Rhizogenesis of Regenerated Shoot in Bangladeshi Sunflower (*Helianthus annuus* L.) Varieties

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Declaration

I hereby declare that the research work embodying the results reported in this thesis entitled **"Rhizogenesis of Regenerated Shoot in Bangladeshi Sunflower (*Helianthus annuus* 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-Dichlorophenoxy acetic acid

BAP - 6-Benzylaminopurine

BARI - Bangladesh Agricultural Research Institute

cm - Centimeter

Conc. - Concentration

ddH_2O - Double-distilled water

EDTA - Ethylenediaminetetraacetic acid

EtOH - Ethanol

Fig. - Figure

GA3 - Gibberellic Acid

gm - Gram

IAA - Indole-3 Acetic Acid

IBA - Indole-3 Butyric Acid

L - Litre

lbs/sq. - *Pound* per square inch

LS - Linsmaier and Skoog (1965) medium NAA - Napthalene acetic Acid

mg - Milli gram

ml - Millilitre

MS - Murashige and Skoog (1962) medium

NAA - Napthalene acetic Acid

NaOH - Sodium hydroxide

pH - Negative logarithm of hydrogen ion concentration

SD - Standard deviation

t/ha - *Tonne per hectare*

w/v - Weight/Volume

Abstract

Sunflower (*Helianthus annuus* L.) is a highly nutritious oil-producing crop of Bangladesh. But the yield of this crop is disrupted by several biotic and abiotic factors. Application of biotechnological techniques is necessary for the improvement of this plant. The purpose of this study was to establish a tissue culture mediated plant regeneration protocol for two Bangladeshi varieties, namely, BARI Surjomukhi 2 and BRAC Hysun 33. Embryonic axis and hypocotyl were the two explants used from these selected varieties. Suitability and regeneration capacity of these explants, optimization of the hormone supplementation for shoot and root formation and following acclimatization stage of the regenerated plantlets were accomplished in this study. Hypocotyl explants were excised from (3-9) days old seedling and for embryonic axis, proximal portion of the seed was excised before culturing on MS medium supplemented with ten different combinations of BAP and NAA. For embryonic axis explants, BAP singly gave direct regeneration and BAP combined with NAA showed indirect regeneration in both the sunflower varieties. The highest shoot regeneration (66.67%) was found in MS media supplemented with 2.0 mg/l BAP in BRAC Hysun 33 variety. In case of BARI Surjomukhi 2 variety the regeneration rate was 46.67%. To obtain proper shoot length, a shoot elongation medium, full strength MS with 2.0 mg/l BAP and 0.1mg/l GA_3 was used. At the rooting stage, three concentrations of IBA were examined, among them 1.0 mg/l IBA showed better root formation capacity. All the rooted plantlets were transferred to soil for hardening and BRAC Hysun 33 variety gave highest survival rate at this stage. So, it can be determined that *in vitro* regeneration potential of BRAC Hysun 33 variety is better compared to BARI Surjomukhi 2 variety. Shoot regeneration efficiencies of another explant, hypocotyls on the same hormonal treatments were found lower in comparison with the embryonic axis explants. Hypocotyl explants of both the varieties produced shoots only on 1.0 mg/l BAP containing MS media and BARI Surjomukhi 2 variety showed better regeneration response than BRAC Hysun variety. Further studies are required to modify the acclimatization stage of the regenerated plantlets of sunflower.

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Chapter 1: Introduction

1. Introduction

Nuts and seeds have always been a major part of human diet. Like other plant foods, they provide a wide range of essential nutrients. Among these nutrients, protein and fats are most important because they provide energy for human body to function. The World Health Organization (WHO) recommends that maximum 30% of total energy should come from oils and fats (WHO, 2015). Soybean, sunflower, rapeseed and peanuts are the major sources of edible seed oils. Due to high oil content (40% - 52%), no cholesterol and high non-saturated fatty acids content, sunflower is considered as world's second most important oil seed crop next to soybean (National Sunflower Association, 2015). It is cultivated in 18 million hectares all over the world for the purpose of extracting edible oil and also as a source of healthy food. The production of this plant is influenced by several biotic and abiotic stresses. Approaches like gene transformation can play a vital role in improving sunflower production by transmitting desired characteristics. However, the success of genetic manipulation depends on the transformation as well as regeneration frequency of the plant. Therefore, determination of an efficient plant regeneration protocol for sunflower is essential.

1.1 Origin

Sunflower (*Helianthus annuus* L.) is a native North American plant. The genus *Helianthus* comprises about seventy species. Most of the species are considered as perennial plants. Because of its strong vibrant color, sunflower was considered only as an ornamental plant until 19th century. It was the Russian breeders who developed ornamental sunflower into oil bearing crop (National Sunflower Association, 2015). Now it is cultivated as an essential oil seed crop all over the world. The European Union, Russia, U.S.A and Argentina are the leading producers of sunflower.

1.2 Plant description

This tall, herbaceous, annual plant, sunflower is recognized worldwide for the high nutritional value as well as its beauty. Sunflowers can grow upto a height of 50 - 400 cm (20 - 157 in). The plant is grown from its seeds and can be grown in different growing conditions. During growth the young flower bud of sunflower plant shows heliotropism.

1.2.1 Seed: Sunflower plant produces oval or drop-shaped, small (6 - 10 mm) seeds containing an outer coat called hull. When de-hulled, the grey-white colored remainder

portion is called the kernel. They are rich in oil and protein and are incredible source of health benefiting nutrients.

1.2.2 Flower: Sunflower head (inflorescence) is made up of 1,000 to 2,000 individual flowers. Two types of floret named ray floret and disc floret typically form the whole flower. The flowers in the center of the head are called disk flowers (Fig. 1.1). Disk flowers are arranged in a spiral pattern (Dedio, 2005). The outer florets, ray florets produce the outer petals, while the inner florets mature into the seeds. Ray florets are sterile as they lack both stamen and pistil. They mainly attract insects for pollination. Sunflowers are normally yellow in color; however, the shading can range from lemon-yellow, orange to reddish.

1.2.3 Leaves: Sunflower plant has large, wide, oval shaped lower leaves which are arranged alternately on the stem. The smaller, narrower upper leaves are attached individually to the stem. The mature dark green leaves are rough in nature and have prominent veins on the leave surface (http://www.motherearthliving.com/natural-health/the-many-uses-of-sunflowers.aspx, date: 15.11.2015).

1.2.4 Stem: The sunflower plant has a thick, hairy, unbranched stem. Depending on the number of internodes the stem can grow upto 3m (10 ft) tall. Sunflower leaves and stem release certain substances that can inhibit the growth of other plants (http://articles.herballegacy.com/sunflower-natures-perfect-plant/, date: 15.11.2015).

1.2.5 Roots: Sunflower plants have strong tap root system with smaller hairy secondary roots. The root can stretch upto 1-3 feet deep in the soil. Though sunflowers grow best in warm, arid climates they require dampness and a loose soil composition for the proper growth of roots (http://homeguides.sfgate.com/kinds-roots-sunflowers-have-60427.html date: 15.11.2015).

1.3 Reproductive biology

The sunflower reproduces sexually through cross pollination method. When the plants are fully flowered they attract insects and use them to spread the reproductive pollen from one flower to another. Alternatively, being a monoecious plant, sunflower can self-reproduce by twisting itself around its own pollen. However, self-pollination can only produce an identical flower, so no hybridization will occur using this method. Anther surrounds the style of the carpel and each anther contains pollens. 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.

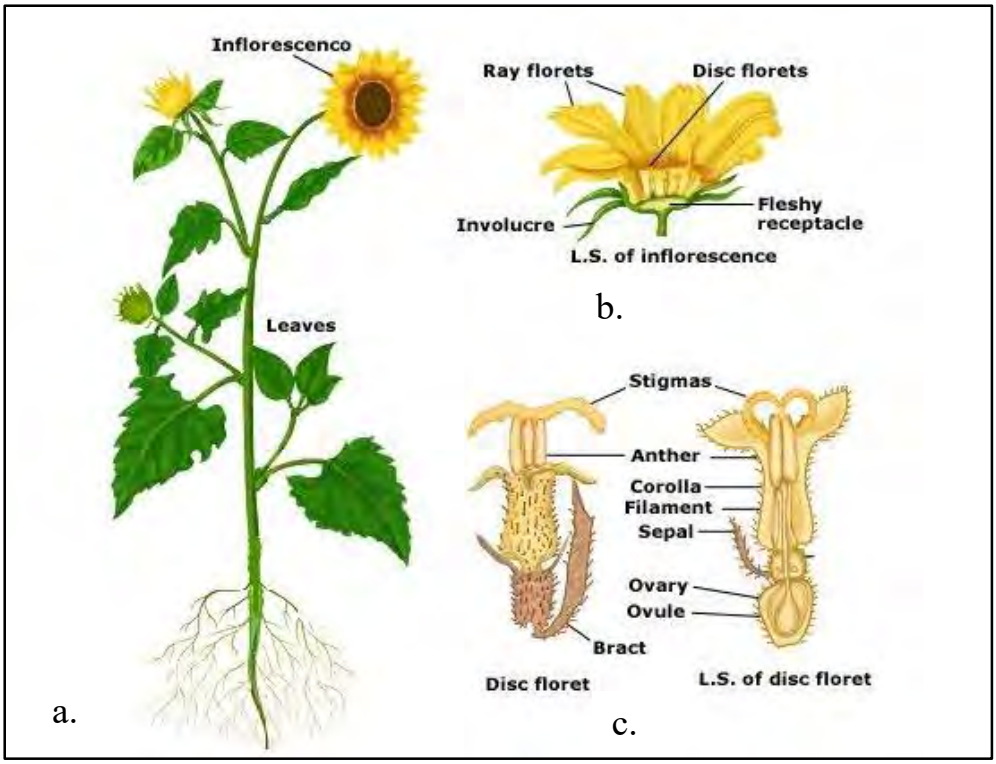

Fig. 1.1: a. A sunflower plant, **b.** longitudinal section of the inflorescence and **c.** longitudinal section of disc floret

1.4 Importance of sunflower

1.4.1 Nutritional value: Sunflowers are valued for their caloric content and abundant health benefits. The seeds of sunflower are consumed as food either by drying or roasted or salted as snacks, like groundnuts. They are a good source of several key nutrients like healthy fats, protein, fiber, minerals, vitamin E. The seeds have been ranked at the top of the nuts and seeds list for their high content of phytosterols, a class of plant chemicals that reduce cholesterol levels. These seeds are also loaded with many essential minerals which play a vital role in bone mineralization, RBC production, hormone production, as well as in the regulation of cardiac and skeletal muscle activities (http://www.nutrition-and-you.com/sunflower-seeds.html, date: 15.11.2015). Sunflower oil has a light taste and used as salad dressings, cooking oil and margarine. The percent of oil in sunflower is higher

(45%-55%) than other oil seed crops (National Sunflower Association, 2015). Moreover, the by-products of the seed is also a rich source of proteins (35%) and carbohydrates (18%-20%) and can be used as animals and poultry feed (Ibrahim, 2012; Aminifar and Galavi, 2014).

1.4.2 Medicinal value: According to USDA Nutrient Database, sunflower seeds are considered as the best source of vitamin E, an antioxidant which plays an important role in the prevention of cardiovascular diseases. Sunflower seeds can be used to treat respiratory diseases like coughs, bronchitis etc. Recently, scientists are focusing on applying sunflower in cancer research as the plant contains histamine, a chemical found predominantly in leukemia patient. The roots of the sunflower plant can be applied externally on swellings, sores, for snakebites and spider bites (https://tjdemogarden.wordpress.com/2011/12/16/plant-profile-sunflower/, date: 15.11. 2015).

1.4.3 Industrial and other uses: Sunflowers are also used in other areas, such as, in textile industry they can be used to make dye for textiles. The ray florets of sunflower can be used for producing dye, especially yellow dye. The stems are used to make a number of things like paper, clothes and microscope slide mounts (https://tjdemogarden.wordpress.com/2011/12/16/plant-profile-sunflower/, date: 15.11.2015). The oil of sunflower is used in cosmetics industry to produce soap, cream, lotion, ointment etc.

1.5 Cultivation of sunflower

As long as it is not water logged sunflowers can be grown on a wide range of fertile soil types; sandy loam to clays. Due to the deep tap root system, well-dug, loose soil is preferable for successful sunflower cultivation. The plant is adapted to almost all climatic regions of the world. They do fine in soils that are slightly acidic to somewhat alkaline (pH 6.0 to 7.5). Sunflowers, however, do 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. The plant is tolerant to both low and high temperatures but more tolerant to low temperatures. Being a photo-insensitive crop it can be grown in any season Rabi (October – March) and Kharif (July – October). As the name implies, sunflowers grow best in a location with direct sunlight. Sunflower is largely produced in Russia or the former Soviet Union. Ukraine, Argentina, the combined European Union, China, South Africa are the top producer countries of sunflower. Russia

and Ukraine are producing almost half of the world sunflower seeds. The statistics of the global consumption of vegetable oils shows that in 2014/2015 sunflower oil consumption amounted to 15.18 million metric tons (The Statics Portals, 2016). Daily 6 to 8 hours of sunlight is required for their proper growth. During germination the seeds do not need light, rather a cool atmosphere is required for satisfactory germination. Once the seedlings reached the four to six leaf stage, warmer weather is required for maturation. Optimum temperature for growth is 21°C-26°C, but they can be grown on a wider range of temperatures (18°C-33°C). Extremely high temperatures have been shown to lower oil percentage, seed fill and germination (Putnam, *et al*., 1990). Depending on the variety and weather condition, total growing period ranges from 70-130 days (http://www.fao.org/nr/water/cropinfo_sunflower.html, date: 16.12.2015). According to the National Sunflower Association, United States, China, Turkey and Spain are the top sunflower oil consuming countries in the world.

1.6 Present scenario of sunflower production in Bangladesh

In Bangladesh, sunflower was first introduced as a garden plant of winter season. Till 1980s, cultivation of sunflower as oilseed crop was not familiar in this country. But later limited initiatives were taken to do research on this plant. Some dwarf varieties were initially introduced to cultivate sunflower as an oil seed crop. However, in recent times sunflower is getting huge popularity among the local farmers of this country.

