Establishment of Tissue Culture Protocol of Two Bangladeshi Sunflower Varieties (*Helianthus annuus* L.)

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

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DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled "**Establishment of Tissue Culture Protocol of Two Bangladeshi Sunflower Varieties (*Helianthus annuus* L.)**" submitted by the undersigned has been carried out under supervision of Dr. Aparna Islam, 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 ABBREVIATIONS

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
ddH_2O	Double-distilled water
EDTA	Ethylenediaminetetraacetic acid
GA_3	Gibberellic Acid
gm	Gram
HSD	Honest Significant Difference
IAA	Indole-3 Acetic Acid
IBA	Indole-3 Butyric Acid
Kn	Kinetin
L	Litre
LS	Linsmaier and Skoog (1965) medium
mg	Milligram
ml	Millilitre
MS	Murashige and Skoog (1962) medium
NAA	Napthalene acetic Acid
NaOH	Sodium hydroxide
psi	Pound per square inch
pH	Negative logarithm of hydrogen ion concentration
SD	Standard deviation
Zn	Zeatin

ABSTRACT

Sunflower (*Helianthus annuus* L.) is an important commercially valuable crop with a high oil content. It is seed propagated, but poor germination, along with other abiotic and biotic factors pose problems in sunflower cultivation. Biotechnological techniques, such as, tissue culture and gene transfer systems have been used for improvement of most crops. This study aims to improve production of sunflower by optimising a tissue culture mediated regeneration protocol, to be used for micropropagation in future. Two Bangladeshi varieties: BARI Surjomukhi-2 and BRAC Hysun 33 were used here. In the present study, the effect of varying combinations of different concentrations of growth hormones on the formation of shoots, roots and flowers were demonstrated, followed by the acclimatisation of regenerated plantlets. Embryonic axes were obtained by removing the testa and excising 3-5 mm pieces from the proximal portion of the seeds. These were inoculated on MS medium supplemented with four different combinations of the hormones BAP and NAA, and were kept in a photoperiod of 18/6 hour dark/light, at a temperature of 26 ± 2°C. BRAC Hysun 33 showed best shoot formation in MS media supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA (97.03%), while BARI Surjomukhi-2 gave best shoot formation in MS media supplemented with 3.0 mg/l BAP (80.00%). The regenerated shoots were transferred to rooting media that contained two different concentrations of the hormone IBA. Root formation occurred best on shoots that had been grown in MS with 1.0 mg/l BAP and 0.5 mg/l NAA for BRAC Hysun 33 and in MS with 3.0 mg/l BAP for BARI Surjomukhi-2. In relation to rooting hormone, there were no difference in percentage rooting between the two concentrations, though roots were bigger and formed faster in ½MS with 0.4 mg/l IBA. All the plantlets that formed roots were transferred to soil for hardening. Plants that formed roots in ½MS media with 0.4 mg/l IBA and underwent shooting on MS with 1.0 mg/l BAP and 0.5 mg/l NAA (for BRAC Hysun 33) or MS with 1.0 mg/l BAP and 0.1 mg/l NAA (for BARI Surjomukhi-2) survived better. BRAC Hysun 33 proved to be the best variety, giving the most surviving plantlets with tallest average height and more numbers of flowers, buds and viable seeds.

TABLE OF CONTENTS

CHAPTER 1

— INTRODUCTION —

1. Introduction

Sunflower (*Helianthus annuus* L.) is a popular oilseed crop. It is an annual dicotyledonous angiosperm (https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id=2065, date: 06.04.18) with a high oil content (40-52%) and is rich in unsaturated fatty acids and vitamins; it also has no cholesterol (Ibrahim, 2012). As such, sunflower oil is favoured throughout Russia, Mexico, most of Europe, and South America. Between 2012-2017, Ukraine and Russia produced over 50% of the world's sunflowers (National Sunflower Association, 2017). However, poor seed germination is a major problem in its cultivation. In recent years, biotechnological techniques have been used for improvements in production. Reports on shoot regeneration from various organs are available, but success has been limited. Hence, a more reproducible tissue culture protocol is necessary.

1.1 Taxonomy

Taxonomy of *Helianthus annuus* L. (Integrated Taxonomic Information System):

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Division	Tracheophyta – vascular plants, tracheophytes
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae – sunflowers, tournesols
Genus	Helianthus L. – sunflower
Species	*Helianthus annuus* L.

1.2 History

H. annuus L. is native to North America (National Sunflower Association, 2018). It was domesticated in central USA in 3000 BC, then taken to Europe by Spanish explorers during the 1500s, where it gained fame as a decorative plant. In the 1700s, sunflower oil became popular in Europe. The plant was commercialised for oil production in Russia in the 1800s. Russian immigrants took sunflower back to America. Sunflower is now grown as an essential oil seed crop all over the world.

1.3 Description of the plant

Sunflowers are annual, dicotyledonous, herbacious angiosperms that reach 100-300 cm in height. Budding sunflowers exhibit heliotropism: during the day, they track the sun from the east to west before returning eastward at night. This is done by motor cells in the pulvinus, a bendable portion of the stem just below the bud. As the blooming stage begins, this region stiffens and the plant is no longer heliotropic; the flowers permanently face east (https://web.archive.org/web/20130523191033/http://www.flowers-org.com/helianthus-sunflower.html, date: 05.04.18).

1.3.1 Reproductive organs (flower and seed)

The inflorescence, characteristic of the Compositae family, can reach 30 cm in diameter. The head shape can be concave, convex or flat. It is pleasantly scented and colourful to attract insects. What is called the flower is actually a composite of 1000-2000 flowers packed spirally together (figure 1.1) (DeBerry, 2017). The outer flowers (ray florets) forming the petals are sterile and can be yellow or orange. The smaller flowers inside the head are disk florets. These mature into the fruits (achene/seed) of the plant (https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id=2065, date: 05.04.18).

