attack. According to National Sunflower Association, around 30 diseases have been identified but most of them do not interfere with the yield of sunflower. Among those, Downy Mildew (*Plasmopara halstedii*), Sunflower Rust (*Puccinia helianthi*), Phomopsis grey spot or stem cancer (*Diaporte helianthi*), Rhizopus head rot (*Rhizopus arrhizus*), Grey mould (*Botryotinia fuckeliana*), Alternaria blight (*Alternaria helianthi*), Phoma black stem (*Phoma macdonaldii*) diseases are most common (National Sunflower Association, 2014). Besides these microbial attacks, sunflower seeds are also destroyed by moths, weevils, black maize beetle, astylus beetle, American bollworm etc. (National Sunflower Association, 2014).

1.7 Production and demand of sunflower in Bangladesh

According to the latest estimate from United Nations, the current population of Bangladesh is 158.5 millions and annual growth rate is 1.6% (https://www.cia.gov/library/publications/the-world-factbook/fields/2002.html date: November 11, 2014). From this data it can be easily assumed that population is increasing very fast day-by-day. With the increase of population, the demand for oil is also increasing because of economic advancement, changes in food habits and rising income of people in rural areas. As stated by Malaysian Palm Oil Council (MPOC), more than 1 million metric tonnes of palm oil are required in Bangladesh to meet up the domestic demand but the production is stuck at only 1.80 lakh tonnes per year. Already we are importing a huge quantity of oil from foreign countries. In 2013 Bangladesh imported 17.77 lakh tonnes of edible oil which is 10.18% higher than the previous year and highest amount so far (http://www.thedailystar.net/business/edible-oil-fat-imports-rise-to-new-high-7106 date: November 15, 2014).

1.8 Cultivation of sunflower crop in Bangladesh

The seed of sunflower is considered as a source of cholesterol free edible oil, still now cultivation of sunflower as oilseed crop is not familiar in whole country. There is limited initiative taken by the Government of Bangladesh to do research on sunflower as economic crop. However, in Bangladesh it is known as ornamental plant in winter season. But the good news is that southern regions of our country have become interested in sunflower cultivation as oilseed crop since last few years. It is gradually gaining popularity. Women are also getting involved in the cultivation of sunflower and thereby increasing women empowerment which ultimately boost up family income. With the help of BRAC, the largest NGO in the world, residents of Patuakhali, Bagerhat, Satkhira, Jhalakati etc. have started cultivating sunflower. BRAC took the preliminary initiative in 1998 but due to lack of fair market value, the cultivation got closed (http://www.thefinancialexpress-bd.com/old/index.php?ref=MjBfMDRfMDdfMTNfMV85MV8xNjU2MDk date: November 5, 2014). After that BRAC has started fresh to expand sunflower cultivation. With a view to promote adaptive technologies among farmers at Sidr (2007) affected area, a two year project “Crop Intensification for Achieving Food Sufficiency in the Coastal Region of Bangladesh” has been taken by them

(http://blog.brac.net/tag/crop-intensification-for-achieving-food-sufficiency-in-the-coastal-region-of-bangladesh/ date: December 1, 2014). Around 1000 hectares of land in 24 upzillas of 17 districts have been brought under sunflower cultivation and they are expecting to obtain 2000 tonnes of oil from these (http://www.thefinancialexpress-bd.com/old/index.php?ref=MjBfMDRfMDdfMTNfMV85MV8xNjU2MDk date: November 5, 2014). In October 2013, BRAC has launched Sunflower oil under the brand name of "Shufola" utilizing sunflower cultivated in southern region of Bangladesh (https://www.cia.gov/library/publications/the-world-factbook/fields/2002.html date: November 11, 2014). In 2012, CSISA-CIMMYT (Cereal Systems Initiative for South Asia-International Maize and Wheat Improvement Centre) Khulna hub has taken initiative for Sunflower production in saline and water limited condition in southern Bangladesh. They collaborated with Bangladesh Agricultural Research Institute (BARI) and Khulna University with the aim of utilizing southern part of Bangladesh which remains uncultivated in the winter rabi season due to rain or irrigation water limitations and soil salinity restrictions (http://www.khulnanews24.com/index.php/local-news/255-initiatives-for-sunflower-production-in-saline-environment-of-southern-bangladesh.html date: November 10, 2014).

1.9 Biotechnological approaches for sunflower

Sunflower is a cross pollinating plant. Because of this property, a diverse range of genotype is observed in sunflower plant. It makes breeding of sunflower quite difficult because true breeding homozygous line becomes difficult to identify. Moreover, this genetic variation causes hitches in applying any biotechnology technique (Bayraktaroglu *et al.* 2011). Guerel and Kajan (1998) suggested that difficulties created by genetic variation in plant regeneration could be reduced to some extent by either utilizing a large number of explants or developing a method for screening plant material to identify those with a low potential for organogenesis in heterozygous plants like sunflower. Conventional improvement methods (introduction, selection and breeding) are used till dates but integration of plant regeneration technique/tissue culture method/plant biotechnological approaches will accelerate both the production of sunflower with desirable characteristics and utilization of broad genotypic variability (Mohmand *et al*. 1994).

It is important to produce homozygous lines for the production of haploids which can be used in breeding method (Mohmand *et al*. 1994). Callus formation from anther of many species has been reported (Bohorova *et al*. 1985).

Though *Helianthus annus* is globally ranked second to soybean (*Glycine max*) as vegetative oil producing crop (Ozyigit *et al*. 2006), but number of studies on sunflower is very limited (Mohmand *et al*. 1994). Moreover, it is regarded as most recalcitrant crop which makes transformation and regeneration through tissue culture more difficult (Patil *et al*. 1993; Badigannavar and Kururvinashetti 1996; Pearson *et al.* 2007; Bayraktaroglu *et al.* 2011).

Before going to apply any transformation technique, it is an essential step to establish an efficient and reproducible tissue culture and plant regeneration protocol (Rao *et al.* 1998; Abdoli *et al*. 2007). In the beginning, prime focus was on delivering desirable traits among different cultivars via conventional breeding method (Rao *et al.* 1998); then tissue culture came under investigation till 1980s but effective regeneration protocol is still hard to establish (Liu *et al*. 2011). However it is deduced that minimization of regenerative tissue culture by applying transformation would be beneficial to sunflower development (Rao *et al.* 1998). As a consequence, integration of desired gene into the genome of sunflower has now taken the position. Though genotypic limitation is a great barrier in *in-vitro* regeneration of sunflower, application of transformation is not getting limited and everyday new approaches are being discovered to overcome these barriers (Bayraktaroglu *et al.* 2011). The primary objectives of sunflower biotechnology are:

1. to enhance resistance to insects
2. to improve disease resistance (Bayraktaroglu *et al.* 2011)
3. to alter the oil composition without disturbing advantageous traits already present (Rao *et al.* 1998)
4. to boost up accumulation of diverse range of heavy metals as it is already reported to be used for soil remediation (Ullah *et al.* 2011).

Till date, several explants are used from different ages of sunflower seedlings of various commercial varieties for the establishment of reproducible tissue culture protocol. Mostly conducted studies focused on cultured stem piths, immature embryos, mature embryos, anthers, buds, meristems, leaves, hypocotyls cotyledons and cotyledonary petioles as explants for both direct and indirect regeneration (Ozyigit *et al*. 2002, 2006; Guerel and Kajan 1998). But Guerel and Kajan (1998) suggested determining the right source of plant material rather than determining the right treatment during optimizing a regeneration protocol is crucial in sunflower tissue culture.

In case of embryo, most of the studies are conducted with immature embryos and they showed better regeneration on Gamborg's B5 medium with high sugar content (Ozyigit *et al*. 2006). Though genotype has remarkable effect on culture, immature embryo is the only explant which constantly gives regenerative culture in all genotypes of sunflower (Witrzens *et al*. 1988; Bayraktaroglu *et al.* 2011). However, it is also identified that shoot regeneration from the callus of both mature and immature embryo is highly dependent on genotypic variance while explant type and culture condition are maintained similar for explored cultivars (Ozyigit *et al*. 2006; Liu *et al*. 2011).