After the two severe cyclones, Sidr (2007) and Aila (2009) attacked in southwestern coastal areas of Bangladesh many farming land have been severely damaged. Salinity levels intensified, which hampered agricultural production. Thus most of the arable land remained fallow due to intrusion of salinity. Bangladesh is constantly facing these challenges of food security in changing climate. To meet up the domestic demand a huge quantity of edible oil is imported from foreign countries. In 2013 Bangladesh imported 17.77 lakh tons 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: 17.12.2015). With a view to promote adaptive technologies among farmers, BRAC has taken an initiative to expand the cultivation of sunflower in these Sidr affected areas. A two year project “Crop Intensification for Achieving Food Sufficiency in the Coastal Region of Bangladesh” has been taken by them. Beside that BRAC has been

providing financial support along with technology in the field of farming through the Borgachasi/Sharecropper Union Programme (BCU) Project. Residents of Patuakhali, Bagerhat, Satkhira, Jhalakati etc. have started cultivating sunflower. Around 1000 hectares of land in 24 upazilas of 17 districts have been brought under sunflower cultivation and they are expecting to obtain 2000 tons of oil from these (http://www.thefinancialexpress-bd.com/old/index.php?ref=MjBfMDRfMDdfMTNfMV85MV8xNjU2MDk, date: 20.4.2016). In addition, BRAC has launched Sunflower oil under the brand name of "Shufola" utilizing sunflowers cultivated in southern region of Bangladesh (https://www.cia.gov/library/publications/the-world- factbook/fields/2002.html date: December 14, 2015). According to Food and Agricultural Organization (FAO), sunflower ranges second subsequent to soybean as an oil crop in the world. Sunflower oil is a good substitute to overcome this shortage when it is difficult to cultivate other crops due to climate hazards. The market value, easy cultivation and extraction method of this crop encourages farmers to cultivate sunflower. A good number of female farmers are also getting involved in sunflower cultivation on their fallow land since cultivation is easier, cheaper and more profitable. Islamic Relief, Bangladesh (IR,B) is providing technical and monetary help to the farmers for sunflower cultivation in several areas. CSISA-CIMMYT (Cereal Systems Initiative for South Asia-International Maize and Wheat Improvement Centre) Khulna hub has also taken initiative for sunflower production in saline and water limited condition in southern Bangladesh, in collaboration with BARI and Khulna University (http://www.theindependentbd.com/magazine/details/24939/Sunflower-production-in-changing-climate:-Insights-from-farmers-field-laboratory, date: 24.2.2016). The goal is to contribute in achieving food security and reducing malnutrition through increased environmentally sustainable agricultural production. Being a thermo neutral plant sunflower is cultivated as both Rabi and Kharif crop in 16 districts of Bangladesh and the average production is about 1.2 t/ha, which is relatively encouraging. Barisal, Khulna, Shatkhira, Bagerhat are the main sunflower cultivation area of this country. It can fill up the gap between production and consumption of edible oil by expanding the area of sunflower production (http://www.dhakatribune.com/feature/2015/nov/04/sunflower-production-changing- climate, date: 26.2.2016).

1.7 Production constraints of sunflower

Sunflower is grown in frost-free subtropical or tropical regions of the world. Hot, humid summer weather increases the risk of fungal diseases. However, the plant is also vulnerable to summer winds and heavy rainfall. The production of sunflower is influenced by several biotic and abiotic factors. Sunflower is more resistant to abiotic stresses, than other field crops (Skoric, 2016). It is very sensitive to biotic stresses. Concerning biotic stresses, the most serious diseases of sunflower is caused by fungal, bacterial and viral attack. Especially during rainy season, when there is high humidity in the nature, sunflower is susceptible to severe damage by fungal diseases. According to National Sunflower Association, around 30 diseases of sunflower crops have been identified. Diseases like Powdery Mildew, Bacterial Leaf Spot, Charcoal Rot, Rhizopus Head Rots, Root and Collar Rots, Phoma Black Stem, Sunflower Rust, Southern Blight, Sclerotinia Wilt etc. are the common diseases of sunflower. Besides these microbial attacks, sunflower seeds are also destroyed by moths, weevils, black maize beetle, Astylus Beetle, American bollworm etc. (National Sunflower Association, 2015).

In addition to biotic stresses, sunflower plants also face the threat of abiotic stresses. The main abiotic stresses that affect sunflower production include drought, salinity, and freezing temperature. Though sunflower plants can be grown at a wide range of temperature, freezing temperatures may injure the crop. Extremely high temperatures have been shown to lower oil percentage, seed fill and germination (Putnam *et al*,. 1990). Haq and coworkers (2013) reported that the morphological, physiological and chemical characteristics of sunflower are radically affected by salinity (NaCl) stress like any other C3 crops (e.g. wheat, soybean, peanut etc.) This consequence is observed beyond genotypes under all levels of salinity (upto 4.8 dS m^{-1}). Normally sunflower is classified as moderately tolerant to salinity. Seed yield is somewhat unaffected by soil salinity upto 4.8 dS m^{-1}. But increased salinity level can reduce its yield by 5% (Francois, 1996). The effects of these stresses can have a devastating impact on plant growth and yield under field conditions.

1.8 Biotechnological approaches

Sunflower is considered as one of the most important oil seed crops in the world. The seed of sunflower is an incredible source of protein and other nutrients. Because of its

commercial value, the necessity of good quality sunflower oil is rapidly increasing. However, certain diseases and other environmental influences can reduce the yield and consistency of this crop (Witrzens *et al*., 1988). There is a need for the development of improved sunflower genotypes. In recent years, biotechnological approaches like: tissue culture and gene transfer systems have been used for improvement of sunflower. But these techniques are mostly limited by the tissue culture response of commercial varieties (Ozyigit *et al*., 2007). Therefore, to overcome such limitations, it is important to establish an efficient and reproducible tissue culture and plant regeneration protocol (Rao *et al*., 1999; Abdoli *et al*., 2007).

Several progresses have been made by different authors regarding *in vitro* regeneration of sunflower but effective regeneration protocol is still limited till date (Liu *et al*., 2011). It may be due to the fact that the regeneration is the most important and difficult part of sunflower tissue culture system (Bayraktaroglu and Dağüstü, 2011). Besides, sunflower 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).

Sunflower regeneration is possible by either organogenesis or somatic embryogenesis (Carter *et al*., 1999; Abdoli *et al*., 2007). *In vitro* regeneration of sunflower depends on many factors, including high variability between genotypes, age and type of explant, culture condition, media composition, gelling agent, sucrose concentration, plant own hormones, cytoplasmic effect and nucleo-cytoplasmic interaction etc. (Ozyigit *et al*., 2002, 2006; Abdoli *et al*., 2007; Liu *et al*., 2011; Aurori, 2011; Bayraktaroglu *et al*., 2011).

Researchers reported different explants including immature embryos, mature embryos, anthers, buds, meristems, leaves, shoot apices, hypocotyls, cotyledons and cotyledonary petioles for both direct and indirect regeneration of sunflower plant (Witrzens *et al*., 1988; Gurel and Kazan 1998; Ozyigit *et al*., 2002, 2006). Response of various explants has been compared by Aurori, (2011) and reported that intact embryonic axis explant has high regeneration potential in sunflower. In most of the studies of sunflower regeneration, immature embryos have been used more frequently than mature embryos (Ozyigit *et al*., 2006). Though genotype has remarkable effect on tissue 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).

Similarly, Bayraktaroglu and Dagustu (2011) performed *in vitro* regeneration with the aim of developing efficient callus and shoot regeneration from different explants of sunflower. They showed that the effect of genotype, explant and interaction between genotype and explant were very important on callus induction and plant development. The effect of genotype on sunflower regeneration was also investigated by Paterson and Everett (1985); Ozyigit *et al*., (2002 and 2007).

Composition of basal medium is also very important on callus induction and plant development Bayraktaroglu and Dagustu (2011). Till date, different types of media, namely, Murashige and Skoog (MS), Linsmaier and Skoog (LS) and Gamborg's B5 were studied for sunflower regeneration; in which MS basal media was reported to be most appropriate for developing plantlets (Mohmand and Quraishi 1994; Ozyigit *et al*., 2005). However, modifications of the MS medium were also tried and successful callus culture was observed on liquid MS medium with BA (1.0 mg/l) alone or in combination with NAA (0.1 mg/l) (Badigannavar and Kururvinashetti 1996). Chraibi *et al*., (1992) recommended the use of liquid media as it permits better contact between tissue and medium. On the other hand, in a similar study of Carter *et al*., (1999) liquid culture procedure resulted in contamination problems and extensive vitrification of shoots.

Different combination of plant growth regulators in various concentrations were used in sunflower regeneration protocol. MS medium containing BAP as the cytokinin was found to be the best medium for regeneration and 1 mg/l is the most used concentration. NAA is the most popular auxin to be used in sunflower tissue culture studies and the preferred concentration is 0.5 mg/l (Mohmand and Quraishi, 1994; Ozyigit *et al*., 2007 and 2014; Bayraktaroglu and Dagustu, 2011). An increase in the concentrations of cytokinin level inhibit root formation since higher cytokinin level produces excessive callus and failed to increase the efficiency of shoot multiplication. The optimal range for multiplication is 0.1-1 mg/l (Peterson, 1984).

Ozyigit *et al*., (2014) established plant tissue culture systems on MS media supplemented with various plant growth regulators. They reported MS media with 1 mg/l BAP, 0.5 mg/l

NAA is the best combination for highest shoot regeneration using hypocotyl explant. MS media supplemented with hormones, like BAP, NAA, 2, 4-D and even GA_3 were found to enhance plant regeneration efficiency (Badigannavar and Kurkurvinnashetti, 1996; Ozyigit *et al*., 2007; Elavazhagan *et al*., 2009). Influence of different levels of Kinetin, IAA and Zeatin were also tested in different experiments of Witrzens *et al*., (1988) but no consistent results were found from the above mentioned hormones. They assessed that in sunflower tissue culture, auxin should be avoided during first stage of callus induction. Addition of auxin stimulates callus growth but it eventually suppresses the regeneration of plantlets. They also mentioned about premature flower-heads formation from immature embryo and occurrence of vitreous plants. They showed that use of phenolic glycosides like phloridzin, naringin and esculin hydrate significantly reduced the above mentioned difficulties.

Effect of Gibberellic Acid (GA_3), a natural plant hormone has also been studied for sunflower tissue culture. GA_3 is known to be associated with cell enlargement and division which leads to shoot elongation and development. Gibberellin (GA_3) and silver nitrate ($AgNO_3$) effects were evaluated by Carter *et al*., (1999). They used MS basal media containing 0.01 mg/l 1NAA, 0.5 mg/l IBA, and GA_3 at (0, 0.1, 0.5 and 1.0 mg/1). Higher concentrations of GA_3 (0.5 and 1.0 mg/1) tends to produce thin, pale, and over-elongated shoots which did not survive when rooted and transplanted to soil. But a low level of GA_3 (0.1 mg/1) appeared to improve shoot elongation and development (Power, 1987; Schrammeijer *et al*., 1990; Dong and Jia, 1991; Malone-Schoneberg *et al*., 1994; Witrzens *et al*., 1998; Carter *et al*., 1999).

Some sunflower genotypes appear to root readily, but others suffer from callusing and vitrification, resulting inhibition of rooting (Knittel *et al*., 1991; Cerani*et al*., 1992; Chraibi *et al*., 1992b; Greco *et al*., 1994; Wingender *et al*., 1996). Sarrafi *et al*., (1996) studied genetic analysis of organogenesis in sunflower and observed the effect of genotype on both shoot and root regeneration. In a study, Carter and his colleagues, (1999) found that root induction is also affected by high auxin and cytokinin concentrations, which in contrast, are required for maximum shoot initiation. Ozyigit *et al*., (2006) stated that for root induction, 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. Rooting were investigated on MS salts with 30 g/l sucrose, 200 mg/l glutamine, 10 mg/l of

thiamine, 0.1 mg/l of GA_3 and 0.5 mg/l NAA containing medium by (Wingender *et al*., 1996). Whereas Carter *et al*., (1999) used a 2-layer rooting medium, with ½ MS plus 1-2mg/l NAA on the top and lower layer was prepared with activated charcoal. Activated charcoal has been used in rooting media by Chraibi *et al*., (1992). Due to its ability to adsorb inhibitory compounds and reduce the availability of plant growth regulators it can prevent callus formation and decrease the time required for rooting, as observed by Witrzens *et al*., (1988) and Carter *et al*., (1999). Activated charcoal 0.5mg/l with ⅓ MS media containing 1 mg/l NAA 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).

1.9 Objectives

Tissue culture technique of sunflower has been under investigation since the 1980s. Different sunflower regeneration protocol has been reported by different researchers. But in Bangladesh, no complete protocol has been established until now for the local sunflower varieties. Therefore, the aim of the present study was to establish an efficient tissue culture mediated plant regeneration protocol for locally grown sunflower varieties of Bangladesh. Hence, two farmer popular local varieties, BRAC Hysun 33 and BARI Surjomukhi 2 were used for *in vitro* regeneration in this study. Embryonic axis and hypocotyl were the two explants used from both of the varieties. Therefore, the specific objectives considered for this study were:

- Determination of the best explant for *in vitro* regeneration
- Determination of optimum hormone supplementation for shoot formation
- Observation of the effect of different hormones on shoot elongation
- Observation of the effect of hormone concentration on root induction
- Optimization of the acclimatization stage of regenerated plantlets

Chapter 2: Materials and Methods

2. Materials and Methods

2.1 Plant materials

Seeds of two locally grown Sunflower (*Helianthus annuus* L.) varieties namely, BARI Surjomukhi 2 and BRAC Hysun 33 were used in this study. BARI Surjomukhi 2 seeds were collected from Bangladesh Agricultural Research Institute (BARI) whereas BRAC Hysun 33 seeds were collected from BRAC-Agricultural Research and Development Centre (ARDC). After seed collection they were preserved at 4°C in the Plant Biotechnology Laboratory, BRAC University, Mohakhali, Dhaka, Bangladesh. Some key features of these varieties are noted in Table 2.1.

Table 2.1: Different features of the sunflower varieties (Chowdhury, 2015)

Sunflower variety	Seed shape and color	Plan height (cm)	Sowing time	Maturation time (days)	Yield (ton/ha)	Yield constraints
BARI Surjomukhi 2	Oval Black	125 - 140	Mid December - mid January	90-100	1.4 - 2.0	Leaf blight disease and hairy caterpillar pest
BRAC Hysun 33	Tear – drop Bluish black	90 - 110	October - December	120-140	1.4 - 1.5	Water logging, rotten diseases

2.2 Methodology

2.2.1 Preparation of stock solutions for MS media

In the present study, Murashige and Skoog (MS) medium (1962) in full or half strengths were used for *in vitro* regeneration. Different components were required for the preparation of stock solutions for MS media. The components and their respective amounts are listed in Table 2.2.

Table 2.2: Different components for preparation of 1 liter MS

Components	Amount
Macro nutrients	**mg/l**
KNO_3	1900
NH_4NO_3	1650
$MgSO_4 \cdot 2H_2O$	370
$CaCl_2 \cdot 2H_2O$	440
KH_2PO_4	170
Inorganic micro element	**mg/l**
KI	0.83
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Fe-EDTA	**mg/l**
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic	**mg/l**
Nicotinic acid	0.5
Pyridoxin HCI	0.5
ThaiminHCl	0.1
Glycin	2.0

2.2.2 Macro nutrients stock solution preparation (10X)

This stock solution of macro-nutrients was made 10 times the concentration of the full strength medium. For this, all the components (Table 2.2) were weighed accurately and serially dissolved in distilled water. The final volume (1000 ml) was made by adding more distilled water. Next, the solution was poured into a labeled glass container and autoclaved (Model: WAC-47, Korea). Once ready, it was stored in a refrigerator at 4ºC for several weeks.