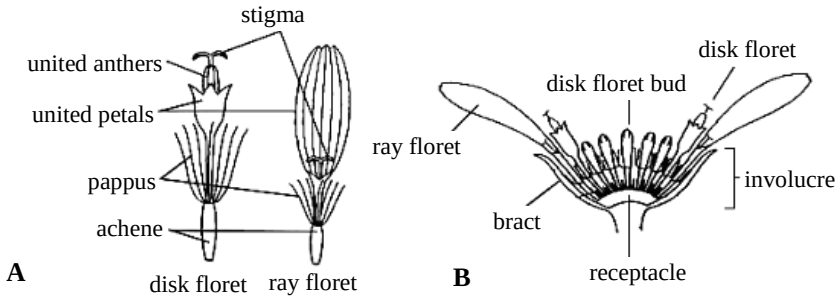

Figure 1.1: A. Disk and ray florets. **B.** A sunflower head showing disk and ray florets

The seeds are 1-seeded achenes that are produced once the disk florets are pollinated. It comprises of one seed (the kernel), and a surrounding pericarp (the hull). Without fertilization, the achenes remain empty. Sunflower achenes can be linear, oval or round and are usually 7-25 mm long and 4-13 mm wide.

1.3.2 Vegetative organs

Sunflower leaves are petiolate, three-veined, and either linear or oval. The leaves are serrated and coarsely-hairy. The stems are mostly unbranched and hairy. The first leaves are always opposite to one another across the stem, but in some varieties they become alternate. Sunflowers have long, white, hairy tap roots with branched out secondary roots that reach 2-3 feet in length.

1.4 Reproductive biology

Sunflowers are seed-propagated crops that can impulsively hybridize with related wild crops or weeds (Burke *et al.*, 2002). Prior to 1970, cultivated crops were cross-pollinated by insects as they were self-incompatible. The new hybrid crops are self-compatible and can reproduce asexually (Snow *et al.*, 1998). This gives an identical flower. However, a seed yield of only 10-20% occurs when the plants self-pollinate, compared to the 90% yield in flowers that pollinated via insects (http://www.pollinator.ca/bestpractices/ sunflowers.html, date: 06.04.18). At the end of flowering, each flower in the head produces its own seed.

1.5 Importance of sunflower

1.5.1 Food value

Sunflower seeds yield edible oil rich in vitamins A, B (pantothetic acid and thiamine), D, E, and K. It also contains high amounts of phosphorus, selenium, and copper. Sunflowers also have high unsaturated fatty acids, fibre, amino acids (especially tryptophan), phytosterols, etc., and are low in trans fatty acids. Sunflower oil accounted for 8-11% of the world's vegetable oil trade between 1999 and 2009 (USDA, 2017).

The edible flowers can be added into salads. The seeds can be eaten raw, roasted, or made into flour (https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id= 2065, date: 06.04.18). The meal left after oil extraction can be used as birdfeed or as a high protein meal for livestock (Dedio, 2015).

1.5.2 Medicinal value

Sunflowers are rich in vitamin E and other antioxidants, which help balance cholesterol levels, reduce hypertension, and lower risk of heart disease. They help prevent different types of cancer, e.g. colon, prostrate, and bladder cancer, and improves skin health. The high selenium content improves thyroid function, prevents breast cancer, aids with DNA repair and removes damaged cells. Sunflower seeds also balance blood sugar levels (Babcock, 2017).

The dried flowers are used to treat burns, while chewed roots are applied on swellings from snake or spider bites. Native Americans used the flowers in teas to treat lung diseases, malaria and high fevers (https://florafaunaweb.nparks.gov.sg/Special-Pages/plant-detail.aspx?id=2065, date: 06.04.18). Moreover, the leaves and seeds are also diuretic and expectorant (http://www.bio.brandeis.edu/fieldbio/medicinal_plants/pages/Sunflower.html, date: 06.04.18)

1.5.3 Industrial value

Sunflower oil is commercially used in production of lubricants, bio-fuel, hydraulic fluids, soaps, lighting and some types of paints, varnishes and plastics. The petals are used to make yellow dyes (Dedio, 2015).

1.6 Suitable growing conditions

Sunflower grows well in full sunlight, especially 6 to 8 hours a day (https://www.almanac.com/plant/sunflowers, date 07.04.18). It is regarded as a moderately drought tolerant plant, but it requires well drained soil. The plants can grow in both sandy and clayey soil, with soil pH ranging from 5.7 to 7.5. Due to their long roots, sunflowers need well dug up soil to allow the roots to grow. Germination occurs best at 14-21°C. Optimum temperatures for plant growth is 21-26°C. Sunflower is vulnerable to high levels of fertilizer, so it should be applied 2.5 cm away from the seeds.

1.7 Cultivation in Bangladesh

In Bangladesh, sunflower was first introduced as a garden plant, but in the 1980s some varieties were launched as oilseed crops. It has gained popularity among local farmers for its easy extraction method. During monsoon, sowing mustard and sesame becomes difficult, and hence production falls drastically. Sunflower is a good substitute that makes up for the gap in production as it contains almost 40-50% oil and is rich in protein. A kilogram of sunflower seeds yields 500-600g of oil, an amount more than that from any other oil seeds (Nahar and Torikul, 2015).

Sunflower is cultivated during kharif (monsoon) and rabi (winter) seasons in Bangladesh. The optimum sowing time is considered to be between 15th October to 15th November. The plants mature within 120-130 days and can be harvested.

Sunflower can be grown in drought and in dry areas (e.g. the Barind tract) as it needs little irrigation, and also in coastal regions due to its salt tolerance. BRAC Hysun 33 variety was first introduced by BRAC project beneficiary farmers in the coastal regions of Barguna, Patuakhali, Jhalakathi, Pirojpur, Satkhira, Bagerhat and Khulna in 2010-2011. Sunflower is also gaining fame among coastal farmers for its good market value (http://greenwatchbd.com/satkhira-farmers-getting-inclined-to-sunflower-cultivation/, date: 07.04.18).