Mohmand and Quraishi (1994) established green and compact callus formation in Linsmaier and Skoog's (LS) medium supplemented with 0.5-1.0 mg/l BAP and 2.0 mg/l NAA from hypocotyl and cotyledons which have been collected from 21 days old seedlings. They found that with the increase of BAP and 2, 4-D levels, callus formation has decreased in both the explants. In addition, they reported to find properly formed embryoids in the callus, formed from both the explants but the embryoids do not produce any plant.

Sunflower regeneration could be possible through either organogenesis or somatic embryogenesis (Liu *et al*. 2011; Abdoli *et al*. 2007). It is found in many previous studies that several parameters, like, organogenesis, high variability between genotypes, age of explant, culture condition and interaction with gelling agent, plant own hormones, reciprocity, cytoplasmic effect and nucleocytoplasmic interaction affect the *in-vitro* regeneration of sunflower (Ozyigit *et al*. 2002 and 2006; Abdoli *et al*. 2007; Liu *et al*. 2011; Bayraktaroglu *et al.* 2011). Haq *et al.* (2013) also reported

that the morphological, physiological and chemical characteristics of sunflower are radically affected by salinity (NaCl) stress like any other C3 crops. This consequence is observed beyond genotypes under all levels of salinity.

Somatic embryogenesis and plant regeneration is also reported to be successful in soybean (*Glycine max* L.) using seven day old embryonic axes (Kumari *et al*. 2005).

Ozyigit and his colleagues (2006) demonstrated regeneration protocol using mature embryos of 5 Turkish varieties of sunflower. They showed that mature embryos produce friable callus on Murashige and Skoog's (MS) media, enriched with 1.0 mg/l 2, 4-D. And in regeneration media, MS supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA, this callus shows regeneration within 2 to 3 weeks. They also illustrated that MS media with/without 1.0 mg/l IBA both stimulates rooting in regenerated shoots but thicker and denser roots are obtained in hormone supplemented media.

In another study of Ozyigit and his co-workers (2002 and 2007), reproducible tissue culture protocol was established using cotyledon and hypocotyl as explants from previously used Turkish varieties. Cotyledons and hypocotyls collected from 10 days old seedlings were subjected to both direct and indirect regeneration. For direct regeneration, explants were first cultured on Murashige and Skoog's (MS) media containing 1.0 mg/l BAP and 0.5 mg/l NAA. On the other hand, for indirect regeneration, explants were first subjected to MS media supplemented with 1.0 mg/l 2,4-D for callus induction. In both the cases, they came to a conclusion that hypocotyl explants were more capable in regeneration response compared to cotyledons.

Being considered as one of the important source of oil, *Brassica* spp. is also subjected to *in vitro* plant regeneration technique. Mollika *et al.* (2011) established genotype independent regeneration protocol for three Bangladeshi local varieties of mustard successfully. They used cotyledonary leaf with or without petiole, petiole without cotyledon, cotyledonary node, hypocotyls, epicotyls and leaf segment etc. as explants.

Using cotyledonary node as explant, Kumari *et al.* (2013) demonstrated that direct organogenesis can be done in soybean using MS medium fortified with BAP and TDZ with the different concentrations ranging from 5-25 μM. For indirect organogenesis, they used half seed excluding embryonic axis as explant under hormonal supplement

of BAP, 2,4-D and NAA in the different concentrations ranging from 5-25 µM. For shoot proliferation, they used BAP and Kn. Use of cotyledonary node as explant in groundnut (*Arachis hypogaea* L.) was also outlined by Venkatachalam and Kavipriya (2012).

Correspondingly, Badigannavar and Kururvinashetti (1996) also worked with hypocotyls and cotyledons as explant using 3 Indian varieties of sunflower. They reported ease of callus culture generation but difficulties of regeneration from that callus in most responsive genotypes. Moreover, Badigannavar and Kururvinashetti (1996) and Ozyigit *et al*. (2002 and 2007) also mentioned genotypic effect on regeneration. Both of these groups found hypocotyls to be genotype independent but cotyledons significantly dependent on genotype in callus induction as well as for regeneration. Guerel and Kajan (1998) evaluated that somatic embryogenesis could be initiated from the proximal part of cotyledon explants without formation of callus. Successful *in vitro* regeneration protocol for soybean was also reported by Joyner *et al.* (2010). They used mature cotyledons and embryo for indirect organogenesis.

It is demonstrated that sucrose is the best carbohydrate source in any culture medium. Presence of other carbohydrate sources such as, glucose, maltose, mannitol generate negative results most of time (Witrzens *et al*. 1988; Liu *et al*. 2011). But recent studies showed osmotic potential of the medium determines the morphogenic response of the *Helianthus annuus* embryo. In such case, mannitol might be used as osmotic agent (Konieczny *et al*. 2007).

Witrzens *et al*. (1988) evaluated that auxin should be absent during 1st stage of callus induction as it eventually restrain the regeneration of plantlets. They also mentioned about formation of premature flower-heads from immature embryo and occurrence of vitreous plants. They showed that use of phenolic glycosides like phloridzin, naringin and esculin hydrate significantly reduced or eliminated the above mentioned problems.

Another such case regarding organogenesis was carried out by Abdoli *et al*. (2007). They focused on reducing the event of hyperhydric shoots in sunflower during organogenesis by using three agar concentrations. Though hyperhydricity is causing failure in transplantation of sunflower, it can be prohibited in various ways like,

enhanced vessel aeration, dropping cytokinin levels, rising agar concentration and altering the concentration of medium constituents. In this study, Abdoli and his colleagues (2007) explained the effect of agar concentration on organogenesis and hyperhydricity of sunflower. They critiqued that occurrence of hyperhydric shoots is reciprocally related to agar concentration of the media. They proposed that the consistency of the medium might affect the availability of water, plant growth regulators and nutrients to plant, thus, cause hyperhydricity.

Konieczny and his co-workers (2007) demonstrated that culture of zygotic embryos of *Helianthus annuus* could be induced to organogenesis and somatic embryogenesis. This induction could be accelerated by altering only sugar concentration in the medium. Low sucrose level induces shoot formation whereas high sucrose quantity persuades somatic embryo development. They identified active oxygen species (AOS) like, hydroperoxyl radical, superoxide radical, hydroxyl radical, hydrogen peroxide etc. and considered them as linkers between osmotic properties of the medium and plant regeneration. During oxidative stress, plant cells developed a complex system of antioxidant enzymes like, superoxide dismutases, catalases, and guaiacol dependent peroxidases to control the level of AOS. Finally, the impact of these antioxidant enzymes during early stages of organogenesis and somatic embryogenesis in further morphogenesis of zygotic embryos of sunflower has been assessed in study of Konieczny *et al*. (2007).

Bayraktaroglu *et al.* (2011) worked with three explants (hypocotyls, cotyledon, and root) obtained from 4 day old seedlings of sunflower in Embryo Induction Media (EIM) supplemented with 1% BAP, 1% NAA and 0.1% GA_3. They found that root explant formed watery callus whereas cotyledon explants gave compact callus along with shoot formation zone. They deduced that root and hypocotyls explants formed highest callus. Moreover they stated about genotypic dependency during rooting on explant.