2.2.3 Micro nutrients stock solution preparation (100X)

The components were weighed 100X of the amount listed in Table 2.2 then added one after another and stirred using a magnetic stirrer. Final volume (1000 ml) was made by adding distilled water. The solution was stored at 4ºC for a few weeks. The resulting solution was 100 times the full strength.

2.2.4 Iron-EDTA stock solution preparation (100X)

The solution was made 100 times of their full strength. $FeSO_4.7H_2O$ (2.78 g/l) was added and stirred in hot plate till dissolved. After that $Na_2EDTA.2H_2O$ (3.73 g/l) was added and stirred till dissolved. The solution was made 1 liter and was preserved at 4ºC in amber bottle as it is light sensitive.

2.2.5 Organic stock solution preparation (100X)

The components (100X of the mentioned amount in Table 2.2) of organic nutrients were added one-by-one. Using magnetic stirrer solution was stirred well for some time before adding next component. Then the final volume (1000 ml) was made and after autoclaving the solution was stored in a refrigerator at 4ºC.

2.2.6 Preparation of stock solution for growth regulators (1 mg/10 ml)

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

i. Auxins
   - Napthalene acetic-acid (NAA)
   - Indole-3-butyric acid (IBA)

ii. Cytokinins
   - 6-Benzyl amino purine (BAP)

iii. Gibberellic Acid (GA_3)

Preparation of all the growth regulators is almost similar. To prepare any of the above mentioned hormonal stock solutions, 10 mg of hormone powder was taken in a clean beaker and dissolved with the complementary solvent listed in Table 2.3. The dissolved mixture was then made upto 100 ml in volume by adding distilled water. This will produce 1 mg/10 ml of hormone stock solution. The solution was then sterilized by syringe filter and preserved in a refrigerator at 4°C for several weeks.

Table 2.3: Growth regulators and their solvents

Growth regulators	Solvents	Molecular weight
NAA	1 N NaOH	186.21
IBA	1 N NaOH	203.24
BAP	1 N NaOH	225.3
GA_3	EtOH	346.4

2.2.7 Preparation of full strength MS medium

Murashige and Skoog (1962) medium (MS) was used as basal tissue culture medium. The components and amounts used are listed in Table 2.4.

Table 2.4: Different components and amount for preparation of 1 liter MS medium

Components	Amounts (for 1000 ml)
Macronutrients (10x)	100ml
Micronutrients (100x)	10 ml
Vitamin/Organic (100x)	10ml
Fe- EDTA (100x)	10 ml
Myo-inositol	0.1g
Sucrose	30g

All the components (Table 2.4) were added in a conical flask and volume was made upto one liter with ddH_2O. The pH was adjusted to 5.8 with 1N NaOH or 1N HCl as needed. For preparing solid media, agar (Sigma) was added in 0.7% (w/v) ratio. The whole mixture was heated in a microwave oven (Model: MH6548SR, LG, China). Next, the medium was distributed into conical flasks and sealed and finally autoclaved. The flasks were stored in the culture room at 25 ± 2°C for few days before use.

2.2.8 Preparation of seed germination media

Hormone-free solid MS medium was used for aseptic seed germination and seedling development. Seeds in the germination medium were kept in a dark chamber to emulate the light less conditions underneath soil which is essential for germination to occur.

2.2.9 Preparation of shoot regeneration media

Preparation of regeneration media is similar to basal MS media preparation except appropriate amounts of hormone stock solutions were added to the MS media before sterilization. To determine the effect of phytohormones on shoot regeneration, MS media was supplemented with varying concentrations and combinations of BAP with/without NAA for each media. The combinations used are noted in Table 2.5.

Table 2.5: BAP and NAA combinations used in the present study

Treatments	BAP (mg/l)	NAA (mg/l)
Treatment 1	0.0	5
Treatment 2	1	0.0
Treatment 3	1	0.1
Treatment 4	1	0.5
Treatment 5	1	1
Treatment 6	2	0.0
Treatment 7	2	0.1
Treatment 8	2	0.5
Treatment 9	2	1
Treatment 10	5	0.0

2.2.10 Preparation of media for shoot elongation

To obtain proper shoot length, a shoot elongation medium was used. To prepare this medium for shoot elongation, here, full strength MS medium with 2 mg/l BAP was further supplemented with 0.1mg/l gibberellic acid (GA_3).

2.2.11 Preparation of media for subculture

Regenerated shoots needed to be transferred into fresh media in 15-30 days intervals. Media with same hormonal composition were prepared to subculture the regenerated shoots.

2.2.12 Preparation of root induction media

The basal medium used for rooting was half strength MS media. The media was supplemented with different concentrations (0.2, 0.5 and 1 mg/l) of IBA. For solidification, 0.6% (w/v) phytagel (Sigma) was used.

2.3 Media sterilization

The media containing conical flasks were autoclaved at 15 lbs/sq. inch pressure at 121°C temperature for 20 minutes. The medium was allowed to cool after sterilization and stored at 25 ± 2°C before use.

2.4 Tissue culture

2.4.1 Precaution to maintain an aseptic culture condition

Sterility is an essential criteria to be met before under taking any plant tissue culture procedures. To this end, all trials were carried out inside the Laminar Air Flow cabinet (Model: SCV-4AI, Singapore), which was sterilized by irradiating the work chamber with the in-built Ultra-Violet (UV) light for at least 30 minutes before use. Before starting any work, the workbench was thoroughly cleaned with 70% ethanol spray. Personal hygiene is also important therefore before opening the cabinet hood after irradiation, hands and forearms were cleaned properly using commercial hand sanitizer. This was followed by washing with antimicrobial hand sanitizer (Hexisol®, ACI Ltd.). Contamination was further minimized through the use of surgical masks in each session.

During inoculation and explant preparation, all the necessary instruments (forceps, scalpel, Petri-dishes etc.) were flame sterilized inside the cabinet. The flask and Petri-dish lids were

flamed once after opening and again before closing. All these precautions were taken to obtain maximum contamination-free condition during the work. Any contaminated culture and older non-regenerative plant parts were autoclaved before disposal in accordance to bio-safety rules.

2.4.2 Seed sterilization

As an initial step of tissue culture, seed sterilization has to be done appropriately. These are the steps followed in the sterilization procedure:

a. Seeds with seed coat were taken in autoclaved conical flask and washed twice with autoclaved ddH_2O.
b. Next, they were stirred within 70% ethanol for 3 minutes followed by ddH_2O wash for three more times.
c. After that, the seeds were completely immersed in 14% commercial bleach (Clorox) for 15 - 20 minutes with continuous shaking by hand.
d. The Clorox solution was disposed and the seeds were again washed 3 - 4 times (1 minute for each wash) using autoclaved distilled water until all the bubbles disappeared.
e. To facilitate water imbibition, seeds were soaked in ddH_2O for 24 hours in dark chamber.
f. After 24 hours of soaking, the surface sterilized seeds were again washed with ddH_2O twice and were de-hulled using sterilized scalpel and forceps.
g. To remove the white waxy film from the seed surface they were given two more autoclaved ddH_2O wash.
h. Finally, the sterilized seeds were dried on a filter paper and inoculated in germination media (MS) containing flasks and sealed properly.

2.4.3 Seed germination and seedling development

The sealed flasks were placed inside dark chamber for 1-2 days to emulate the proper conditions for germination (Fig. 2.1). Seeds germinated within 2 days in the dark chamber. Then flasks were shifted to culture room having 25 ± 2°C day-light temperature in a 16 hour photoperiod under white fluorescent light to allow seedling development after germination. Finally, they developed into seedlings (Fig. 2.1) which became capable of yielding explants. The explants were collected from seedlings at different ages (3 - 9 days).

2.4.4 Explant preparation and inoculation

Embryonic axis and hypocotyl were the two explants used from both the varieties. To collect embryonic axis, sterilized seeds were kept soaked in ddH_2O for 24 hours in a dark chamber. After de-hulling, 3mm portions were sectioned out from the proximal part of the seeds and inoculated in regeneration media. The explant hypocotyl was collected from 3, 5, 7 and 9 days-old seedlings. They were cut into 3-5mm pieces and then transferred to media containing flasks. These explants were cultured in MS media with various concentrations and combinations of plant growth hormones (Table 2.5) to achieve both direct and indirect regeneration.

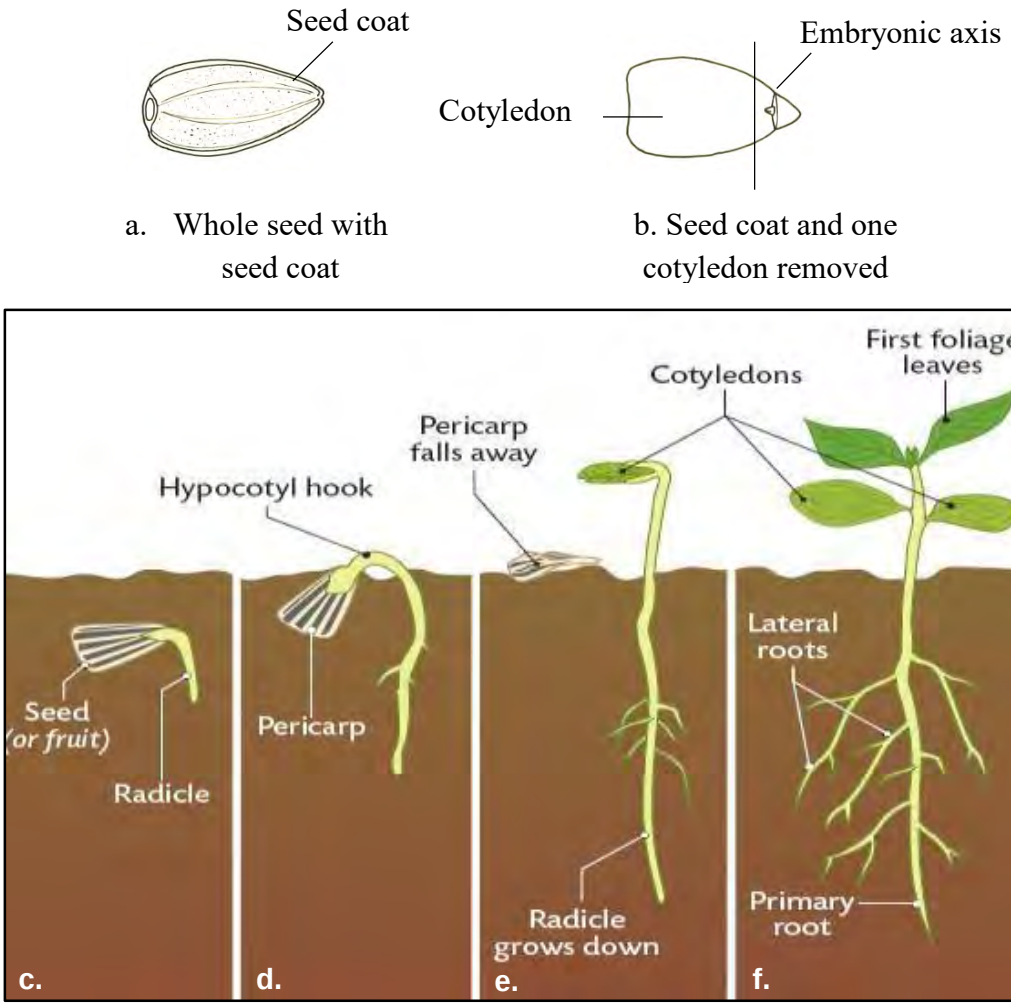

Fig. 2.1: Germination stages of Sunflower. **a-b.** Seed **c-f.** Germination and seedling development. Source: (http://www.biology-resources.com/plants-seeds.html date: 24.3.2015).

2.4.5 Shoot induction

Shoot induction was brought about by inoculating the regenerated explants in shoot regeneration media prepared according to the procedure detailed in Section 2.2.9. Ten different growth combinations were used for each sample. A total of 50 explants per treatment were used, with 5 explants in each flask. For assessment this experiment was repeated four times.

2.4.6 Shoot development

To obtain well developed shoots with proper height, an elongation medium prepared according to the methodology in Section 2.2.10 was used. After shoot induction, explants which were very small in height were transferred to the elongation media and cultured for 3-4 weeks. For assessments, regenerated shoots were monitored regularly.

2.4.7 Subculture

Depending on the regeneration response, all the regenerated explants were transferred to fresh elongation media within 15 to 30 days. This was done to provide sufficient nutrients to the developed shoots for their further development. Cultures were regularly monitored to observe any morphological changes and data collection.

2.4.8 Root induction

Well-developed shoots of 3cm or longer were selected for rooting. Four weeks after initiation, *in vitro* grown shoots were excised and inoculated in test tubes containing rooting media (Section 2.2.12). They were separated by a single sharp cut at the node before inoculation. The basal medium used for rooting was half strength MS medium, which was supplemented with different concentrations (0.2, 0.5 and 1 mg/l) of IBA. Cultures were monitored daily to determine the appearance of roots. Within 2-3 weeks from the initiation, rooted plants became ready to be transferred to the soil.

2.4.9 Transplantation and acclimatization in natural environment

Plants grown in a protected culture condition needed a period of acclimatization for adjustment to the natural environment. It is a critical stage for plantlet regeneration. The following steps were implemented in this procedure:

a) Using large forceps, the regenerated shoots with sufficient roots were carefully separated from the rooting media. To minimize contamination due to attached media remnants, the roots were washed under running tap water. This was done very carefully to keep the root system intact.
b) Next, the plantlets were transferred to small pots containing a sterilized mixture of ground soil and sand at the ratio of 1:2.
c) Pots were covered with transparent perforated plastic bags with water sprayed inside to maintain proper humidity.
d) The pots were kept in the culture room for a period of two weeks to reduce sudden environmental shock. The plastic covering was removed in the second week.
e) In the next week, the plantlets were exposed to natural environment for 2-7 hour intervals and returned to the culture room.
f) Once the plantlets reach maturity (around three weeks after transplantation), they were transferred to larger pots and introduced to the natural environment. The growth and development of the plants was routinely observed afterwards.

Chapter 3: Results

3. Results

The aim of the present study was to establish an efficient tissue culture mediated plant regeneration protocol for locally grown sunflower varieties of Bangladesh. In this experiment, two explants namely, embryonic axis and hypocotyl were used from two varieties namely, BRAC Hysun 33 and BARI Surjomukhi 2. Suitability and reproducible capacity of these explants for *in vitro* regeneration and optimization of different hormonal supplementation were accomplished through repeated trials. Finally, *in vitro* regenerated plantlets were acclimatized in soil following proper hardening stage.

3.1 Analysis of regeneration capacity of two different explants

3.1.1 Explant: Hypocotyl

Hypocotyl explants collected from aseptically germinated seedlings were used to perform *in vitro* shoot regeneration. They were cut into 3-5mm pieces and then the excised explants were inoculated in ten different media compositions for shoot induction and development.