Sunflower is grown in 16 districts of Bangladesh with an average production of about 0.7-1.2 ton/ha, though with irrigation, fertilisers and insecticides, it can be increased to 3 ton/ha. In 2014, 30-35 maunds of sunflower seeds were produced per acre in Satkhira at a market price of about Tk. 50,000. In a CSISA-IRRI report from Barisal, BARI Surjomukhi-2 gave an 11 mounds/bigha yield (Nath, 2013). BRAC Hysun 33 gave a better yield than BARI Surjomuhki-2, however. BRAC Hysun 33's efficiency had also been observed previously in 2011 (Biswas *et al.*, 2012), when BRAC's sunflower variety screening program evaluated the varieties Aguara-4, BRAC Hysun 33, and Kironi for agronomic purposes, with BRAC Hysun 33 giving the highest yield.

Various steps have been taken to encourage the farming of sunflower. The International Rice Research Institute (IRRI) operates a USAID-funded initiative called Expansion of

Cereal Systems Initiative for South Asia in Bangladesh (CSISA-BD) in the Patuakhali and Barguna districts. The oil and food company 'Amrita' has opted to buy sunflower seeds from local farmers instead of importing (Hossain, 2014). Also, BRAC launched sunflower oil brand Shufola, which is extracted from sunflower grown in southern Bangladesh (http://www.thedailystar.net/news/brac-to-produce-sunflower-oil, date: 07.04.18).

According to Hossain (2014), Bangladesh produces 0.4 million tons of edible oil, though demand is 1.6 million tons, while the rest is imported. Bangladesh spends Tk. 1,38,141 million yearly to import this oil; growing sunflower locally reduces this expenditure (Bangladesh Bank, 2014). Also, sunflower can make considerable profit for farmers, as the investment cost is about Tk. 4,000 per hectare for seeds along with other production costs, with a return of about Tk. 45,000 per hectare. If cultivation of sunflower is going through a larger scale, an improved production and better profits can be achieved, which will ensure an economic improvement and sustainability of the local people.

1.8 Limitations of cultivation

Sunflower yields can be effected by many factors. Fungi, weeds and pests are the biotic factors; abiotic factors include temperature, water levels, and salinity.

Diseases are caused mainly by fungi. In Bangladesh, *Alternaria sp.* (leaf blight), *Botrytis cinerea* (head rot), *Colletotrichum sp.* (anthacnose), and *Sclerotium rofsii* (stem and coller rot) are major problems. *Aspergillus spp.*, *Penicillium sp.*, *Rhizopus spp.*, *Cladosporium spp.*, etc. are also problematic (Fakir and Rahman, 2007). Sunflowers can also be attacked by many insects, like, worms and beetles, which cause problems during the emerging and budding stages (National Sunflower Association, 2018).

Temperature is one of the main factors affecting growth of sunflower in Bangladesh. High amounts of heat accumulation while growing can cause tissue damage, which is a reason for the stem toppling over (National Sunflower Association, 2018).

Sunflower is categorized as a low to medium drought sensitive crop (Rauf, 2008). It has a highly branched taproot that helps it get water from 2 metres below the ground, allowing

it to survive during water stress. However, sunflower needs 500-1000 mm rainfall annually; it has been found that a worldwide reduction in sunflower yield has been associated with drought (Dragovic and Maksimovic, 1995).

Another abiotic factor affecting growth of sunflower is salinity. McKenzie (1988) stated that sunflowers have a salt tolerance of 8 dSm^{-1}. As soil salinity levels increase, the stress on germinating seedlings also increases. Tests show that hybrid plants grown in high salinity soils were 50% shorter than control plants, had lower number of leaves and also a lower seed yield (Francois, 1996).

1.9 Biotechnological approach

Conventional breeding and enhanced management have both been tried for increasing yield, but they have resulted in limited commercial success. Over the years, biotechnological techniques, such as, tissue culture and gene transfer systems have been attempted, but these techniques are mainly limited by the tissue culture response of commercial varieties (Ozyigit *et al*., 2007). Though positive results have been achieved by using biotechnological techniques on other plants, attempts at increasing production of sunflowers with specific traits have been unsatisfactory (Ivanov *et al*., 2002). Different parts of the plant have been used as explants for tissue culture techniques, such as, immature and mature embryos, embryonic axes, anthers, buds, meristems, leaves, shoot tips, hypocotyls, cotyledons and cotyledonary petioles, roots, and stems (Witrzens *et al*., 1988; Gürel and Kazan, 1998; Ozyigit *et al*., 2002, 2006, 2007; Aurori *et al*., 2011; Inoka and Dahanayake, 2015; Abd Elaleem *et al*., 2015; Badigannavar and Kururvinashetti, 1996). Intact embryonic axis explant has shown high regeneration potential in sunflower for all genotypes, especially immature embryos. However, tissue culture methods often face problems like precocious flowering, vitrification or poor rooting. Hence, in order to increase sunflower yield, developing an effective and replicable protocol is imperative. An extensive summary of attempted tissue cultures for sunflower over the years has been written by Mohammad Sharrif Moghaddasi (2011). Some of these are mentioned in table 1.1.

Table 1.1: A summary of some of the attempted tissue cultures for sunflower over the years.