1.10 Objective

In Bangladesh, previously no report has been found on sunflower. But sunflower can enhance our healthy lifestyle in addition to aesthetic value. To solve edible oil problem, sunflower can serve as a great source. However, due to cross pollinating nature, it is highly heterozygous which makes considerable challenge to obtain true breeding homozygous line. Under this circumstance, plant biotechnological approach may be an option for improvement. For such approaches, establishing reproducible tissue culture protocol is the basic step. Hence, two farmer popular local varieties BRAC Hysun 33 and BARI Surjomukhi 2 were subjected to *in vitro* regeneration in this study. Cotyledon and embryonic axis were used as explants for both the varieties. Therefore, the aim of this study is to-

1. establish optimum seed sterilization methodology with highest germination rate
2. find out efficient explants for BRAC Hysun 33 and BARI Surjomukhi 2 varieties
3. determine the most suitable age and size of explant for regeneration
4. determine optimum hormone supplementation for shoot regeneration
5. check the capacity of root induction in regenerated shoots

Chapter 2:
Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Plant material

In the current experiment, two farmer popular locally grown sunflower (*Helianthus annuus* L.) varieties were used. The seeds were collected from two institutions for this experiment BRAC and BARI. Seeds were preserved at 4°C temperature in Plant Biotechnology Laboratory, BRAC University, Mohakhali, Dhaka, Bangladesh.

2.1.1.1 Bari Surjomukhi 2

This black colored seed was collected from Bangladesh Agricultural Research Institute (BARI) Joydebpur Gazipur. It was released in 1982 and takes usually 90-100 days for maturation. The sowing and harvesting time of this variety are Mid December-mid January (Rabi) and March-April respectively. This seed provides 125-140 cm long plant with unicum broad leaf. The major constraints of cultivation of this variety are leaf blight disease, hairy caterpillar pest and parrot (Fig 2.1).

2.1.1.2 BRAC Hysun 33

This bluish seed was collected from BARDC (BRAC-Agricultural Research and Development Centre) Joydebpur, Gazipur. This seed has a life expectancy of 110-115 days. The sowing time of this variety is October to December but shows best cultivation result in mid October to mid November. Germination rate of this seed is 100% and it has 8 ds/mol salt tolerance. This plant grows well in loamy or sandy loam soil and needs 2 to 3 times irrigation for better yield. The plant grows up to 90 to 110 cm with its flower having diameter of 21 to 22 cm. Each flower approximately contains 800 to 1200 seeds. This variety harvests around 1.4 to 1.5 ton per acre. The major constraint of this plant is water logging which ultimately leads to Downy Mildew disease (Fig 2.1).

Fig 2.1: A. BARI Surjomukhi 2 variety **B.** BARI Surjomukhi 2 variety

2.2 Methods

2.2.1 Media preparation

In this study, full strength MS medium (Murashige and Skoog 1962) was used. For seed germination, only MS medium was used whereas for shoot induction and development, regeneration medium was prepared by adding different concentration and combination of BAP and NAA to MS medium.

To prepare MS medium, several constituents are required in different quantities. For convenience, separate stock solutions of macro-nutrients, micro-nutrients, organics, Na-Fe-EDTA stock solutions and hormone solutions were prepared before media preparation.

2.2.1.1 Preparation of stock solutions required for MS medium

To prepare the appropriate stock solutions required for MS media preparation, different components were added to ddH_2O according to following calculations:

Table 2.1: Components of Macro nutrient stock solution (10X strength; 1 Liter):

Components	**Amount (mg/L)**
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.2H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170

Table 2.2: Components of Minor salts stock solution (100X strength; 1 Liter):

Components	**Amount (mg/L)**
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Table 2.3: Components of Iron EDTA stock solution (100X strength; 1 Liter):

Components	**Amount (mg/L)**
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3

Table 2.4: Components of Organics stock solution (100X strength; 1 Liter):

Components	**Amount (mg/L)**
Nicotinic acid	0.5
Pyridoxin HCl	0.5
Thaimin HCl	0.1
Glycine	2.0

2.2.1.2 Preparation of stock solution of growth regulatory hormones

In the current study, auxin NAA (Naphthalene acetic acid), IBA (Indolebutyric acid) and cytokinins BAP (6-Benzyl amino purine) were used in the regeneration media. Both hormones dissolve readily in NaOH, so in order to prepare 100 ml 10X stock solutions of each hormone, 10 mg of hormone powder was first dissolved in a few drops of NaOH. After that, the double distilled water was added to volume. Upon requirement, specific quantity of hormone stock solution was added to media.

2.2.1.3 Preparation of media for seed germination

The MS media (Murashige and Skoog 1962) was used for aseptic seed germination. The media was prepared according to following calculation:

Table 2.5: Components with their quantity to prepare 1 litre MS media:

Stocks (Stock concentration)	Amount (for 1000 ml)
Macro nutrients (10x)	100 ml
Minor salts (100x)	10 ml
Iron EDTA solution (100x)	10 ml
Organics (100x)	10 ml
Myo-inositol	0.1 g
Sucrose	30 g

All the components were taken into a conical flask and mixed thoroughly. Then the final volume was made 1000 ml by adding ddH_2O. The pH of the media solution was adjusted to 5.8 using 1N NaOH or 1N HCl depending on how acidic/alkaline the solution was. Agar was then added in 0.8% (w/v) ratio and melted in the oven. Finally, molten media was distributed into conical flasks, which were then sealed properly using aluminum foil and sterilized by autoclaving at 15 psi pressure at 121ºC temperature for 20 minutes.

2.2.1.4 Preparation of media for multiple shoot regeneration

In order to inoculate explants for shoot induction and development, eight different types of regeneration media were used. These were full strength MS media (section 3.1.3) supplemented with varying concentrations and combinations of plant hormones BAP or NAA or both. As such, preparation of regeneration media is similar to that of MS media preparation except that the appropriate amount of hormone stock solutions were added into the MS media before sterilization.

2.2.1.5 Preparation of media for root induction

For induction of roots from the *in vitro* grown shoots, well developed shoots were placed on root induction media. To prepare, half MS (half strength of macro and micronutrients of MS) medium was supplemented with 0.2 mg/l IBA.

2.2.2 Seed sterilization

Initially the seeds with testa were taken into an autoclaved conical flask and washed with autoclaved ddH_2O for two times for 1 minute each. Then they were washed with 70% ethanol for 3 minutes followed by ddH_2O wash for three more times. Next the seeds were immersed in 30% commercial bleach (Clorox) for 20 minutes while shaking continuously by hand. After this to remove bleach, seeds were washed in ddH_2O for 4-5 times. Seeds were then soaked in ddH_2O in a sealed flask to facilitate water imbibitions.

Next morning seeds of both varieties were transferred on the germination media (Murashige and Skoog 1962). Seeds of both the varieties were finally washed with ddH_2O twice. After completing the wash, the seeds were dehulled using sterilized scalpel and forceps. These seeds were then again cleaned with ddH_2O for three times and then dried with a filter paper. Once dry, seeds were inoculated in germination media. Then the sealed flasks were kept inside the dark chamber for 1-2 days to emulate proper environment for seed germination.

2.2.3 Seedling development

Seeds germinated within 2 days in the dark chamber. Flasks were then shifted to culture room having photoperiod (16-8 hour day-night cycle) incubation for seedling development. Finally, they developed into seedlings which became capable of yielding explants. The explants were collected from seedlings at different ages.

2.2.4 Explant collection and inoculation

For explant growth and development, a regeneration media was used. This is essentially Murashige and Skoog (MS) media containing the plant hormones NAA and BAP. As part of the experiment, the concentrations of the afore-mentioned hormones were varied for different explants. Embryonic axis and cotyledon were collected as explants. Cotyledon was collected from 5, 7 and 9 day-old seedlings of both varieties. The cotyledon explants were halved to yield two pieces each, which were transferred to regeneration media containing varied amounts of the afore-mentioned plant hormones. To collect embryonic axis explants from both varieties, sterilized seeds were kept soaked in double distilled water for 24 hours in a dark chamber. After dehulling, 3 mm portions were sectioned from the proximal part of the seeds and inoculated in regeneration media with varying concentrations of NAA and BAP. The inoculated media-containing flasks were sealed and kept under light.