3.1.1.1 Effect of explant age

Explant age refers to the number of days from seed inoculation on germination medium to excision of the hypocotyl explants. In this study, hypocotyl explants were collected from different ages (3, 5, 7 and 9 days) of seedlings to determine the optimum age for regeneration. Collection of 7 and 9 days old explants were difficult because by then the plant parts were no longer young and soft, and hypocotyls matured into hard stems. Still, both 7 and 9 days old explants had been investigated for both the varieties. In BARI Surjomukhi 2 variety, 7 and 9 days old explants only increased in size, but did not show any shoot regeneration response. Enlarged callus with adventitious roots (Fig. 3.1) were observed but shoot or bud formation was not attained in any of the ten treatments. On the other hand, explants of BRAC Hysun 33 variety remained almost unchanged. After 30 days of inoculation, they gradually grew bigger and turned in brown colour and died. The 5 days old explants also showed very poor and slow response and were non-regenerative in both the varieties. Among these three explant ages, in both the varieties, 3 days old hypocotyl showed the highest rate of response with less time requirement for response initiation (Table 3.1).Therefore, 3 days old hypocotyl explants were taken for further experiments.

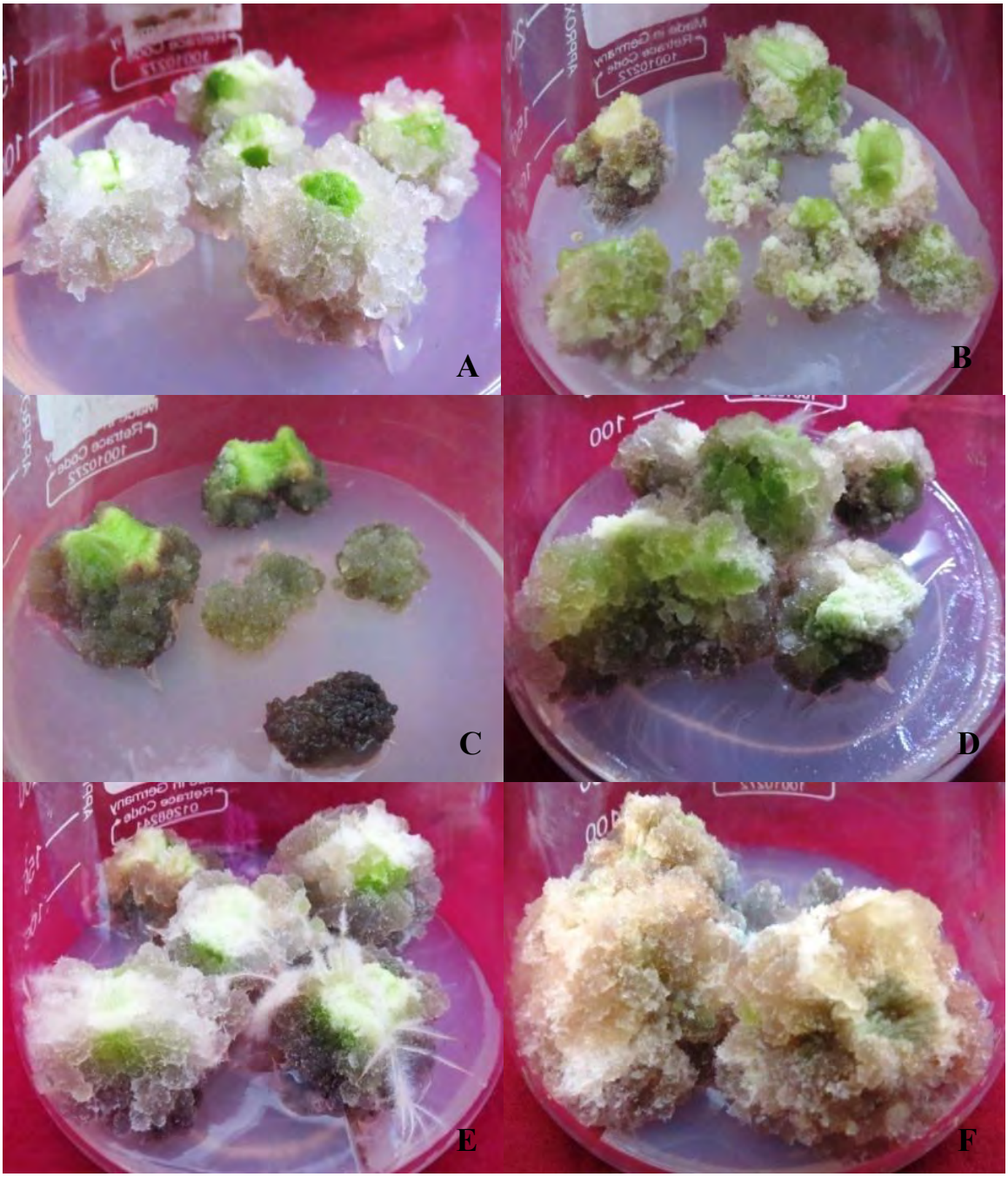

Fig. 3.1: Hypocotyl explant of different ages from BARI Surjomukhi 2 (A,C,E) and BRAC Hysun 33 (B,D,F) varieties. **A-B.** 5 days old hypocotyls inoculated in MS + 5 mg/l BAP supplemented media (Photographed 30 days after inoculation) in BARI Surjomukhi 2 variety. **C-D.** 7 days old hypocotyl inoculated in MS + 2 mg/l BAP **C.** Showing black dead callus; **D.** BRAC Hysun 33 variety formed friable callus (Photographed 45 days after inoculation). **E-F.** 9 days old hypocotyl explants in **E.** MS + 1 mg/l BAP + 0.1 mg/l NAA formed adventitious roots on white callus. **F.** Callus formation in MS + 2 mg/l BAP and 0.5 mg/l NAA (Photographed after 45 days of inoculation).

Table 3.1: Determination of optimum age of hypocotyl explants in relation to *in vitro* response

Varieties	Explant age (Days)	Responsive explant (%) ± SD	Days required for response initiation ± SD	Average length in 30 days (cm) ± SD
BARI Surjomukhi 2	3	94.44 ± 0.58	3.00± 1.00	1.35 ± 0.13
	5	11.11 ± 1.15	_	_
	7	72.22 ± 0.58	10.67 ± 1.53	_
	9	38.89 ± 1.53	_	_
BRAC Hysun 33	3	88.89 ± 1.15	5.33 ± 1.53	0.75 ± 0.25
	5	16.67± 1.00	_	_
	7	55.56 ± 0.58	13.00± 2.00	_
	9	27.78 ± 0.58	19.67 ± 1.53	_

Values obtained from triplicate trials. In each trial 48-50 explants per variety were tested on MS media in ten hormonal combinations.

3.1.1.2 Effect of various hormones on hypocotyl explants

Same hormonal treatments were applied for BARI Surjomukhi 2 and BRAC Hysun 33 varieties. Hypocotyl explants of BARI Surjomukhi 2 produced callus in all the hormone supplemented media. Both types of callus, translucent and friable were formed. Only few of them successfully developed into shoot but the rest remained as callus. In this variety, explants did not show any consistent regeneration response in any of the hormone supplemented media except MS + 1 mg/l BAP where multiple shoot developed through callus. In BARI Surjomukhi 2 variety, shoot regeneration was also observed using 2 mg/l BAP alone and 1 mg/l BAP combining with 0.1 mg/l NAA, but the responses were very low (6.67% and 13.33%). So, ten hormonal combinations, only three showed regeneration response and the rest remained non-regenerative (Table 3.2).

Similar to BARI Surjomukhi 2, explants of BRAC Hysun 33 variety showed almost similar response against most of the hormonal treatments. In MS + 5 mg/l BAP, MS + 1

mg/l BAP + 0.1 or 0.5 mg/l NAA and in MS + 2 mg/l BAP + 0.5 mg/l NAA, explants produced callus but shoot initiation was not observed from them (Fig. 3.2, 3.4). Here, media having 1 mg/l BAP alone produced single shoot within 6 days of inoculation. The regenerated shoot had an average height of 0.5 cm to 1.0 cm. MS medium with this hormonal composition gave the most shoot regeneration response for both the varieties (Fig. 3.2 and 3.3).

If the hypocotyl explants are compared between the two varieties, it can be said that BARI Surjomukhi 2 variety is a better one as the regeneration rate is higher and it also produced healthier shoots in lesser days compared to BRAC Hysun 33 variety (Table 3.2).

3.1.2 Explant: Embryonic axis

To collect this explant, 3mm portions were sectioned out from the proximal part of a sterilized seed and then inoculated in regeneration media for shoot induction. In each trial 50 explants per variety were investigated on MS media with ten hormonal combinations. Using embryonic axis explants the role of various hormonal supplementations upon *in vitro* regeneration was analyzed.

3.1.2.1 Influence of various hormone supplementation on shoot formation

Shoot formation was found to be dependent on hormone concentrations. During this experiment both direct and indirect regenerations were observed in response to different hormonal treatments. Depending on the variety and hormonal supplementation, regeneration initiation from this explant took place within 9 to 12 days.

Good response was found in BRAC Hysun 33 variety, when MS media contained BAP alone. Addition of a low concentration of NAA led to callus formation. It was observed that explants showed less regeneration response when the concentration of NAA was increased (Table 3.3). Single shoot formation was observed in media containing 1 mg/l BAP alone or in combination with 0.1 mg/l NAA. But highest shoot regeneration was found in MS medium supplemented with 2 mg/l BAP (66.67%). Although the shoot regeneration response rate was high but the height of these shoots was short. Highest length recorded was less than 2.0 cm 20 days after inoculation.

Table 3.2: Shoot regeneration efficiency of 3 days old hypocotyl explants from two sunflower varieties in various hormonal treatments

Varieties	Hormones		Responsive explant (%) ± SD	Type of response	Shoot regenerative explant (%) ± SD	Average shoot length in 30 days (cm) ± SD
	BAP (mg/l)	NAA (mg/l)				
BARI Surjomukhi 2	0.0	5	16.67 ± 1.00	Indirect	0	_
	1.0	0.00	94.44 ± 0.58	Indirect	46.67 ± 1.15	1.30 ± 0.26
		0.1	72.22 ± 0.58	Indirect	13.33 ± 0.58	0.70 ± 0.13
		0.5	66.67 ± 1.00	Indirect	0	_
		1.0	61.11 ± 0.58	Indirect	0	_
	2.0	0.0	55.56 ± 0.58	Indirect	6.67 ± 0.58	0.17 ± 0.29
		0.1	38.89 ± 0.58	Indirect	0	_
		0.5	44.44 ± 1.53	Indirect	0	_
		1.0	83.33 ± 1.73	Indirect	0	_
	5.0	0.0	33.33 ± 1.00	Indirect	0	_
BRAC Hysun 33	0.0	5	5.56 ± 0.58	Indirect	0	_
	1.0	0.00	88.89 ± 0.58	Direct	33.33 ± 1.15	0.85 ± 0.38
		0.1	77.78 ± 0.58	Indirect	0	_
		0.5	94.44 ± 0.58	Indirect	0	_
		1.0	72.22 ± 1.15	Indirect	0	_
	2.0	0.0	77.78 ± 0.58	Direct	0	_
		0.1	50.00 ± 1.00	Indirect	0	_
		0.5	88.89 ± 0.58	Indirect	0	_
		1.0	72.22 ± 0.58	Indirect	0	_
	5.0	0.0	61.11 ± 1.53	Indirect	0	_

Values obtained from triplicate trials.

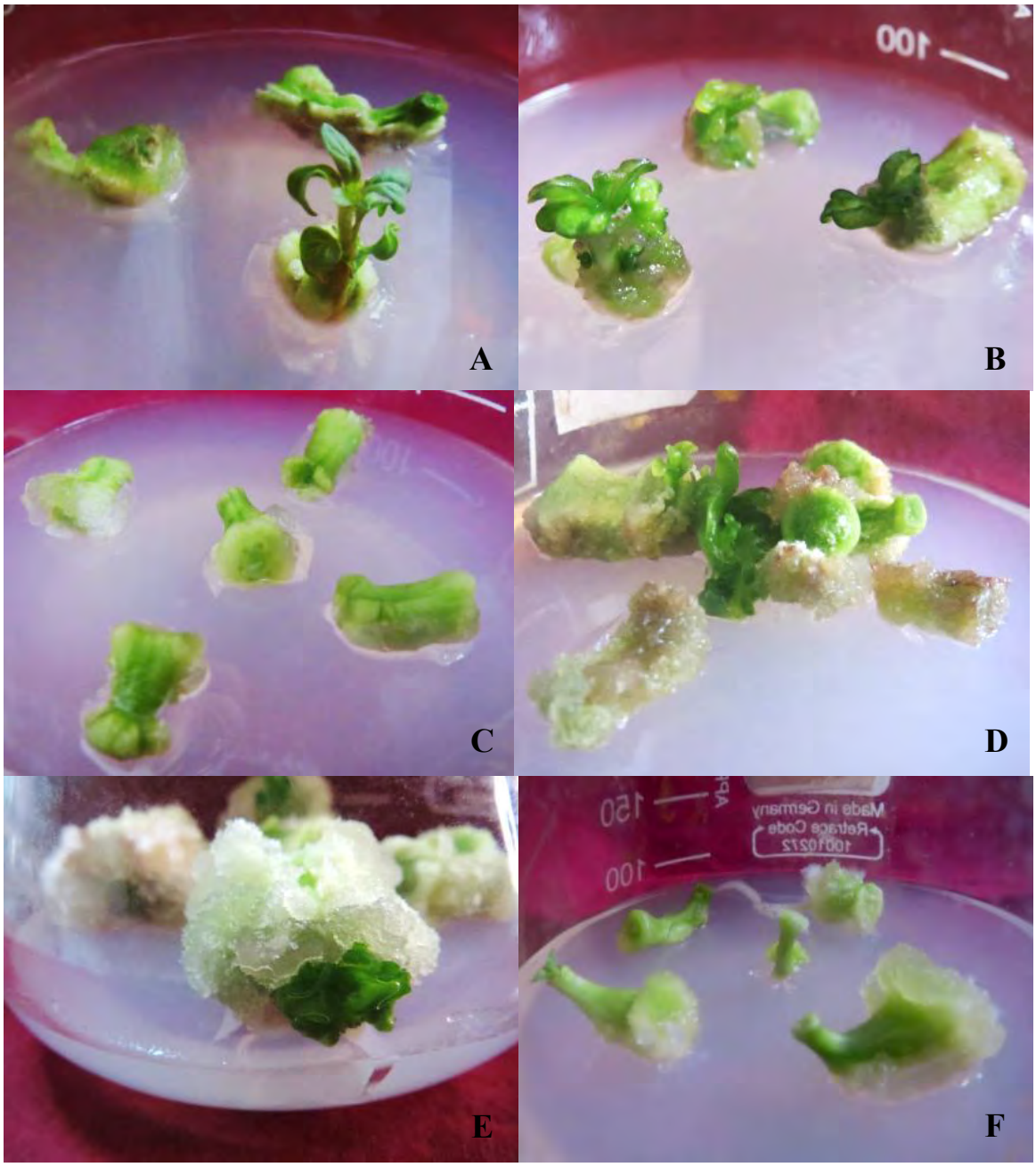

Fig. 3.2: Hypocotyl explants of BARI Surjomukhi 2 variety on various hormone supplemented media. **A.** Successful shoot formation on MS media supplemented with 1 mg/l BAP within 30 days of inoculation. **B.** Indirect shoot regeneration initiation on MS + 1 mg/l BAP and 0.1 mg/l NAA **C.** Callus development on MS media supplemented with 1 mg/l BAP and 0.5 mg/l NAA within 7 days of inoculation. **D.** Indirect regeneration and shoot initiation on MS media with 2 mg/l BAP (Photograph taken 30 days after inoculation). **E.** White callus formation and indirect regeneration in MS media + 2 mg/l BAP and 0.1 mg/l NAA within 10 days of inoculation. **F.** Callus formation in MS media + 5 mg/l BAP.