Explant	Plant hormones used	Findings	Reference
• Leaf, • Cotyledon, • Shoot apices, • Hypocotyl segments.	**Shoot induction:** MS + 0.0-5.0 mg/l 2,4-D and BAP.	• BAP induced callus from different explants. • 2,4-D alone produced poorly developed, nodular callus. • BAP alone induced abundant growth of compact, green callus. Several of these regenerated many shoots, some of which flowered *in vitro*.	Greco *et al*. (1984)
• Cotyledons.	**Shoot induction:** Modified MS + 0.0-1.0 mg/l of NAA and BAP. **Root formation:** Hormone free ¼MS media.	• As BAP increased, shoot formation increased with increasing number of shoots per explant. • As NAA increased, shoot formation decreased with decreasing number of shoots per explant. • Shoot formation decreased from 90 to 40% if basal half of cotyledons were cut into smaller portions. • Regeneration efficiency found to be genotype dependent.	Ceriani *et al.* (1992)
• Mature cotyledons.	**Shoot induction:** Modified MS + 1.0 mg/l NAA and 1.0 mg/l BAP (first liquid media, then solid).	• Shoots formed in 19 recalcitrant genotypes. • Ethylene inhibited shoot formation. • Ethylene production by explants increased in solid medium but decreased in liquid medium. • Reduction of ethylene allowed regeneration in all genotypes, implying regeneration may be general for different genotypes.	Chraibi *et al.* (1992a)

Explant	Plant hormones used	Findings	Reference
• 2, 4 day old cotyledons.	**Shoot induction:** Liquid basal media + different concentrations of NAA and BAP; Solid basal media + different combinations of NAA, IBA, IAA and BAP. **Root formation:** ½MS media + 0.5 mg/l NAA, GA_3 and 0.5% activated charcoal.	• Age of explant affected shoot regeneration. • Best shoot formation occurred with 1.0 mg/l NAA and 1.0 mg/l BAP. • Liquid medium gave more shoot induction than solid medium.	Chraibi *et al.* (1992b)
• Shoots.	**Root formation:** ⅓ MS media + 1 mg/l NAA, 1% sucrose and 0.5mg/l activated charcoal.	• Obtained 68-88% root formation. • Determined that transfer to rooting media should be done before flowering or right when it starts.	Patil *et al*. (1993)
• 21 day old hypocotyls, • Cotyledons.	**Shoot induction:** LS media + 0.5-1.0 mg/l BAP and 2.0 mg/l NAA.	• Compact, green callus had formed. • As BAP increased, callusing decreased for both explants.	Mohmand and Quraishi (1994)
• Bracts.	**Callus formation:** MS media + 0.5-1.0 mg/l NAA, 0.5-1.0 mg/l BAP and 1.0-2.0 mg/l 2,4-D.	• For all genotypes, 100% callus induction seen in media containing only 1.0 mg/l NAA, only 2.0 mg/l 2,4-D and media containing a combination of 1.0 mg/l each of NAA and BAP. • Cytokinin effect on organogenesis was genotype specific.	Badigannavar and Kuruvinashetti (1996a)
• Cultured anthers.	**Callus formation:** MS media + 0.5-1.0 mg/l NAA, 1.0 mg/l BAP and 1.0-2.0 mg/l 2,4-D.	• High callus induction found in MS medium with 2.0 mg/l 2,4-D and 1.0 mg/l BAP. • Need of growth regulators was genotype dependent.	Badigannavar and Kuruvinashetti (1996b)

Explant	Plant hormones used	Findings	Reference
• Hypocotyl protoplasts.	**Protoplast culture:** Liquid mKM media. **Shoot induction:** Differenciation media + 1.0 mg/l BAP and 0.02 mg/l NAA. **Root formation:** Dipped in 1.0 mg/l NAA and placed on modified hormone free SE_{20} media.	• An initial plating density of $2.0x10^4$ protoplasts per ml agarose was required for callus development. • Reduction of osmolarity was essential for normal shoot development during culture on solid medium. • Plating efficiency varied between genotypes. • 90% of the shoots produced roots when dipped in NAA; no roots were formed if shoots weren't dipped. However, supplying NAA in rooting medium resulted in callusing.	Wingender *et al.* (1996)
• Shoot-tips, • Hypocotyl segments, • Cotyledons, • Cotyledonary petioles.	**Germination:** MS + 0.1 mg/l BAP. **Somatic embryogenesis medium:** MS + 1.0 mg/l BAP, 1.0 mg/l NAA, 0.1 mg/l GA_3. **Regeneration medium:** MS + 1.0 mg/l BAP, 0.5 mg/l NAA, 0.1 mg/l GA_3.	• Amount of callus increased with increasing BAP, but callus formation was best without NAA. • Shoot tips and hypocotyls gave most callusing and were most responsive to shoot formation. Cotyledons, cotyledonary petioles gave no shoots. Hybrid cultivars gave better shooting. • BAP prevents root induction, and cotyledons were least responsive to rooting effect of NAA. • Somatic embryogenesis could be induced on cotyledons, especially from basal (proximal) portions. • Genotypic variation appeared to be the most critical factor for both somatic embryo and root production. • Thin cell layers from hypocotyl segments were not found to be successful for plant regeneration.	Gürel and Kazan (1998)

Explant	Plant hormones used	Findings	Reference
• Immature embryos	**Shoot induction:** MS + 0.5 mg/l each NAA, GA_3, and Kn, and 1.0-3.0 mg/l Zn. **Root formation:** ½MS + 0.5 mg/l NAA and 0.5 mg/l GA_3.	• Development of calli was influenced by age of the immature embryos, genotype and media. • Best response seen with 2 mg/l Zn. • Distinct differences in regenerable calli production were observed among different genotypes.	Nedev *et al.* (1998)
• 1-5 day old cotyledon.	**Shoot induction:** Basal media + 1.0, 2.0, and 5.0 mg/l BAP + 0, 0.1, 0.5 and 1.0 mg/l NAA. **Root formation:** 2 layer rooting media. ½MS + 1.0-2.0 mg/l NAA on top; Activated charcoal below.	• Shoot initiation needed brief exposure hormones, • Less exposure to high levels of hormones improved shoot development. • Rooting was improved by incorporating activated charcoal in the lower layer of a 2-layer rooting medium.	Baker *et al*. (1999)
• 2 day old hypocotyls.	**Germination:** Hormone free ½MS media. **Shoot induction:** MS + 1.0 mg/l BAP and NAA.	• Organogenesis was genotype dependent. • Proximal explants gave more shoots per explant than distal explants.	Abdoli *et al.* (2003)
• Mature embryos.	**Callus formation:** MS + 1.0 mg/l 2,4-D. **Shoot induction:** MS + 1.0 mg/l BAP and 0.5 mg/l NAA. **Root formation:** MS +1.0 mg/l IBA; Hormone free MS media.	• MS medium supplemented with 1.0 mg/l 2,4-D was suitable for callus induction. • Shoot regeneration from calli was genotype dependent. • Use of mature embryos instead of immature embryos or other explants are better due to seasonal independence, easy handling, and relative sterility of tissue.	Ozyigit *et al.* (2006)