2.2.5 Subculture of explants

Generally, all the cultures were subcultured within 15 to 30 days depending on regeneration response. Regular monitoring was also done to collect data on any morphological changes observed.

2.2.6 Root induction

Well developed shoots were collected and then placed into 0.2 mg/l IBA containing media to observe their capacity to produce sufficient root.

Chapter 3: Results

3. Results

In this study, two farmer popular Bangladeshi sunflower varieties, named, BRAC Hysun 33 and BARI Surjumukhi 2 were used to determine the most suitable and reproducible explant for *in vitro* regeneration. During this experiment, two explants were used. One is cotyledon and another one is embryonic axis. Several trials were performed to optimize the sterilization method and check suitability of explants under various hormone supplements to establish shoot regeneration.

3.1 Optimization of sterilization process

Several types of treatments were performed and germination rate along with time required for germination were noted. Treatments consist of 70% ethanol, followed by different concentration of Clorox treatments for varied duration. Among all treatments, considering these two parameters, washing with 70% ethanol for 3 minutes followed by vigorous hand shaking with 14% Clorox for 20 minutes are found to be optimum for sterilizing seeds of both the varieties. Among these two varieties, BRAC Hysun 33 variety showed better germination rate than BARI Surjomukhi 2 variety (Table 3.1 and Table 3.2).

3.2 Analysis of overall response of two different explants of BRAC Hysun 33 and BARI Surjumukhi 2 varieties

Two explants were used in this experiment to determine the most suitable explant for BRAC Hysun 33 and BARI Surjomukhi 2 varieties. Cotyledon was collected from different ages of seedlings and embryonic axis was excised from ungerminated mature embryo. These explants were used under various hormonal supplements to check their regeneration ability.

3.2.1 Explant: Cotyledon

In the present experiment, cotyledon explants of different ages were collected. They were transversely sectioned into 2 pieces. Excised cotyledons were then inoculated into eight different hormone supplemented media for shoot initiation and development.

Table 3.1: Optimization of seed treatment method of BRAC Hysun 33 variety in relation to germination rate and time requirement

Treatments	Seed conditions	Contamination in germinated seeds	% of seed germination	Average time required for germination (Days)
70% ethanol for 3 minutes+14% Clorox for 20 minutes	With testa	Yes	93.33 (1)	1.67 (0.58)
	Without testa	No	37.78 (0.58)	7.33 (0.58)
70% ethanol for 3 minutes+30% Clorox for 20 minutes	With testa	Yes	82.22 (0.58)	2.33 (0.58)
	Without testa	No	48.89 (0.58)	8.67 (0.58)
70% ethanol for 3 minutes+14% Clorox for 20 minutes. Testas removed before transferring to germination medium	With Testa	No	95.56 (0.58)	2.67 (0.58)

SD value within parenthesis

Table 3.2: Optimization of seed treatment method of BARI Surjomukhi 2 variety in relation to germination rate and time requirement

Treatments	Seed conditions	Contamination in germinated seeds	% of seed germination	Average time required for germination (Days)
70% ethanol for 3 minutes+14% Clorox for 20 minutes	With testa	Yes	84.44 (0.58)	2.67 (0.58)
	Without testa	No	64.44 (0.58)	7.33 (0.58)
70% ethanol for 3 minutes+14% Clorox for 15 minutes	With testa	Yes	71.11 (1.53)	5.67 (0.58)
	Without testa	No	55.56 (0.58)	8.33 (0.58)
70% ethanol for 3 minutes+14% Clorox for 10 minutes	With testa	Yes	35.56 (1.53)	8.67 (0.58)
	Without testa	No	No germination	No germination
70% ethanol for 3 minutes+14% Clorox for 20 minutes. Testas removed before transferring to germination medium	With testa	No	93.33 (1)	3.67 (0.58)

SD value within parenthesis

3.2.1.1 Effect of hormonal supplementation on cotyledon explant

In BRAC Hysun 33 variety, the cotyledon explants increased in size and most of them survived up to 45 days after inoculation. Largest cotyledon was obtained in 1.0 mg/l BAP in combination with 0.1 mg/l NAA supplemented MS media. They were greenish brown in color. Under 1.0 mg/l BAP combined with 1.0 mg/l NAA hormone condition, explants increased in size within 15 days of inoculation. But after that they started growing brownish and within 25 days of inoculation, they died. They did not form any callus. In media containing 2.0 mg/l BAP and 0.1 mg/l NAA, greenish translucent callus was formed within 20 days of inoculation. Media supplemented with 2.0 mg/l BAP with 0.5 mg/l NAA and 2.0 mg/l BAP with 1.0 mg/l NAA produced brownish green friable and translucent callus, respectively. Excessive adventitious rooting was observed only in media containing 1.0 mg/l BAP and 0.5 mg/l NAA. Rooting was initiated all over the callus after 20 days (Fig. 3.1).

On the other hand, in BARI Surjomukhi 2 variety, most of the cotyledon explants survived up to 40 days of inoculation. In media containing 1.0 mg/l BAP in combination with 0.1 mg/l NAA, explants increased in size and friable callus was formed. Largest friable callus was obtained in 2.0 mg/l BAP containing MS media. In response to 1.0 mg/l BAP and 0.5 mg/l NAA containing media, it enlarged and remained green in color. Adventitious rooting on the surface of the explant was found in much higher in number in this variety and was seen in most of the media compositions. Among them, 2.0 mg/l BAP with 0.1 mg/l NAA and 2.0 mg/l BAP with 0.5 mg/l NAA showed rooting initiated 25 days after inoculation.

So, in conclusion of this experiment, it can be said that cotyledon explant of BRAC Hysun 33 and BARI Surjomukhi 2 varieties were found to grow larger more or less in all the media composition within 7 days of inoculation (Table 3.3). Some of these explants showed callus regeneration in presence of hormonal supplementation like, 1.0 mg/l BAP and 0.5 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l NAA, 2.0 mg/l BAP and 0.5 mg/l NAA etc. Friable and translucent callus were formed but remain non-regenerative as no shooting was initiated from these calluses. However, spontaneous rooting was seen in most of these calluses under various hormonal concentrations more commonly in BARI Surjomukhi 2 variety but in one hormone supplementation in BRAC Hysun 33 variety (Fig. 3.1).

Table 3.3: Shoot regeneration efficiency of cotyledon explant in various hormonal supplements

Varieties	Hormone concentration		Responsive explants (%)	% of shoot regenerating explants
	BAP (mg/l)	NAA (mg/l)		
BRAC Hysun 33	1.0	0.0	93.75 (0.71)	0
		0.1	100 (0)	0
		0.5	93.75 (0.71)	0
		1	87.5 (1.41)	0
	2.0	0.0	87.5 (0)	0
		0.1	81.25 (0.71)	0
		0.5	93.75 (0.71)	0
		1	81.25 (0.71)	0
BARI Surjumukhi 2	1.0	0.0	93.75 (0.71)	0
		0.1	93.75 (0.71)	0
		0.5	93.75 (0.71)	0
		1	87.5 (1.41)	0
	2.0	0.0	93.75 (0.71)	0
		0.1	87.5 (0)	0
		0.5	87.5 (1.41)	0
		1	75 (0)	0

SD value within parenthesis

3.2.1.2 Effect of age of cotyledon explant

Cotyledon explant was collected from 5, 7 and 9 days old seedlings of both the varieties to determine the optimum age of explant for regeneration. Among these, cotyledons obtained from 7 days old seedlings showed highest response in callus formation in both the varieties though no shoot was formed on any cotyledon explant (Table 3.4; Fig. 3.1). Five days old cotyledon explant died within 3 days of inoculation in all media concentrations for both the varieties. In BRAC Hysun 33 variety, nine days old cotyledon explant grew larger in size in most of the media composition but did not form any callus or shoot. But in BARI Surjomukhi 2 variety, nine days old cotyledon remained almost unchanged. After 30 days, they gradually developed brown color and died. Therefore, for further studies 7 days old cotyledon explants were taken for regeneration experiments.