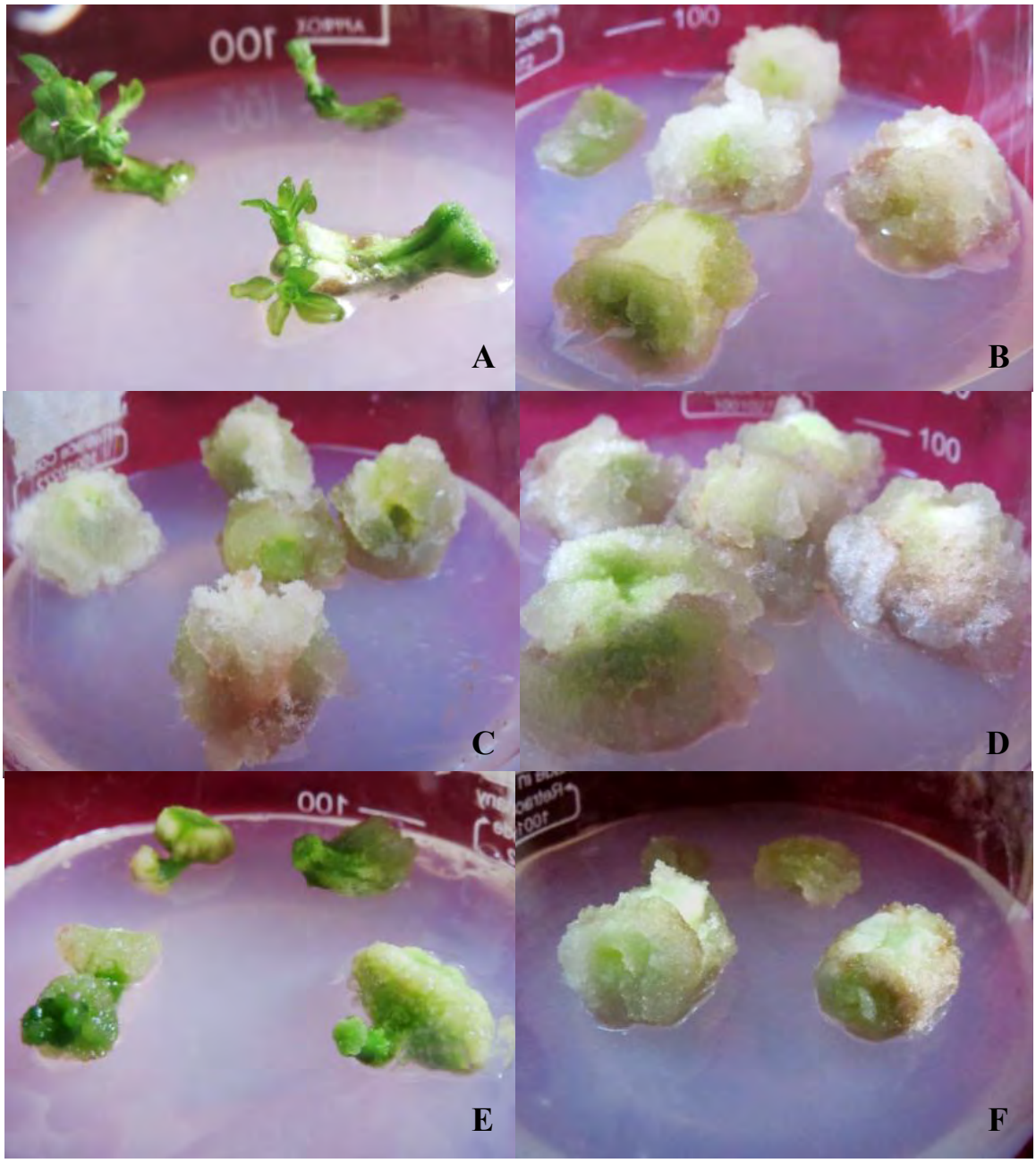

Fig. 3.3: Hypocotyl explants of BRAC Hysun 33 variety on various hormonal treatments. **A.** Direct shoot regeneration initiation in MS media with 1 mg/l BAP within 30 days of inoculation; **B.** Explants size enlargement in MS + 1 mg/l BAP and 0.1 mg/l NAA **C-D.** White, translucent callus produced on 1 mg/l BAP + 0.5 mg/l NAA, and 1 mg/l BAP + 1mg/l NAA supplemented media. (Photographed 35 days after inoculation); **E.** Callus development in MS media supplemented with 2 mg/l BAP; **F.** In MS + 2 mg/l BAP + 0.1 mg/l NAA supplemented media explants grew bigger and formed white callus BAP (Photograph taken 40 days after inoculation).

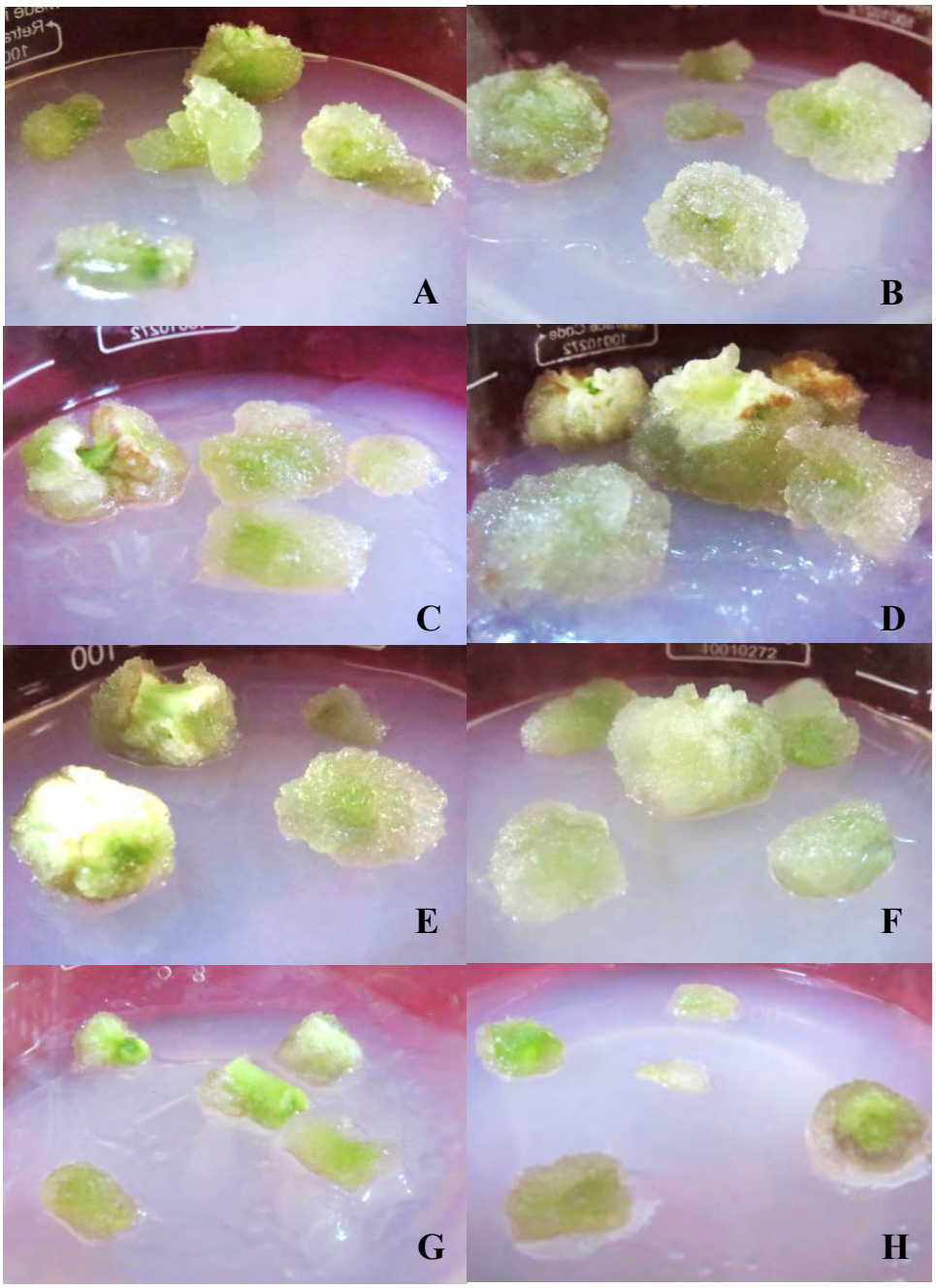

Fig. 3.4: Callus development from hypocotyl explants of BARI Surjomukhi 2 (A,C,E,G) and BRAC Hysun 33 (B,D,F,H) varieties. **A.** 1 mg/l BAP+ 1 mg/l NAA ; **B - C.** 2 mg/l BAP + 0.5 mg/l NAA; **D - E.** 2 mg/l BAP+ 1 mg/l NAA; **F.** 5 mg/l BAP; **G - H.** 5 mg/l NAA hormonal supplementation in MS media (Photographs taken 45 days after inoculation).

Shoot initiation also occurred in other hormonal combinations, such as, MS + 1 mg/l BAP + 1 mg/l NAA and 2 mg/l BAP + 0.1 mg/l NAA. But the regeneration and elongation was very slow. Hormonal treatments containing 2 mg/l BAP combined with 1 mg/l NAA produced only white callus. Callus formation initiated within 10 days. The calluses increased in size and formed a white foam-like layer around them. In both the varieties, explants which were cultured in 1.0 mg/l BAP with 0.5 mg/l NAA and 2.0 mg/l BAP with 0.5 mg/l NAA and 5 mg/l NAA alone formed some undefined structures (Fig. 3.7) of approximately 2 - 4 cm within 30 days of inoculation. They only grew bigger in size but no shoot regeneration was achieved from those.

On the other hand, explants of BARI Surjomukhi 2 variety produced more than 2 cm long shoots in presence of 2 mg/l BAP containing MS medium. Using this hormone composition, 46.67% regeneration was obtained which was initiated 10 days after inoculation (Fig. 3.6). Second best result was found in treatments with 1 mg/l BAP + 0.1 mg/l NAA and only in 5 mg/l BAP. Shoot formation was also initiated in 1 mg/l BAP with or without 0.5 mg/l NAA and 2 mg/l BAP with 0.1 mg/l NAA supplemented media. But the shoot regeneration rate in these compositions was very low (Table 3.3). Both of the varieties formed white, translucent or jelly-like callus during regeneration stage (Fig. 3.5-3.7). Variation was observed in BARI Surjomukhi 2 variety, where low BAP concentration initiated direct regeneration, but higher BAP supplementation gave callus formation.

If compared, embryonic axis explants of BRAC Hysun 33 variety, showed higher regeneration frequency than BARI Surjomukhi 2 variety (Table 3.3).

Table 3.3: Effect of different hormonal supplementations on *in vitro* shoot regeneration from embryonic axis explants of two Sunflower varieties

Varieties	Hormones		Responsive explant (%) ± SD	Type of response ± SD	Shoot regenerative explant (%) ± SD	Average length in 30 days (cm)± SD
	BAP (mg/l)	NAA (mg/l)				
BRAC Hysun 33	0.0	5	40.00 ± 1.00	Indirect	0	_
	1.0	0.00	86.67 ± 0.58	Direct	33.33 ± 0.58	0.75 ± 0.05
		0.1	80.00 ± 1.00	Direct	46.67± 0.58	2.18 ± 0.35
		0.5	73.33 ± 0.58	Indirect	0	_
		1.0	73.33 ± 0.58	Indirect	6.67 ± 0.58	_
	2.0	0.0	93.33 ± 0.58	Direct	66.67± 0.58	1.75 ± 0.25
		0.1	73.33 ± 1.15	Both	26.67 ± 0.58	1.75 ± 0.05
		0.5	80.00 ± 1.00	Indirect	0	_
		1.0	86.67 ± 1.15	Indirect	0	_
	5.0	0.0	93.33± 0.58	Indirect	53.33 ± 0.58	1.42 ± 0.14
BARI Surjomukhi 2	0.0	5	13.33 ± 0.58	Indirect	0	_
	1.0	0.00	66.67± 0.58	Both	40.00 0.00	1.58 ± 0.38
		0.1	73.33 ± 1.53	Indirect	13.33 ± 0.58	2.02 ± 0.23
		0.5	53.33 ± 0.58	Indirect	6.67 ± 0.58	_
		1.0	46.67 ± 0.58	Indirect	0	_
	2.0	0.0	86.67± 0.58	Direct	46.67± 0.58	2.50 ± 0.25
		0.1	33.33 ± 0.58	Indirect	26.67 ± 0.58	0.58 ± 0.38
		0.5	40.00 ± 0.00	Indirect	0	_
		1.0	53.33 ± 1.15	Indirect	0	_
	5.0	0.0	86.67 ± 0.58	Indirect	33.33 ± 0.58	1.28 ± 0.65

Values obtained from triplicate trials.