Explant	Plant hormones used	Findings	Reference
• 10 day old hypocotyls • Cotyledon.	**Germination:** Hormone free MS media. **Callus formation:** MS + 1.0 mg/l 2,4-D. **Shoot induction:** MS + 1.0 mg/l BAP and 0.5 mg/l NAA; Hormone free MS. **Root formation:** MS + 1.0 mg/l IBA; Hormone free MS.	• Genotype, explant type affected regeneration response. • Callus induction from hypocotyls was faster than cotyledons. • MS medium supplemented with 1.0 mg/l 2,4-D was suitable for callus induction from these explants. • Shoot formation was greater in hypocotyls than cotyledons; No shoot formation seen on hormone free MS medium. • Rooting was seen in both media, but roots were thicker and denser in presence of IBA.	Ozyigit *et al.* (2007)
• 2 day old hypocotyls.	**Germination:** Hormone free ½MS media. **Shoot induction:** MS + 1.0 mg/l BAP and 1.0 mg/l NAA.	• Organogenesis, optimum agar level were genotype dependent. • Shoot formation increased as agar concentration decreased. • Hyperhydric shoots increased as agar concentration decreased.	Abdoli *et al.* (2007)
• 7 day old hypocotyls, • Cotyledons, • Immature leaflets.	**Callus formation:** MS + 0.5-2.0 mg/l BAP and NAA + 0.5 mg/l GA_3. **Shoot induction:** MS + 0.5-5.0 mg/l BAP. **Shoot multiplication:** MS + 2.0-3.5 mg/l BAP and 0.1 mg/l NAA. **Root formation:** MS + 1.0-3.0 mg/l IBA.	• Cotyledon showed the highest percentage of response. • Highest callus induction was observed on MS medium with 2.0 mg/l BAP and 0.5 mg/l GA_3. • Most multiple shoot bud induction was observed on MS medium with 5.0 mg/l BAP and 0.5 mg/l IBA. • Highest root induction was observed on MS medium with 3.0 mg/l IBA.	Elavazhagan *et al*. (2009)

Explant	Plant hormones used	Findings	Reference
• 10 day old cotyledon, • Hypocotyls, • Root segment.	**Callus formation:** MS + 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l of 2.4-D and NAA.	• MS media with 2,4-D was good for callus induction. • Highest callusing was obtained with cotyledons in 1.5 mg/l 2,4-D. • Callus obtained with NAA by all explants gave high rooting.	Abd Elaleem *et al.* (2015)
• Leaf, • Root • Stem.	**Callus formation:** MS + 0.1 mg/l BAP and 0.0-3.0 mg/l 2,4-D. **Shoot induction:** MS + 0.1 mg/l NAA and 0.0-3.0 mg/l BAP. **Root formation:** MS + 0.01-1.0 mg/l IBA.	• Greatest number of shoots was observed in MS medium with 0.5 mg/l BAP and 0.1mg/l NAA. • Root induction was best at 1.0 mg/l IBA. • Highest diameter of callus was observed with 2.0 mg/l 2, 4-D and 0.1 mg/l BAP. • Most shoot regeneration and callus production was seen with stem.	Inoka and Dahanayake (2015)

1.10 Objectives

Since the 1980s, various researchers have been trying to flesh out an efficient regeneration protocol for sunflower. While some protocols have been developed in other countries, no complete protocol has been ascertained yet in Bangladesh for local varieties. In this study, two popular native varieties (BRAC Hysun 33 and BARI Surjomukhi-2) were used to develop a reproducible tissue culture mediated plant regeneration protocol. Embryonic axis was used as explant from both of the varieties.

The specific objectives for this study were:

1. Determination of most favourable hormone supplements for shoot formation,
2. Determination of most favourable hormone supplements for root induction,
3. Optimization of the acclimatization stage of regenerated plantlets,
4. Determination of the reproducibility of the seeds produced.

CHAPTER 2

— MATERIALS AND METHODS —

2. Materials and Methods

2.1 Plant materials

In the current investigation, two local varieties of sunflower, BRAC Hysun 33 and BARI Surjomukhi-2, were used (figure 2.1). BRAC Hysun 33 was collected from BRAC Agricultural Research and Development Center (BRAC ARDC). the latter was collected from Bangladesh Agriculture Research Institute (BARI). Both organisations are in Joydebpur, Gazipur. The seeds were kept at 4°C in the Plant Biotechnology and Biosafety Laboratory of BRAC University, located in Mohakhali, Dhaka, Bangladesh.

2.1.1 BARI Surjomukhi-2

Released by BARI in 1982, these black seeds produce plants of 125-140 cm in height. Seeds are sown in December and January. Flowering takes 57-65 days; the plants take 90-100 days to mature. Flowers have diameters of 15-18 cm and produce 450-650 seeds. Successful seed yields are 1.5-2.3 ton/ha. Seeds have a 1000 Seed Weight of 65-70 g and an oil content of 42-44%. The variety is tolerant towards *Alternaria* leaf blight disease.