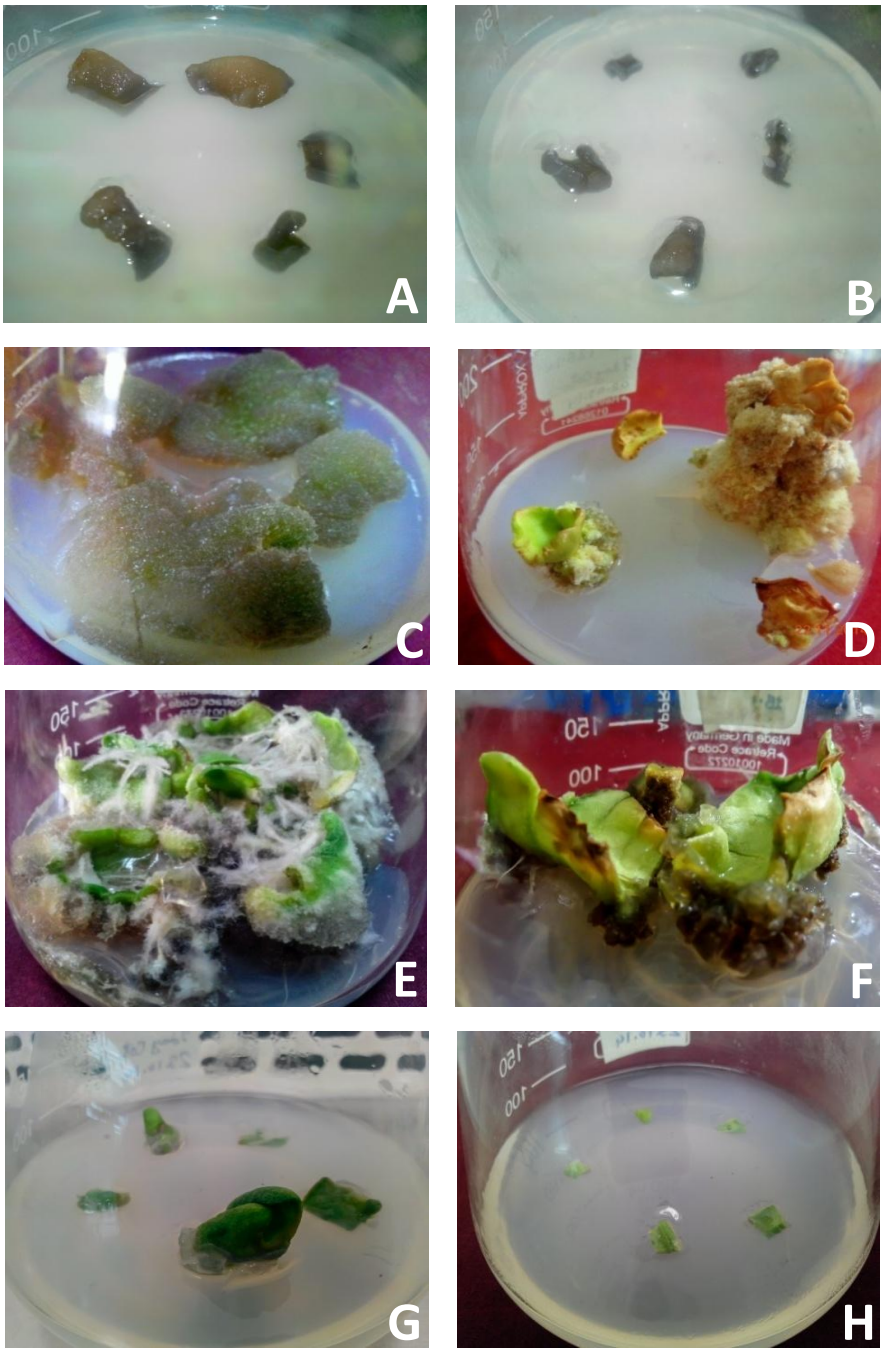

Fig. 3.1: Cotyledon explant of different ages from BRAC Hysun 33 (A, C, E and G) and BARI Surjomukhi 2 (B, D, F and H) varieties. **A-B**: 5 days old cotyledon inoculated in MS + 1.0 mg/l BAP + 0.5 mg/l NAA supplemented media (Photographed 7 days after inoculation). **C-F**: 7 days old cotyledon inoculated in MS + Various concentration BAP and NAA supplemented media (Photographed 30 days after inoculation). **C-D**: In 2.0 mg/l BAP, BRAC Hysun 33 variety showing translucent callus formation and BARI Surjomukhi 2 variety showing friable callus. **E-F**: MS + 1.0 mg/l BAP + 0.5 mg/l NAA, BRAC Hysun 33 variety having spontaneous root on whitish callus and BARI Surjomukhi 2 variety, having direct organogenesis. **G-H**: 9 days old cotyledon inoculated in MS + 1.0 mg/l BAP + 0.5 mg/l NAA supplemented media (Photographed after 45 days of inoculation).

Table 3.4: Determination of suitable age of cotyledon explant for regeneration (based on callus formation capacity)

Varieties	Age of cotyledon explants (days)	% of responsive explants	Days required for response (callus formation)	Shoot regenerative explants
BRAC Hysun 33	5	0	Died	----------
	7	89.84 (0.75)	8.38 (1.02)	0
	9	75.78 (0.85)	15.13 (1.15)	0
BARI Surjomukhi 2	5	0	Died	----------
	7	89.06 (0.81)	10.19 (1.11)	0
	9	0	Died	----------

SD value within parenthesis

3.2.2 Explant: Embryonic axis

After seed sterilization, they were imbibed in autoclaved ddH_2O overnight in dark chamber. The next day, testas were removed and from proximal portion of the seed, a 3 mm piece was excised and cultured in MS media with eight different hormonal supplements.

3.2.2.1 Effect of hormonal supplementation on 1 day old embryonic axis explant

The explant embryonic axis of BRAC Hysun 33 variety showed higher regeneration frequency compared to BARI Surjomukhi 2 variety (Table 4.5). Both of the varieties formed translucent white callus in all the regeneration media.

In BRAC Hysun 33, with the increase of hormone concentrations, this explant showed less regeneration response. In media containing 1.0 mg/l BAP in combination with 0.1 mg/l NAA, highest regeneration (58.33%) was obtained with an average of one single shoot formation within 5 to 7 days of inoculation. Approximately 3.2 cm long shoot length was recorded in the explant within 16 days of inoculation. It was observed that the shoot elongation in this media was faster than any other media composition. At this stage, the shoots were transferred to rooting media instead of subculture (Fig. 4.4). Shoot initiation also occurred in 1.0 mg/l BAP in combination with 0.5 mg/l NAA. Single shoots were formed within 10 days but the elongation rate was very

slow. After 20 days, their length ranged from 0.5-1.0 cm. In media containing 2.0 mg/l BAP, single shoot was initiated within 10 days of inoculation following indirect regeneration. Among the eight types of media, four showed no shooting response. In 2.0 mg/l BAP with 1.0, 0.1 and 0.5 mg/l NAA and in presence of 1.0 mg/l BAP with 1.0 mg/l NAA hormonal supplementation, translucent callus were formed but no shoot initiation was observed from these calli (Fig. 3.2).