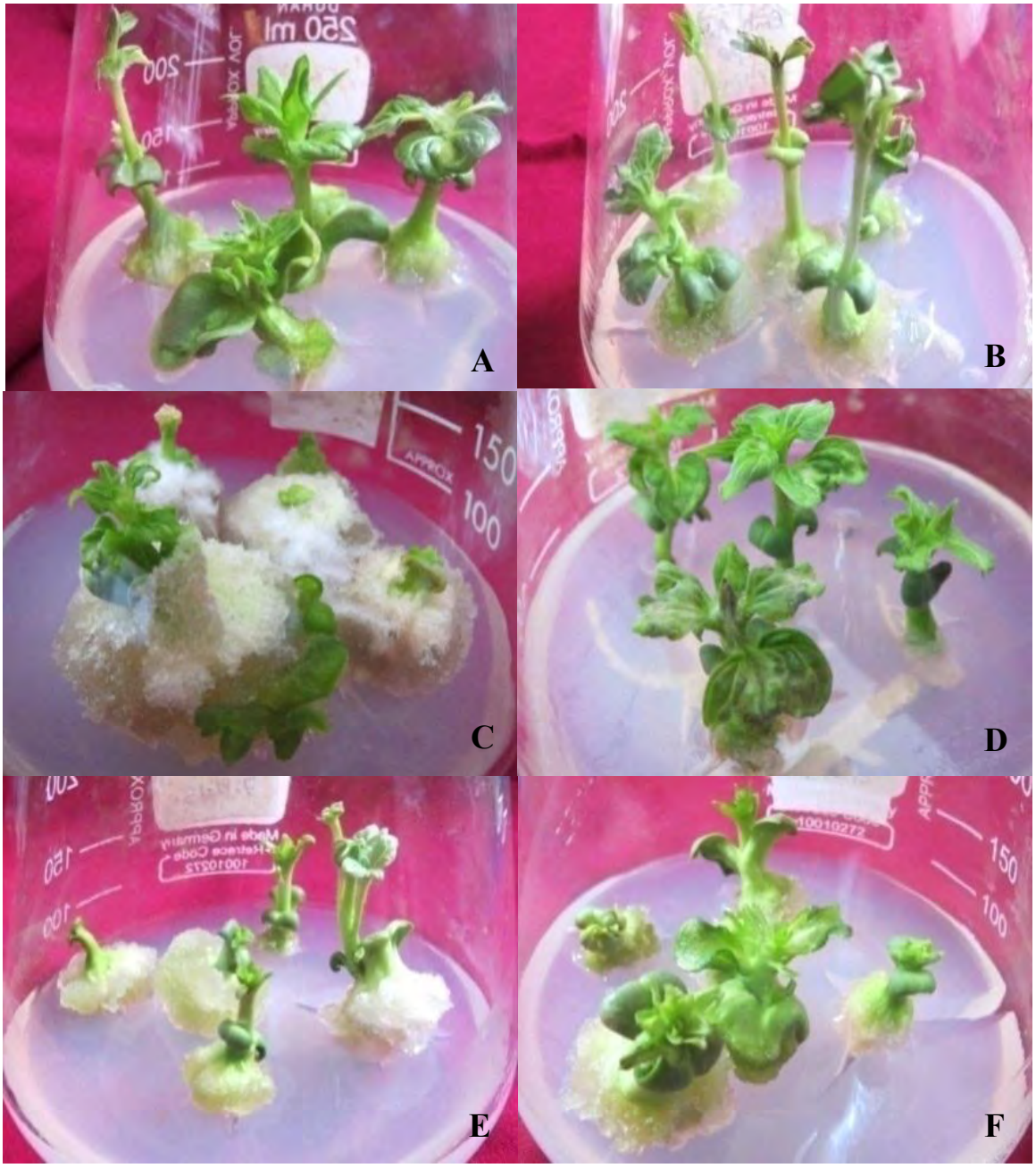

Fig. 3.5: Embryonic axis explant of BRAC Hysun 33 variety on different hormone supplemented media. **A.** Shoot formation at 1mg/l BAP **B.** Long shoot was obtained on MS + 1 mg/l BAP + 0.1 mg/l NAA; **C.** Indirect shoot regeneration on MS + 1 mg/l BAP + 1 mg/l NAA **D.** Shoots regenerated directly at 2 mg/l BAP; **E.** White callus and initiation of shoot on MS + 2 mg/l BAP + 0.1 mg/l NAA **F.** Initiation of multiple shooting on MS + 5 mg/l BAP (Photographs were taken after 20 days of inoculation)

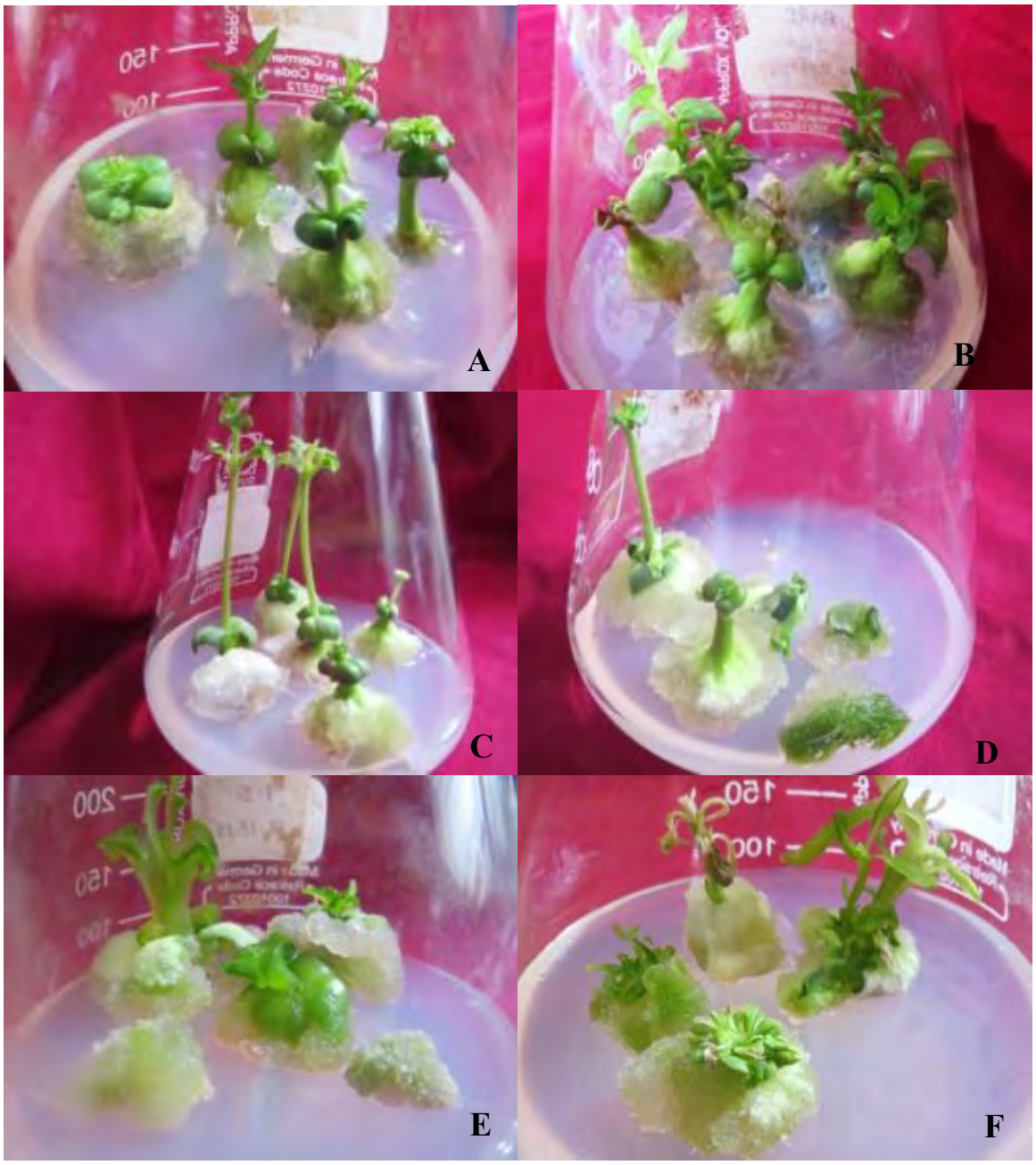

Fig. 3.6: Embryonic axis explant of BARI Surjomukhi 2 variety in various hormone supplemented media. **A-B.** Successful shoot formation at **A.** 1 mg/l BAP; **B**. 2 mg/l BAP **C-E**. Formation of translucent callus and vitrified shoots **C**. 1 mg/l BAP and 0.1 mg/l NAA; **D.** 2 mg/l BAP and 0.1 mg/l NAA **E.** 1 mg/l BAP and 0.5 mg/l NAA; **F.** Multiple shooting initiated on callus at 5 mg/l BAP(Photographs were taken 20 days after inoculation).

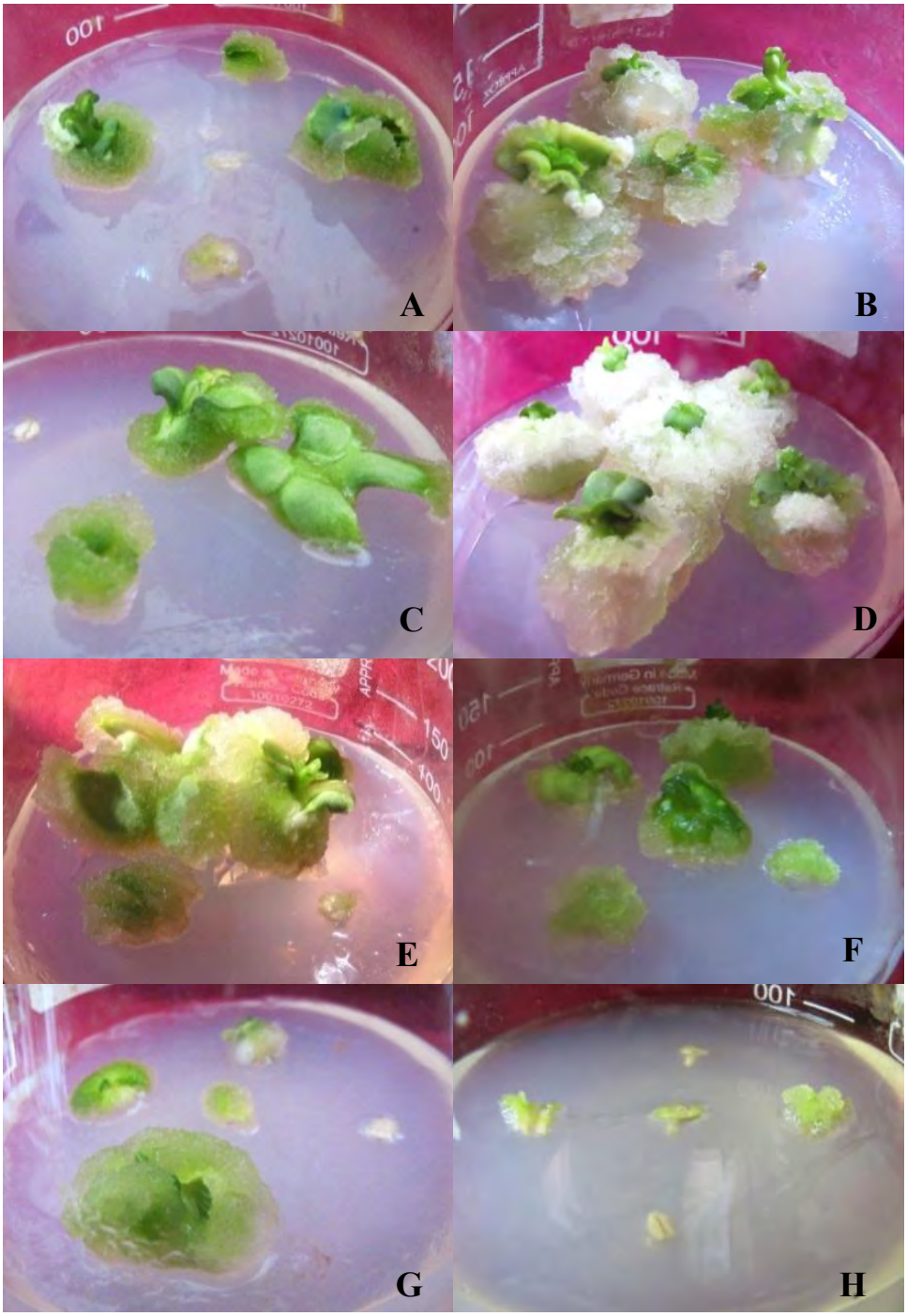

Fig. 3.7: Callus formation from embryonic axis explants of BRAC Hysun 33 (A,C,E,G) and BARI Surjomukhi 2 (B,D,F,H) varieties. **A-B.** Translucent callus in **A.**MS + 1 mg/l BAP and 0.5 mg/l NAA; **B**. MS + 2 mg/l BAP and 0.5 mg/l NAA**; C.** Formation of undefined large structures in MS media supplemented with 2 mg/l BAP and 0.5 mg/l NAA; **D.** White callus formation on MS + 1 mg/l BAP and 0.5 mg/l NAA. **E-F.** Callus formation at MS + 2 mg/l BAP and 1 mg/l NAA **G-H.** On MS + 5 mg/l NAA (Photographs were taken after 45 days of inoculation).

3.2 Elongation and development of regenerated shoots

Shoots regenerated at the previous stage could not produce satisfactory length of the shoots. At this point, to obtain proper shoot length, the regenerated shoots were transferred to a shoot elongation medium. The previous regeneration medium, MS + 2 mg/l BAP was further supplemented with GA_3 at a concentration of 0.1 mg/l. When the explants were cultured in this media composition, the length of stunted stem was enhanced. The presence of GA_3 helped stem elongation, especially in the shoots raised which were usually shorter in size but had a high regeneration frequency (Table 3.4). It was found that, for both the varieties, addition of a low level (0.1 mg/l) of GA_3 to development media did not only affect shoot elongation but also appeared to improve shoot production (Table 3.4). Approximately 3.0- 3.5 cm long, healthier and thicker shoots were produced using this elongation medium. Interestingly, spontaneous root occurred at this treatment along with enhancement of shoot length. This length was recorded after 20 days of inoculation. However, variation was noticed between the varieties. In BRAC Hysun 33, all the explants showed similar response to this treatment, whereas vitrified, over-elongated or malformed shoots were found for some explants of BARI Surjomukhi 2 variety (Fig. 3.8).

3.3 Seasonal influence on initiation of multiple shoots

A notable response was found regarding multiple shoot formation. At the end of December, MS media containing either 2 mg/l BAP or 5 mg/l BAP gave multiple shooting for these two Bangladeshi sunflower varieties (Fig. 3.5, 3.6). But using the same hormonal supplementation significant variation was seen in different seasons of the year. During mid-December to January, in both of the varieties, frequent multiple shoot formation was found but no consistent response was observed during the other time of the year.

Table 3.4 Response of embryonic axis explants from two sunflower varieties on elongation media

Varieties	Media composition	Responsive explant (%) ± SD	Shoot regenerative explant (%) ± SD	Average length in 30 days (cm) ± SD	Spontaneous rooting
BRAC Hysun 33	MS + 2 mg/l BAP	93.33 ± 0.58	66.67 ± 0.58	1.75 ± 0.25	_
	MS +5 mg/l BAP	86.67 ± 0.58	60.00 ± 1.00	1.42 ± 0.14	_
	MS + 2 mg/l BAP + 0.1 mg/l GA_3	93.33 ± 0.58	53.33 ± 0.58	3.02 ± 0.23	Thick tap root
BARI Surjomukhi 2	MS + 2 mg/l BAP	86.67 ± 0.58	46.67 ± 0.58	2.50 ± 0.25	_
	MS +5 mg/l BAP	80.00 ± 0.00	33.33± 0.58	1.28± 0.36	_
	MS + 2 mg/l BAP + 0.1 mg/l GA_3	86.67± 0.58	53.33 ± 0.58	3.10 ± 0.65	Thin lateral root

Values obtained from triplicate trials.