2.1.2 BRAC Hysun 33

These blue seeds were introduced in 2010 and give plants of 90-150 cm. Seeds are sown in October-November. Flowering takes around 92 days and the plants live for 110-120 days. Diameters of the flowers are 21-22 cm and hold 800-1500 seeds. They give a yield of 2.6-4.2 ton/acre, with an average 1000 Seed Weight of 65.7 g and oil content of 40%. They also have a salt tolerance of 8 ds/mol, and grow well in loamy soil. Excessive moisture leads to *Fusarium* spp. contamination and Downy Mildew disease.

Figure 2.1: A. Seeds: BARI Surjomukhi-2 variety. **B.** Seeds: BRAC Hysun 33 variety.

2.2 Methodology

2.2.1 Media preparation

In this study, Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with different combinations of BAP and NAA hormones was used for shoot regeneration. Half strength MS media enhanced with the hormone IBA was used for rhizogenesis.

2.2.1.1 Preparation of MS media

To prepare MS medium, firstly, four different stock solutions were made by dissolving their respective constituents one-by-one in distilled water (table 2.1). These stocks were then mixed together with other components, as needed, to prepare MS medium or ½MS medium (table 2.2).

Table 2.1: Components and their amounts for preparing 1 L of respective stocks.

MACRONUTRIENTS		**MICRONUTRIENTS**	
Compound	**Amount (mg/l)**	**Compound**	**Amount (mg/l)**
KNO_3	1900	KI	0.83
NH_4NO_3	1650	H_3BO_3	6.2
$MgSO_4.7H_2O$	370	$MnSO_4.4H_2O$	22.3
$CaCl_2.2H_2O$	440	$ZnSO_4.7H_2O$	8.6
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	0.25
ORGANIC		$CuSO_4.5H_2O$	0.025
Compound	**Amount (mg/l)**	$CoCl_2.6H_2O$	0.025
Nicotinic acid	0.5	**Na-Fe-EDTA**	
Pyridoxine.HCl	0.5	**Compound**	**Amount (mg/l)**
Thiamine.HCl	0.1	FeSO4.$7H_2O$	27.8
Glycine	2.0	$Na_2EDTA.2H_2O$	37.3

Table 2.2: Components and their quantities used to produce 1L basal MS medium:

Components	**Amount for 1 L MS medium**	**Amount for 1 L ½MS medium**
Macronutrients (10x)	100 ml	50 ml
Micronutrients (100x)	10 ml	5 ml
Organic (100x)	10 ml	5 ml
Na-Fe-EDTA (100x)	10 ml	5 ml
Sucrose	30 g	30 g
Myo-inositol	0.1 g	0.1 g
Agar	8.0 g	4.0 g

2.2.1.2 Preparation of plant growth regulatory hormones

The effects of the following hormones on the regeneration of sunflower were examined:

1. *Auxins:* Napthalene acetic-acid (NAA); Indole-3-butyric acid (IBA),
2. *Cytokinins:* 6-Benzyl amino purine (BAP).

To prepare these hormones, 20 mg of the powdered hormone was dissolved in 2-5 drops of 1N NaOH. Volume was made 200 ml by adding distilled water. The hormone stocks were added to MS media to prepare shooting and rooting media.

2.2.1.3 Media sterilisation

The conical flasks or test tubes containing the media were autoclaved at 121°C and 15 psi for 20 minutes (Model: WAC-47, Korea). The media was cooled and stored at 25±2°C.

2.2.2 Seed sterilisation

Seeds were washed in autoclaved ddH_2O for 1 minute two times, then in 70% ethanol for 3 minutes. Afterwards, they were washed again with ddH_2O for 1 minute, twice, followed by 14% Clorox for 20 minutes. They were next washed with distilled water for 1 minute 4-5 times to remove any Clorox, and then submerged in ddH_2O and stored overnight in a box to aid water uptake. The next day, soaked seeds were washed thrice, dehulled, and washed 5-6 more times with ddH_2O to remove the white waxy layer on the seed surface. Every step was carried out inside a laminar air flow cabinet (Model: SCV-4AI, Singapore).

2.2.3 Explant preparation and inoculation

All dehulled seeds were cut 3 mm away from their proximal regions (figure 2.2) and inoculated, cut portion upwards, into hormone supplemented MS media. The flasks were sealed and kept at 25°C with a photoperiod of 16 hours light and 8 hours dark. After 30 days, cultures were transferred to fresh media with the same hormone composition.

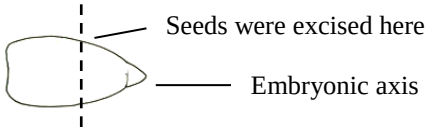

Figure 2.2: How to obtain embryonic axis from sunflower seeds.

2.2.4 Transfer of shoots to rooting media

Once 2-5 cm long, shoots were transferred to rooting media. Shoots were cut at the node where the cotyledons joined the shoots to separate the cotyledon and shoot (figure 2.3). Shoots were transferred to rooting media with the base well inserted in the media.

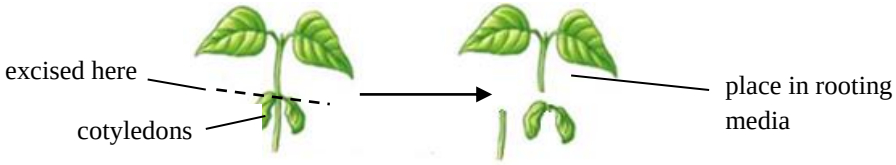

Figure 2.3: obtaining shoots to place in rooting media.

2.2.5 Acclimatisation of plants that formed roots

The shoots that had developed sufficient roots in the rooting media were then transferred to soil for hardening, or acclimatisation. The plantlets were removed from the media and roots were washed carefully under running tap water to remove any media. The water flow had to be sufficiently low and roots were gently pressed to avoid breakage. Once the roots were cleaned, plantlets were transferred to plastic cups containing autoclaved soil mixed with fertiliser at a ratio of 4:1. They were watered just enough to moisten the soil. The pots were covered with perforated clear plastic bags, and water was sprayed on the inside of the bag. After 2 weeks, the plastic bags were daily removed and replaced, with the duration of removal gradually increasing until they were no longer needed.