On the other hand, in BARI Surjomukhi 2 variety highest length of shoot was obtained in 2.0 mg/l BAP containing media. In this media composition, 46.67% shooting was obtained which was initiated after 10 days of inoculation and the length of shoot ranged up to 3 cm. Media having 2.0 mg/l BAP with 1.0 mg/l NAA and 1.0 mg/l BAP also showed shoot regeneration which ranged in length from 0.4 to 1.1 cm within 15-18 days of inoculation. Media concentration 1.0 mg/l BAP in combination with 1.0 mg/l NAA, 2 mg/l BAP in combination with 0.1 or 0.5 mg/l NAA, produced only translucent callus. Callus appeared within 8 to 10 days of inoculation. In presence of 1.0 mg/l BAP with 0.5 mg/l NAA explants became green in color but no shooting was initiated. The explant increased in size and formed green structure of approximately 4 cm within 20 days of inoculation. Interestingly, 1.0 mg/l BAP in combination with 0.1 mg/l NAA containing media showed varied result and giving only 2% of shoot regeneration (Fig. 3.3).

Table 3.5: Regeneration response of embryonic axis explant in various hormonal supplements

Varieties	Hormone concentrations		Responsive explants (%)	Regenerative explants (%)	Shoot length after 20 days (cm)
	BAP (mg/l)	NAA (mg/l)			
BRAC Hysun 33	1.0	0.0	50 (0.82)	12.5 (0.71)	0.57 (0.12)
		0.1	95.83 (0.5)	58.33 (0.58)	2.35 (0.54)
		0.5	87.5 (0.96)	33.33 (0.82)	0.76 (0.22)
		1.0	91.67 (0.58)	0 (0)	---------
	2.0	0.0	83.33 (1.15)	45.83 (0.5)	0.83 (0.31)
		0.1	75 (0.58)	0 (0)	---------
		0.5	87.5 (0.5)	0 (0)	---------
		1.0	70.83 (0.5)	4.17 (0.5)	---------
BARI Surjomukhi 2	1.0	0.0	53.33 (0.58)	33.33 (0.58)	1.1 (0.26)
		0.1	86.67 (0.58)	2 (1.15)	0.8 (0.28)
		0.5	66.67 (0.58)	0	---------
		1.0	40 (1)	0	---------
	2.0	0.0	93.33 (0.58)	46.67 (0.58)	2.61 (0.54)
		0.1	46.67 (0.58)	0	---------
		0.5	86.67 (0.58)	0	---------
		1.0	93.33 (0.58)	13.33 (1.15)	0.4 (0.14)

SD value within parenthesis

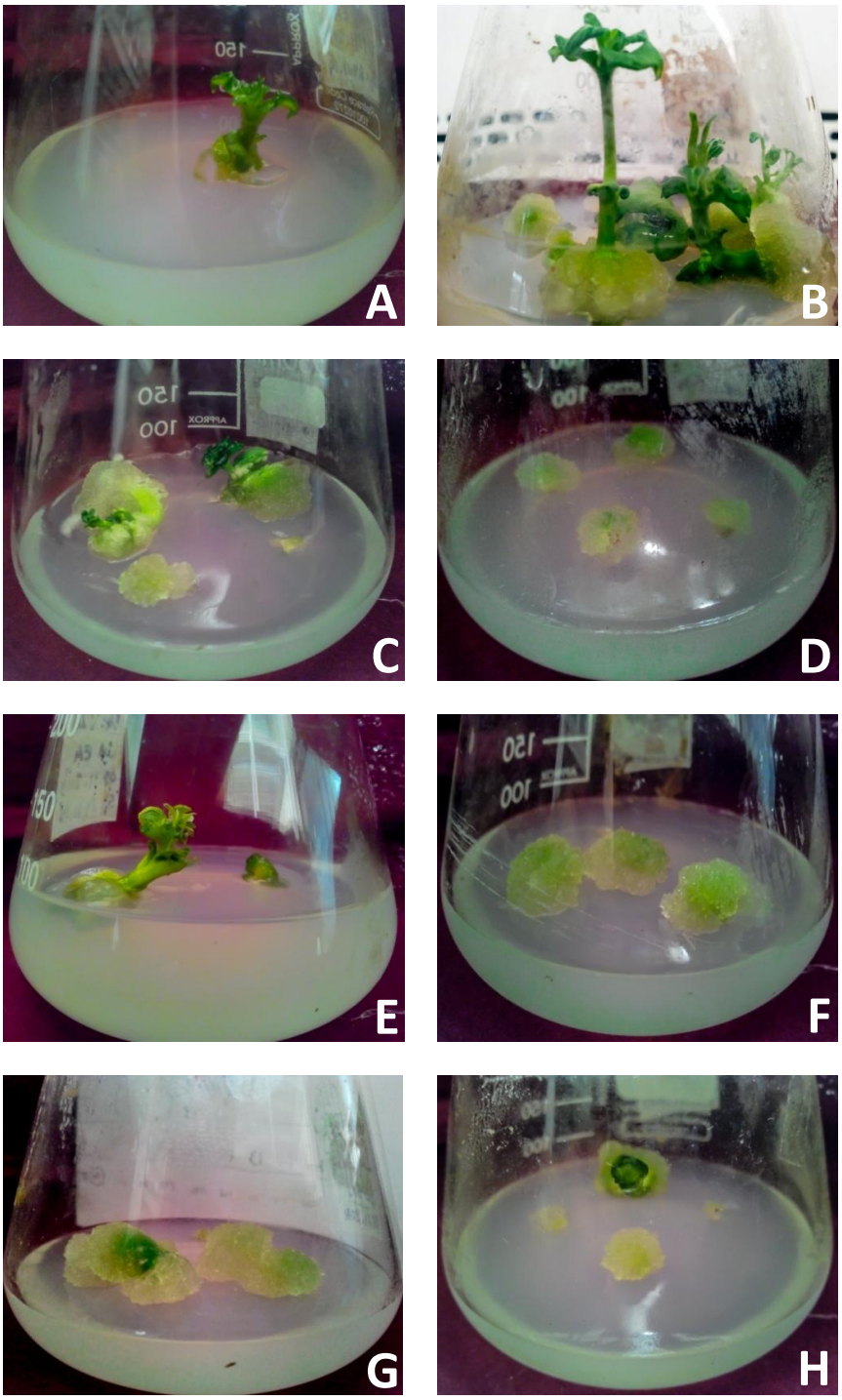

Figure 3.2: Embryonic axis explant of BRAC Hysun 33 variety on different hormonal supplemented media. **A.** Shoot formation at 1.0 mg/l BAP **B.** Highest length of shoot was obtained at 1.0 mg/l BAP + 0.1 mg/l NAA **C.** Shoot formation at 1.0 mg/l BAP + 0.5 mg/l NAA **D.** Formation of callus at 1.0 mg/l BAP and 1.0 mg/l NAA **E.** Initiation of shooting at 2.0 mg/l BAP **F-H:** Callus formation at **F.** 2.0 mg/l BAP + 0.1 mg/l NAA; **G.** 2.0 mg/l BAP + 0.5 mg/l NAA; **H.** 2.0 mg/l BAP + 1.0 mg/l NAA. (Photographs were taken after 20 days of inoculation)