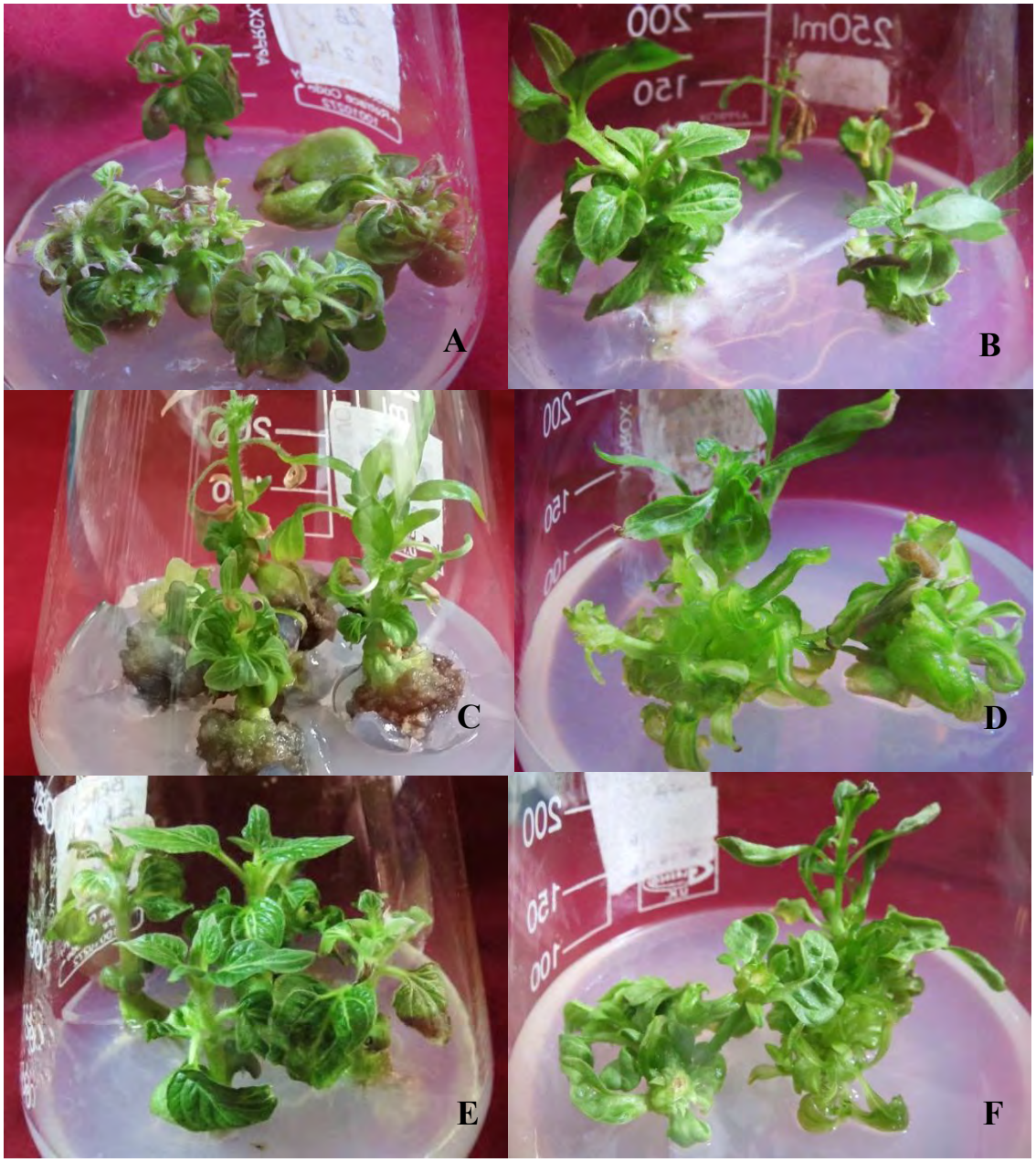

Fig. 3.8: Shoot regeneration from embryonic axis explants of BRAC Hysun 33 (A,C,E) and BARI Surjomukhi 2 (B,D,F) variety on various hormone treatments. **A-B.** Direct shoot regeneration at 2 mg/l BAP; **A.** Showing stunted growth of BRAC Hysun 33 variety; **B.** Formation of white lateral roots in BARI Surjomukhi 2 variety. **C.** Indirect shoot regeneration at 5 mg/l BAP. **D.** Vitrified shoots formation at 5 mg/l BAP; **E**. Elongated, healthier shoot on MS + 2 mg/l BAP+ 0.1 mg/l GA_3**. F.** Abnormal morphology of BARI Surjomukhi 2 explants in MS + 2 mg/l BAP+ 0.1 mg/l GA_3 (Photographed after 30 days of inoculation).

3.4 Effect of various hormone concentrations on root formation

With regard to the rooting abilities of the explants, it was found that, shoots derived from 2 mg/l BAP and 0.1 mg/l GA_3 containing media, rooted readily on the regeneration medium. In this media composition, strong tap roots were observed in BRAC Hysun variety, while BARI Surjomukhi 2 formed some thin lateral roots (Fig. 3.9). In contrast, the shoots regenerated at 2 mg/l BAP supplementation (without GA_3) did not root spontaneously. Some of them suffered from callusing and vitrification, resulting inhibition of rooting. Therefore, to induce root formation, well-developed shoots were cultured on three different concentrations (0.2, 0.5 and 1 mg/l) of IBA.

In presence of root inducing media, no significant differences were found among the varieties during rooting but variation was observed among the concentrations of IBA tested (Table 3.5). Plants formed fibrous and tap roots depending on the hormone concentrations. In BRAC Hysun 33 variety, thin, white tap roots were observed in 0.2 mg/l and 1 mg/l IBA supplemented media (Fig. 3.10). Some long and slender root formation was also observed with this variety using 0.2 and 0.5 mg/l IBA. But the quality of those roots was very poor; as a result these plantlets could not survive when transferred to the soil. Using these IBA concentrations root formation was also attained in BARI Surjomukhi 2 variety. Most of them were fibrous roots. Some of the plantlets of this variety produced callus in the rooting media, thus root formation was not achieved from those shoots. At 1 mg/l IBA supplementation, both the varieties gave many thick roots. Among the two varieties BRAC Hysun 33 variety rooted in lesser time (Table 3.5).

Table 3.5: Effect of different concentrations of IBA on *in vitro* root formation

Varieties	Conc. of IBA (mg/l)	No. of shoots inoculated	Root producing shoots (%) ± SD	Days required for root initiation ± SD	Type of root	Average root length in 20 days (cm) ± SD
BRAC Hysun 33	0.2	10	55.56 ± 0.58	8.33 ± 1.53	Tap root	0.42 ± 0.72
	0.5	10	86.67 ± 0.58	5.00 ± 0.58	Fibrous	2.00 ± 0.25
	1.0	15	93.33± 0.58	5.67 ± 0.58	Both	3.25 ± 0.43
BARI Surjomukhi 2	0.2	9	33.33 ± 1.00	15.6 ± 1.15	Tap root	1.68 ± 0.39
	0.5	15	73.33 ± 0.58	11.33 ± 1.53	Fibrous	2.42 ± 0.38
	1.0	15	86.67± 0.58	12.00 ± 0.00	Fibrous	3.18 ± 0.35

Values obtained from triplicate trials. Shoots derived from MS + 2 mg/l BAP.

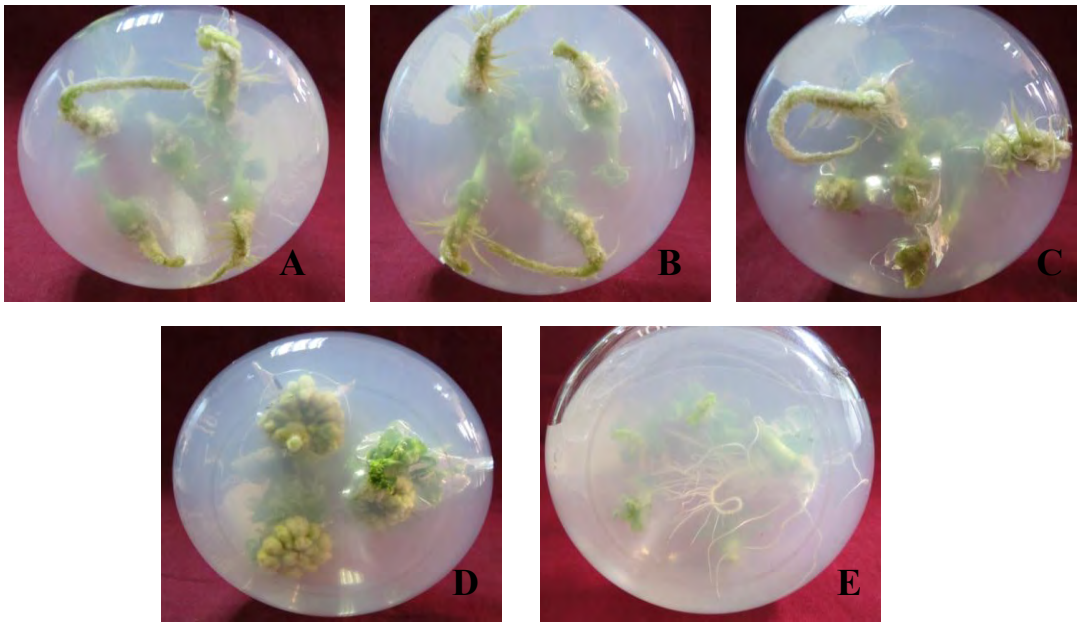

Fig. 3.9: Spontaneous root formation in 2 mg/l BAP and 0.1 mg/l GA_3 containing media. **A-C.** Thick tap roots formed in BRAC Hysun variety. **D-E.** BARI Surjomukhi 2 variety formed callus and thin lateral roots.

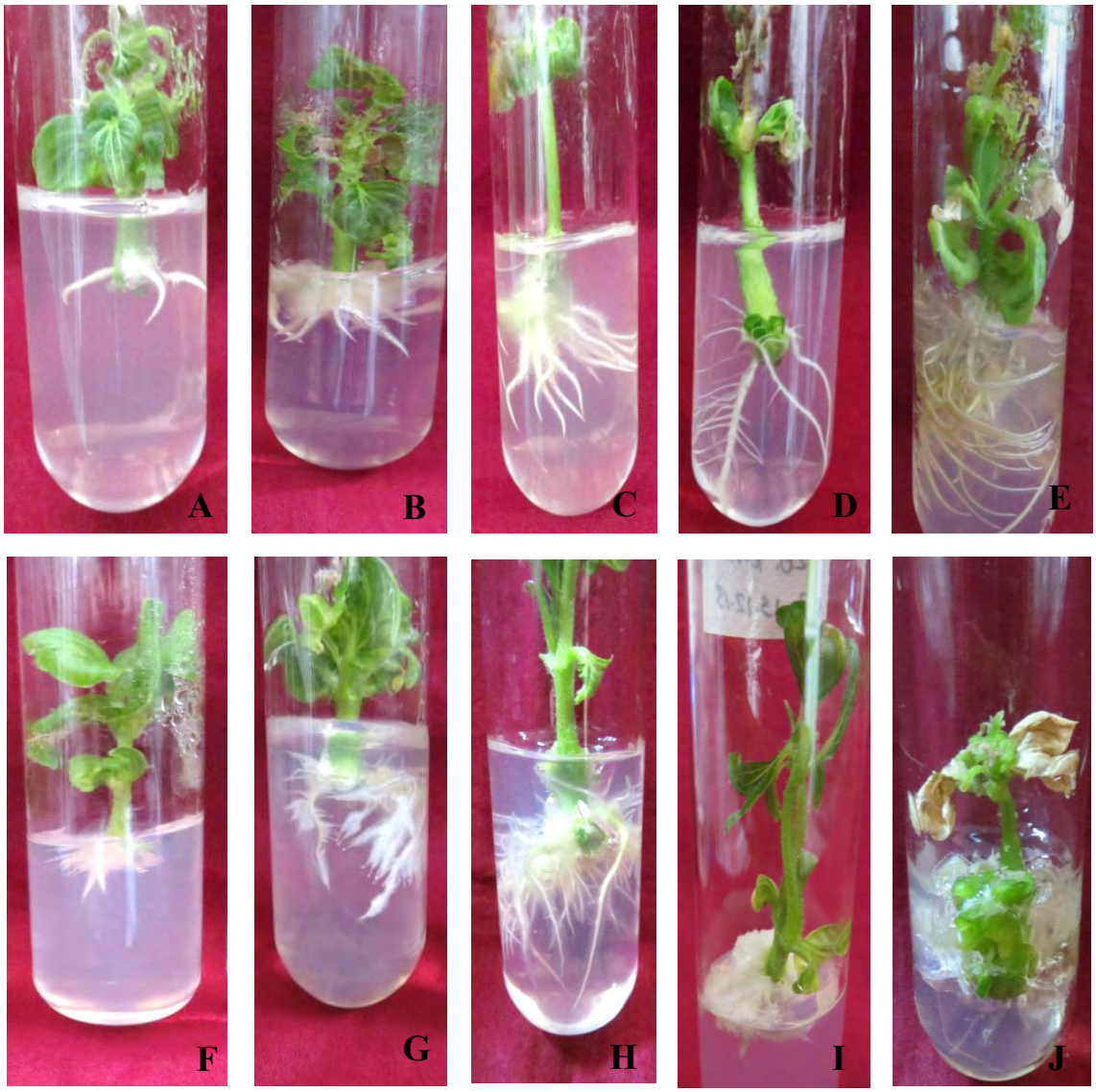

Fig. 3.10: Effect of IBA on root formation in BRAC Hysun 33 (A-E) and BARI Surjomukhi 2 (F-J) varieties. **A.** Induction of tap roots in ½ MS + 0.2 mg/l IBA, **B.** Fibrous roots formation from BRAC Hysun 33 variety in ½ MS + 0.5 mg/l IBA; **C.** Showing white tap roots in ½ MS + 1 mg/l IBA; D-E. Thin slender root formation **D.** 0.2 mg/l IBA; **E.** 0.5 mg/l IBA. **F-H**. Fibrous root formed from BARI Surjomukhi 2 variety at **F.** 0.2 mg/l IBA; **G.** 0.5 mg/l IBA and **H**. 1 mg/l IBA. **I.** Vitrification in BARI Surjomukhi 2 variety in ½ MS + 0.5 mg/l IBA, **J.** Callus formation in 0.2 mg/l IBA.