2.2.6 Seed collection

Seeds from *in vitro* regenerated plants were collected once they were ripe enough (figure 2.4). This occurred once the scales of the buds and the back of the flower turned brown, ovaries and stigmas had dried and fallen off, most leaves had senesced and the stem became dry. The seeds became loose and came off when pulled. The number of seeds were counted and then the seeds were stored in an envelope.

Figure 2.4: Ideal state of sunflower plant for harvesting seeds.

2.2.7 Reproducibility of collected seeds

The collected seeds were checked for their viability in order to determine the reproducibility of the tissue culture. After collection, they were placed on a Petri dish containing a wet filter paper, and kept in a cool dark box to stimulate germination. The seeds that germinated were considered viable and were placed in a cup of soil and allowed to grow. The total number of viable seeds were recorded to determine the percentage of reproducibility of the plants.

2.3 Statistical analysis

Statistical analyses were performed using Microsoft Excel 2007. A 1-way Analysis of Variance (ANOVA) test was performed at the $p<0.05$ significance level to determine whether the differences are due to chance or due to the differences in treatment. If the p-value was less than 0.05, the result was taken to be statistically significant.

If the ANOVA results were found to be significant, Tukey's Honest Significant Difference post-hoc test was carried out in order to find out where the differences lay by finding the HSD value of each pair of results (i.e., the results between hormone treatments 1 and 2, 1 and 3, and so on.). The HSD for each pair of means was calculated using the following formula:

$$\mathrm{HSD} = \frac{\mathrm{M_1} - \mathrm{M_2}}{\sqrt{\frac{\mathrm{MSW}}{\mathrm{n}}}}$$

Where:

- $M_1 – M_2$ is the difference between the pair of means. to calculate this, M_1 should be larger than M_2;
- MSW is the Mean Square Within, and n is the number in the group or treatment.

If the HSD result was found to be greater than the critical value from the Tukey's critical value table (determined using the degrees of freedom and number of treatments), then the results of the two treatments were found to be significantly different.

CHAPTER 3

— RESULTS —

3. Results

This study aimed to develop an effective *in vitro* regeneration protocol for two local sunflower varieties: BRAC Hysun 33 and BARI Surjomukhi-2, with the embryonic axes attached to the seeds as the explant. Multiple trials were carried out in order to determine the best hormonal concentrations and/or combinations for growth of shoots and roots, followed by acclimatisation of the plantlets by hardening. Seeds were finally collected from the flowers produced on the regenerated plants to determine the reproducibility of the tissue culture protocol.

3.1 Analysis of the regenerative capacity of embryonic axes from both varieties

For four hormone treatments, 400 embryonic axes per variety (to obtain 100 explants per treatment for each variety) were taken.

3.1.1 Influence of various hormone supplementation on shoot formation

In terms of regeneration response, overall response of BRAC Hysun 33 was better compared to BARI Surjomukhi-2, as was seen after a period of 25 days post-inoculation (table 3.1). In BRAC Hysun 33, best result (97.03%) was seen with MS media containing 1.0 mg/l BAP and 0.5 mg/l NAA. For BARI Surjomukhi-2, response was best in media having only 3.0 mg/l BAP (80.00%), though the second best response was seen with 1.0 mg/l BAP and 0.5 mg/l NAA (56.00%). For both varieties, lowest response was seen in media containing only 2.0 mg/l BAP (86.14% for BRAC Hysun 33 and 50.00% for BARI Surjomukhi-2).

Media containing both cytokinin and auxin produced an average of 1 shoot per explant, though white callusing was seen at the base of the shoots after shoot formation (figures 3.1B, 3.1E, 3.2B, and 3.2G). These shoots were tall. Media with only cytokinin, however, gave both single and multiple shoots and were shorter than shoots grown in combination media. All media compositions gave shoots via direct regeneration, though some non-regenerative calli were formed too (figures 3.1F and 3.2G). BARI Surjomukhi-2 gave a greater amount of non-regenerative calli. Some of these gave bud-like growth, but they did not grow into shoots (figures 3.2E and 3.2F).

A single factor ANOVA (Analysis of Variance) was conducted to compare the effects of the different hormonal treatments on shoot regeneration for both varieties. For BRAC Hysun 33 (table 3.2), there was a significant effect of hormone concentration on shoot regeneration at the $p<0.05$ level among the three conditions [$F(3, 12) = 3.77$, $p = 0.041$]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for the treatment containing 1.0 mg/l BAP with 0.5 mg/l NAA was significantly higher than the treatment containing 2.0 mg/l BAP. However, there were no significant differences when these treatments were compared with the other treatments.

For BARI Surjomukhi-2 (table 3.3), a single factor ANOVA showed that there was a significant effect of hormone concentration on shoot regeneration at the $p<.05$ level for the three conditions [$F(3, 12) = 6.81$, $p = 0.006$]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for the treatment containing 3.0 mg/l BAP was significantly higher than all the other treatments. However, there were no significant differences when the other three treatments were compared with each other.

These results suggest that different hormonal treatments did affect shoot regeneration. Moreover, 1.0 mg/l BAP and 0.5 mg/l NAA favours better shoot regeneration in BRAC Hysun 33 while 3.0 mg/l BAP favours BARI Surjomukhi-2.

Table 3.1: Effect of different combinations of hormonal supplementations on the in vitro shoot regeneration from embryonic axis explants of two sunflower varieties: BRAC Hysun 33 and BARI Surjomukhi-2.