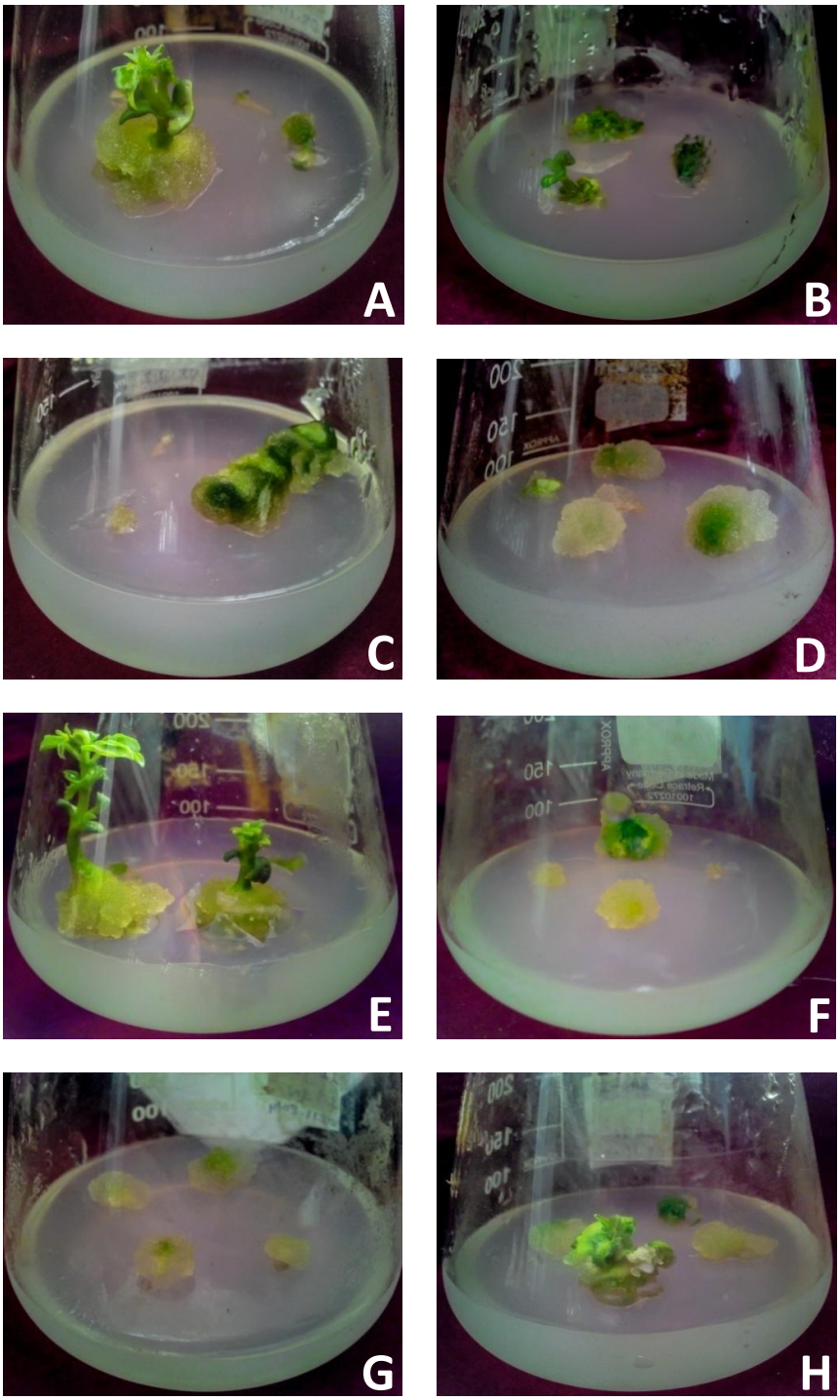

Fig. 3.3: Embryonic axis explant of BARI Surjomukhi 2 variety in various hormone supplemented media. **A-B:** Shoot formation at **A.** 1.0 mg/l BAP; **B.** 1.0 mg/l BAP + 0.1 mg/l NAA **C.** Formation of green structure at 1.0 mg/l BAP + 0.5 mg/l NAA **D.** 1.0 mg/l BAP and 1.0 mg/l NAA: callus formation **E.** Highest shoot length was obtained at 2.0 mg/l BAP. **F-H:** Callus formation at **F.** 2.0 mg/l BAP + 0.1 mg/l NAA; **G.** 2.0 mg/l BAP + 0.5 mg/l NAA; **H.** 2.0 mg/l BAP + 1.0 mg/l NAA.
(Photographs were taken after 20 days of inoculation)

3.2.2.2 Initiation of multiple shooting on 1 day old embryonic axis explant

In BARI Surjomukhi 2 variety, multiple shooting was initiated in embryonic axis explant in mid December to January. The explant was inoculated in MS media supplemented with 2.0 mg/l BAP and within 20-25 days of inoculation. The shoots elongated up to 2.5 to 3 cm. BRAC Hysun 33 variety also showed multiple shooting from embryonic axis explant under 2.0 mg/l BAP supplementation. This was recorded at the end of December to January.

3.2.2.3 Rooting response of regenerated shoots

Shoot regenerated at 1.0 mg/l BAP in combination with 0.1 mg/l NAA media concentration were collected and rooting was initiated. For rooting, 0.2 mg/l IBA hormonal supplement was used. In this medium, 42.85% shoot responded and rooting initiated within 5-7 days after inoculation. The developed roots were white and thin (Fig. 3.4). This part of the experiment was performed to check the root initiation capacity of the regenerated shoots.