3.5 Transplantation and acclimatization in natural environment

After sufficient root development, mature plantlets of both the sunflower varieties were transplanted to small pots containing a sterilized mixture of ground soil and sand. Pots were covered with transparent perforated plastic bags and kept in the culture room to reduce sudden environmental shock. A total of 45 plantlets were taken for hardening, where BRAC Hysun 33 variety showed better survival rate than BARI Surjomukhi 2. Following transplantation, maximum success was obtained in BRAC Hysun 33 variety (Fig. 3.11, Table 3.6). The survived plantlets grew to maturity within 2-3 weeks and lived more than one month. After that matured plantlets were transferred to larger pots for their further growth and development. Some of the plantlets of both the sunflower varieties showed premature flower bud. Addition to this some shoots of BARI Surjomukhi 2 variety showed top dying nature when transferred to soil. As a result they could not survive.

Table 3.6: Survival rate of regenerated plantlets in natural environment

Sunflower varieties	**No. of plantlets taken for hardening**	**Successfully hardened plantlets**	**Survival rate in hardening** (%)	**No. of plantlets transferred to the soil**	**Survival rate in natural environment** (%)
BRAC Hysun 33	25	11	45.83	4	36.36
BARI Surjomukhi 2	20	7	36.84	1	14.29

Fig. 3.11: Hardening and acclimatization stage of regenerated plantlets. **A-C.** Plantlets of BRAC Hysun 33 variety; **D-F.** Top dying in BARI Surjomukhi 2 variety. **G**. Vitrified plantlet. **H-I**. Flower formation and matured plants of BRAC Hysun 33 variety.

3.6 Comparative analysis of regeneration efficiency of two sunflower varieties

In the present study the whole regeneration system was established for BARI Surjomukhi 2 and BRAC Hysun 33 varieties using hypocotyl and embryonic axis as explants. Shoot regeneration rate and also root forming capacity is better in BRAC Hysun 33 variety compared to BARI Surjomukhi 2 variety. It also produced healthier shoots in lesser days which survived longer period of time in the natural environment. Among the two explants shoot regenerated from hypocotyl explants failed to generate any roots. Though rhizogenesis was tried with three different concentrations of IBA. However, shoot regenerated from embryonic axis explants rooted in both the varieties. But shoots raised from BRAC Hysun 33 variety had to be treated in elongation medium (MS + 2 mg/l BAP + 0.1 mg/l GA_3) to attain sufficient length before rhizogenesis treatment. Interestingly, spontaneous root occurred at this treatment along with enhancement of shoot length. After analyzing overall regeneration responses it was found that BRAC Hysun 33 variety showed better response compared to BARI Surjomukhi 2 variety. So, it can be concluded that *in vitro* regeneration potential of BRAC Hysun 33 variety is better compared to BARI Surjomukhi 2 variety.

In future further investigation is required to overcome the difficulties that was faced in the present study during acclimatization stage. This will lead to better adaptation of the transplanted plantlet and reproductive development. Thus, complete a full life cycle.

Table 3.7: Comparative analysis of regeneration efficiency of two sunflower varieties

Parameters	BARI Surjomukhi 2		BRAC Hysun 33	
	Hypocotyl	**Embryonic axis**	**Hypocotyl**	**Embryonic axis**
Best media for shooting	MS + 1 mg/l BAP	MS + 2 mg/l BAP	MS + 1 mg/l BAP	MS + 2 mg/l BAP
Responsive explants	94.44 % Including NR-callus	86.67 %	88.89 % Including NR-callus	93.33 %
Time required for shoot initiation	4-11 days	9-15 days	6-20 days	7-12 days
Shooting response	46.6 %	46.67 %	33.33 %	66.67 %
Type of shooting response	Single shoot	Multiple shoots in December. While single throughout the year	Single shoot	Multiple shoots in December. While single throughout the year
Average shoot length in 20 days ± SD	1.30 cm ± 0.26	2.5 cm ± 0.25	0.85 cm ± 0.38	1.75 cm ± 0.25
Elongation medium (RM + 0.1 mg/l GA_3)	Not applied	Applied	Not applied	Applied
Spontaneous rooting	N/A	Thin roots appeared	N/A	Strong tap roots formed
Best media for rooting	Failed rooting	½ MS + 1 mg/l IBA	Failed rooting	½ MS + 1 mg/l IBA
Rooting response	_	86.67 %	_	93.33 %

Parameters	BARI Surjomukhi 2		BRAC Hysun 33	
	Hypocotyl	**Embryonic axis**	**Hypocotyl**	**Embryonic axis**
Time required for root initiation	_	15 days	_	8 days
Survival in hardening	_	36.84 %	_	45.83 %
Survival in soil	_	14.29 %	_	36.36 %
Reproductive duration	_	N/A as they did not survive more than 6 weeks	_	More than 6 weeks

NR = Non-regenerative, RM = Regeneration Media

Chapter 4: Discussion

4. Discussion

The current study was performed in three phases with aim to establish a regeneration protocol for two Bangladeshi sunflower (*Helianthus annuus* L.) varieties. At first, two explants were compared to find out the most suitable and efficient one for *in vitro* regeneration. Then in the second phase optimum hormone supplementation for shoot and root induction were examined. Finally, the adaptation capacity in nature was tested for the regenerated plantlets at the acclimatization stage.

Successful *in vitro* culture of sunflower has been reported from explants, like, immature and mature embryos, hypocotyl, cotyledon, shoot-tip meristem, and root (Gurel and Kazan 1998; Abdoli *et al*., 2007; Aurori, 2011; Bayraktaroglu and Dagustu, 2011; Chowdhury, 2015; Oshin, 2015) etc. Among all the explants, embryos and hypocotyls are most commonly used for *in vitro* regeneration. While working with two Bangladeshi varieties BARI Surjomukhi 2 and BRAC Hysun 33, three explants namely, cotyledon, hypocotyl and embryonic axis were reported Chowdhury (2015), Oshin (2015). Among them hypocotyl and embryonic axis showed response. Therefore, in this study, hypocotyl and embryonic axis explants were selected for analyzing the overall *in vitro* regeneration response of the chosen Bangladeshi varieties (BRAC Hysun 33 and BARI Surjomukhi 2).

Aurori *et al.,* (2011) explained the role of explant age on *in vitro* plant regeneration in sunflower. They reported organogenesis in sunflower is greatly influenced by the developmental stage of the explants. As the age of the explants increases, the morphogenetic capacity reduces drastically. A tissue culture protocol has been reported by Ozyigit *et al*., (2014) using 10 days old hypocotyl explants. Mohmand and Quraishi (1994) also used hypocotyl explants in their experiment, where they have used 21 days old seedlings to collect the explant. Using 3 Indian varieties of sunflower, Badigannavar and Kururvinashetti (1996) conducted a similar research with 2-3 days old hypocotyl explants. Recently at Plant Biotechnology lab of BRAC University, a similar project with 5, 7 and 9 days old cotyledon and hypocotyl explants from sunflower varieties BRAC Hysun 33 and BARI Surjomukhi 2 has been initiated by Chowdhury (2015) and Oshin (2015). They reported 7 days old hypocotyls to be the best explants while cotyledon failed to give any regeneration. In the present study 3, 5, 7 and 9 days old hypocotyl explants were tested. In

both BARI Surjomukhi 2 and BRAC Hysun 33 varieties, shooting was obtained with 3 days old hypocotyl explants. Explants collected from 5, 7 and 9 days old seedlings remained non-regenerative on the rest of the media compositions. This is contrasting to previous report. Using the same sunflower varieties, Oshin (2015) also observed similar response for 5 and 9 days old hypocotyl explants. Variation of response may be due to difference of handling.

In addition to explants age, regeneration potential of sunflower is greatly influenced by media composition, culture condition, growth regulators and genotype (Abdoli *et al*., 2007; Aurori *et al*., 2011; Bayraktaroglu *et al*., 2011; Ozyigit *et al*., 2014). Keeping these in mind, the effects of different combinations and concentrations of growth regulators on callus, shoot and root formation was analyzed in the second phase of the study. Shoot regeneration from hypocotyl explants of the tested varieties was ~30% (BARI Surjomukhi 2) to 45% (BRAC Hysun 33). Hypocotyl explants produced shoots only on 1.0 mg/l BAP containing media, showing no regeneration response with either higher cytokinin alone (2.0 and 5.0 mg/l BAP) or in any other treatments with NAA (0.1, 0.5, 1.0, 5.0 mg/l). In these media compositions, explants gradually increased in size and formed callus which further did not show any shoot regeneration response. Using the same sunflower varieties Oshin (2015) observed a different result. In her experiment the best regeneration was obtained on MS medium containing 1 mg/l BAP and 0.1 mg/l NAA. In a similar study, using five Turkish varieties Ozyigit *et al*., (2005) observed shoot regeneration on MS media with 1 mg/l BAP and 0.5 mg/l NAA and 1 mg/l 2, 4-D. In their another study in 2014, they stated that MS media with 1 mg/l BAP and 0.5 mg/l NAA is the best combination for highest shoot regeneration using hypocotyl explant. Although the current study shows dissimilar results but the findings by Bayraktaroglu and Dagustu (2011) and Ozyigit *et al*., (2007) can shed light on this. They demonstrated that callus production depends on genotype and interactions between genotype and explant. Therefore, genetic dependency could be a reason for this deficiency of regeneration in case of the two selected sunflower varieties. Moreover, the present hormonal supplementation is much simple and easy.

In current study, both direct and indirect regeneration were seen using the hypocotyl and embryonic axis explants. Direct regeneration achieved when MS media was supplemented

with only BAP. But addition of a low concentration of NAA led to callus formation. It was also observed that explants showed less regeneration response as the concentration of NAA increased. Indirect shoot formation was observed on MS + 5 mg/l BAP and MS media with 5 mg/l NAA were non regenerative for both of the varieties. This finding is consistent with the known inhibitory effect of auxins on shoot induction reported by Ceriani *et al*., (1992). Earlier reports with Bangladeshi varieties showed that, embryonic axis from BRAC Hysun 33 variety showed 58.33% regeneration on MS media with 1.0 mg/l BAP + 0.1 mg/l NAA and BARI Surjomukhi 2 variety gave maximum regeneration at only 2 mg/l BAP (Chowdhury, 2015). However, the successful conditions demonstrated by the previous studies differ from the current study. Here, the best condition for shoot regeneration was found to occur on MS media with 2.0 mg/l BAP using embryonic axis explants, BRAC Hysun 33 variety gave the best regeneration response (66.67%). On the other hand, with same media composition, BARI Surjomukhi 2 variety gave highest length of shoots with the regeneration rate 46.67%. The connection between callus formation and BAP/NAA combinations in embryonic axis explants shows identical response observed by previous studies (Peterson and Everett, 1985; Lupi *et al*., 1987; Ceriani *et al*., 1992). Carter *et al*., (1999) stated that minimizing the duration of exposure to high levels of plant growth regulators improved shoot development. They also applied GA_3 in the development media at 0.1, 0.5, 1.0 mg/l concentrations and reported that higher level of GA_3 produced thin and pale shoots in sunflower plant. In a similar study Witrzens *et al*., (1998) found that presence of 0.1 mg/l GA_3 and 0.5 mg/l NAA helped in stem elongation in immature embryo explants. These findings resemble the data obtained in the current study with BRAC Hysun 33 and BARI Surjomukhi 2 varieties. Addition of 0.1 mg/l GA_3 with 2.0 mg/l BAP containing media improved shoot elongation and also stimulated shoot production. Besides, a spontaneous rooting system was observed at this treatment along with enhancement of shoot length. In BRAC Hysun 33 variety, shoots raised on this elongation medium (MS + 2 mg/l BAP + 0.1 mg/l GA_3) produced strong tap roots. However, some shoots vitrified, malformed shoots were also found in some cases of BARI Surjomukhi 2 variety. Vitrification or hyperhydricity is a frequent problem in sunflower tissue culture systems (Fauguel *et., al* 2008). The affected shoots fail to root and survive

further stage (Witrzens *et al*., 1988; Knittel *et al*., 1991; Ceriani *et al*., 1992; Baker *et al*., 1999). A future effort has to be made to avoid this undesirable phenomenon.

Among the two explants, in vitro rooting was induced only in embryonic axis explants. Rooting media consisted of half strength of MS media with three concentrations (0.2, 0.5, 1.0 mg/l) of IBA. Hypocotyl explants failed to generate any root in these IBA concentrations, therefore, in future other auxins, like, IAA and NAA may be tried to achieve rooting. Studies on rooting of *in vitro* cultured shoots showed no significant difference between the varieties. Though from embryonic axis explants rooting was initiated in all these three IBA concentrations but highest frequency of rooting (93.33%) was observed at 1.0 mg/l IBA in BRAC Hysun 33 variety. On the same medium composition BARI Surjomukhi 2 showed 86.67 % rooting response. Comparing five different concentrations of IBA, Elavazhagan *et al*., (2009) have observed a significant correlation between auxin concentration and rooting response. With increasing IBA concentration, they found increased percentage of rooting. Inoka and Dahanayake (2015) have reported highest rooting response in MS + 1.0 mg/l IBA on *Helianthus annuus*. According to Ozyigit *et al*, (2007) roots were thicker and denser on MS medium supplemented with 1.0 mg/l IBA than hormone free MS medium. Similarly, in the current study, using the same hormonal concentration, thick roots were found in both the varieties. Moreover, IBA (1.0 mg/l) containing half strength MS media was determined to be the best for root induction.

During this stage some shoots developed translucent callus at the bottom which inhibited the rooting system. In addition to this, formation of premature flower bud was also encountered in the current study. These shoots did not survive when transplanted to the soil. In a similar study, comparing ten different hormonal combinations Patil *et., al* (1993) also observed *in vitro* flowering during rooting stage. They have suggested that development of a suitable rooting media can be a solution for this problem. Therefore, *in vitro* flowering can be taken as an advantage in terms of reduced duration instead of treating it as a problem (Patil *et., al* 1993).

All *in vitro* rooted plantlets were transferred to soil to check their adaptation capability in the natural environment. Following transplantation, maximum success was obtained in

BRAC Hysun 33 variety compared to BARI Surjomukhi 2 variety. Almost all of the plantlets survived in hardening stage but only few of them grew further. The plantlets grew to well-developed plantlets within 2 to 3 weeks and survived in the nature for more than one month. During the hardening stage, the variety BARI Surjomukhi 2 showed top dying in some of the regenerated shoots; as a result they could not survive in nature. Future studies are required to explore such obstacle hence improve the acclimatization stage for these varieties.

Result of this study is the evident that optimum conditions required for tissue culture of hypocotyl and embryonic axis explants of sunflower varieties, BRAC Hysun 33 and BARI Surjomukhi 2 has been explored. Analyzing the effects of growth regulators on shoot regeneration, elongation and root formation, it can be determined that BRAC Hysun 33 variety is a better one than the BARI Surjomukhi 2 variety. In addition it was also found embryonic axis to be better explant for *in vitro* regeneration. Further research is required for hypocotyl explants to establish a complete *in vitro* regeneration protocol. Once that is established, further biotechnological approaches can be taken with these varieties to successfully improve and enhance the genetic characteristics for better productivity.

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