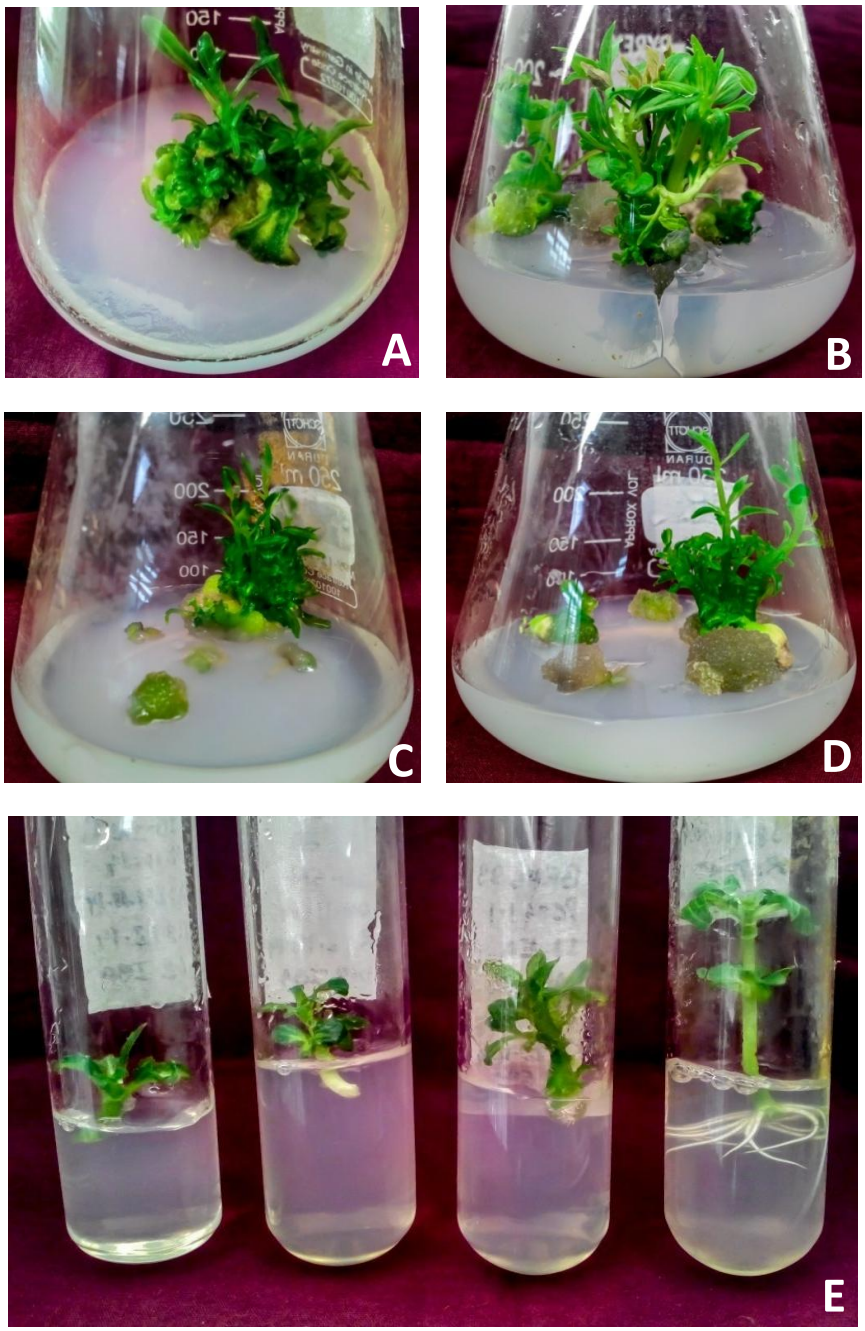

Fig. 3.4: In BRAC Hysun 33 variety, **A.** Formation of bushy shoot from embryonic axis explant was observed within 15 to 18 days of inoculation in 2.0 mg/l BAP media concentration at the end of December (photographed after 35 days of inoculation). In BARI Surjomukhi 2 variety (**B**, **C** and **D**), multiple shooting was initiated in 2.0 mg/l BAP supplemented media within 20 days of inoculation. Shoot elongated up to 2.5 to 3 cm within 35 days of inoculation. This was recorded in mid December to January (photographed after 35 days of inoculation). In BRAC Hysun 33 variety, **E.** the shoots, from embryonic axis explant, were initiated and elongated in 1.0 mg/l BAP and 0.1 mg/l NAA media concentration. They were transferred to MS media supplemented with 0.2 mg/l IBA for root induction.

Chapter 4: Discussion

4. Discussion

In context of our country, sunflower has still not been reported to be subjected to any biotechnology application. Keeping that in mind, the current study focused on determining the most suitable explant of two farmer popular sunflower varieties of Bangladesh, named, BRAC Hysun 33 and BARI Surjomukhi 2 as a step towards establishing regenerative tissue culture protocol. To find out the most regenerative explant, the age of explant and optimum hormonal supplement in regeneration medium were largely examined. In this study, the used explants were cotyledon and embryonic axis from mature ungerminated embryo.

Ozyigit *et al*. (2002) reported reproducible tissue culture protocol using cotyledon as explant in five Turkish varieties. They used 10 days old cotyledon. In another study, Mohmand and Quraishi (1994) used 21 days old seedlings to collect cotyledon explant. However, Aurori *et al*. (2011) mentioned that with the increase of age of explant, the morphogenetic capacity is reduced drastically and developmental stage is affected highly significantly in sunflower organogenesis. Considering all these findings, in current study, five, seven and nine days old cotyledons were used as explant for both BRAC Hysun 33 and BARI Surjomukhi 2 varieties. However, the cotyledon explant for these two Bangladeshi varieties was found to be non-regenerative irrespective to its age. In these varieties, with the increase of age of explant, they found to survive for longer. Though this survival capacity supports most of the studies conducted with cotyledon explant, this contradicts the fact found by Aurori *et al*. (2011).

To check the regeneration capability of cotyledon explant, eight types of regeneration media were used where MS media was supplemented with various combinations of NAA and BAP. Both the varieties formed translucent or friable callus and further did not show any shoot formation. In most of the earlier experiments using sunflower where cotyledon was used as explant under various combinations of auxins and cytokinines, cotyledon was found to be regenerative (Badigannavar and Kururvinashetti, 1998; Ozyigit *et al*. 2002 and 2007; Mohmand and Quraishi1994). Though current result is different from most of the studies of sunflower, it can be explained from findings of studies by Badigannavar and Kururvinashetti (1998) and Ozyigit *et al*. (2002 and 2007). They demonstrated that explant cotyledons

significantly depended on genotype in callus induction as well as regeneration. In this experiment, several combinations of NAA and BAP were used. However no shoot regeneration was obtained though callus formation found. Therefore, genetic dependency could be considered for absence of regeneration in case of these two Bangladeshi varieties.

Recalcitrance in sunflower is the major constraints behind its resistance to genetic improvement. This experiment was mainly focused to determine such an explant which would be capable of regeneration for diverse range of genotypic varieties. Aurori *et al*. (2011) outlined embryonic axis from ungerminated mature embryo as the best regenerative explant rather than embryonic axis from germinated mature embryo. It is considered that immature zygotic embryo is the explant which is quite independent of genetic diversity but it is very difficult to obtain due to seasonal dependency. Moreover, it requires extensive time and effort (Aurori *et al*. 2011). Considering all these obstacles, in the present study embryonic axis from ungerminated mature embryo was used as explant for this experiment.

Ozyigit *et al*. (2006) demonstrated that under hormonal supplement 1.0 mg/l BAP in combination with 0.5 mg/l NAA, mature embryo collected from Turkish varieties gave approximately 44% shoot regeneration. In this present study, embryonic axis from ungerminated mature embryo explant of BRAC Hysun 33 variety showed 58.33% regeneration in media composition of 1.0 mg/l BAP combined with 0.1 mg/l NAA. On the other hand, BARI Surjomukhi 2 variety gave highest regeneration in media containing only 2 mg/l BAP. Although 1.0 mg/l BAP combined with 0.5 mg/l NAA hormonal supplement induced shoot initiation in case of BRAC Hysun 33 variety, shoot elongation rate was very slow compared to 1.0 mg/l BAP combined with 0.1 mg/l NAA composition. In BARI Surjomukhi 2 variety, 1.0 mg/l BAP combined with 0.5 mg/l NAA hormonal supplement induced neither callus formation nor shoot initiation. While replicating the results demonstrated by Ozyigit *et al*. (2006), gave successful regeneration, these conditions did not prove to be optimum for two varieties under investigation.

In case of BRAC Hysun 33 variety, it can be outlined that with the increase of BAP and NAA concentrations, embryonic axis explant showed stunted growth and only BAP could not induced further growth of initiated shoot. This indicates that the concentration of auxins in embryonic axis explant in this variety is not sufficient enough for shoot elongation. Thus, 0.1 mg/l of NAA (auxin) supplement was required along with 1.0 mg/l BAP (cytokinin) for the growth of initiated shoot. On the other hand, in BARI Surjomukhi 2 variety, it was observed that with the increase of BAP upto certain level, regeneration efficiency of this explant improved but presence of NAA did not influence their growth. This indicates the presence of sufficient amount of auxin already in the explant. Thus, presence of auxin gave stunted growth or only callus formation in BARI Surjomukhi 2 variety. So, it can be considered that application of only BAP is optimum for the regeneration from embryonic axis explant in BARI Surjomukhi 2 variety. Therefore, hormonal requirement for *in vitro* regeneration in Bangladeshi sunflower varieties is genotype dependent.

To check the seasonal independency of this explant, complete seed of both varieties were inoculated in germination media. Surprisingly, it was found that seeds which gave 100% germination in period of June to August month, they stopped germinating in October to December. During this off season even if germination occurred, germination time increased upto 7 days. While seeds were inoculated for germination, explant embryonic axis from ungerminated mature embryo was also inoculated in hormonal media. It was found that this explant gave regeneration in October to December month whereas seed germination response declined. Moreover, after mid December, explant embryonic axis from ungerminated mature embryo showed different result. During this period, multiple shoots were initiated and their elongation rate increased than before. Besides, the shoots were healthier than previously obtained shoots. Further research needs to be done to see the seasonal dependency on *in vitro* response.

In the current study, embryonic axis explant gave better regeneration response than cotyledon explant for both the varieties. So, it can be concluded that the regeneration potential of embryonic axis is better than cotyledon as explant for tissue culture of sunflower varieties BRAC Hysun 33 and BARI Surjomukhi 2. This result can be explained and supported from the point of Aurori *et al*. (2011) as well. They mentioned that regeneration ability of explants remain unchanged if they are obtained from embryos rather than plantlets. Further research is required to establish regeneration protocol with these explants for both BRAC Hysun 33 and BARI Surjomukhi 2 varieties. Once regenerative tissue culture protocol is established, efficient transformation can be conducted successfully of this sunflower varieties. And this will open the door of genetic engineering to deliver desirable characteristics, such as, better yield and biotic and abiotic stress tolerance.

Chapter 5: References

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