Varieties	Hormones		Type of response	Shoot regeneration (%)	Average length in 25 days (cm) ± SD
	BAP (mg/l)	NAA (mg/l)			
BRAC Hysun 33	1.0	0.1	Direct	92.08	3.50 ± 1.02
		0.5	Direct	97.03	3.35 ± 1.33
	2.0	-	Direct	86.14	3.24 ± 1.00
	3.0	-	Direct	89.11	2.71 ± 0.83
BARI Surjomukhi -2	1.0	0.1	Direct	52.00	3.67 ± 1.01
		0.5	Direct	56.00	3.48 ± 0.91
	2.0	-	Direct	50.00	3.25 ± 0.84
	3.0	-	Direct	80.00	3.11 ± 1.03

values obtain from quadruplicate trials

Table 3.2: ANOVA results for shoot regeneration of BRAC Hysun 33 explants.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	16.5	3	5.5	3.771429	0.040699	3.490295
Within Groups	17.5	12	1.458333			
Total	34	15				

Table 3.3: ANOVA results for shoot regeneration of BARI Surjomukhi-2 explants.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	144.75	3	48.25	6.811765	0.006211	3.490295
Within Groups	85	12	7.083333			
Total	229.75	15				

3.1.2 Seasonal influence on initiation of shoots

During plant tissue culture, factors like temperature, light, water level and humidity are all controlled, so no differences ought to be observed based on season. However, it is likely that the time of the year did affect the regeneration response. For BARI Surjomukhi-2, explants that were inoculated during January to March did not perform as good as those inoculated during other months (table 3.4). For BRAC Hysun 33, explants were mostly inoculated between July and October, so seasonal impact could not be determined. Additional research to determine seasonal impact on regeneration is required.

Table 3.4: Performance of BARI Surjomukhi-2 explants over different months.

Month	Jan-Feb	Mar-Apr	May-Jun	July-Aug	Sep-Oct	Nov-Dec
Explants inoculated	20	174	100	-	25	45
Shoots formed	3	73	71	-	18	28
Response (%)	15.00	49.95	71.00	-	72.00	62.22

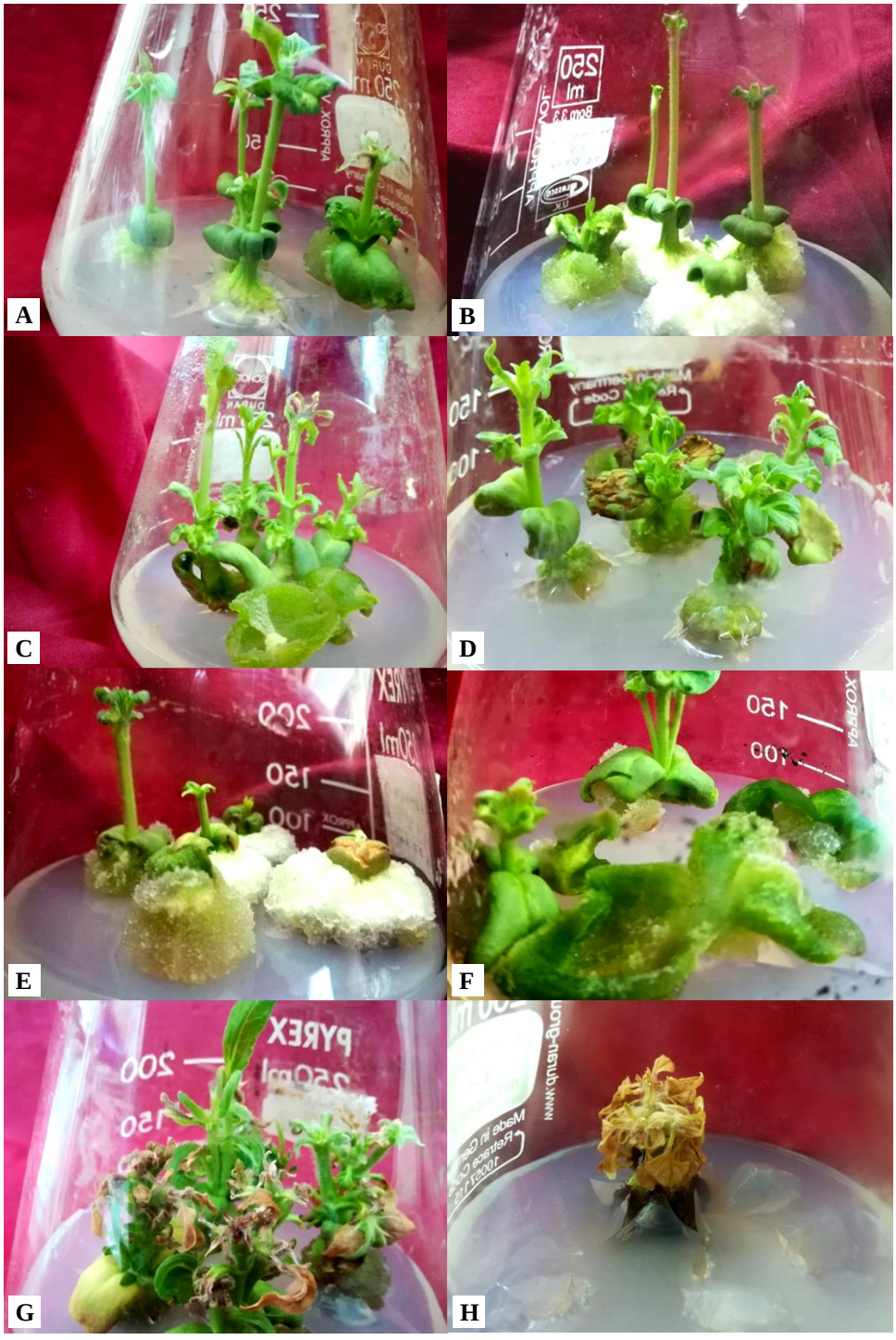

Figure 3.1: Shoots grown from embryonic explant of BRAC Hysun 33 variety on different hormone supplemented MS media after 25 days. **A-D:** Shoots obtained with **A.** 1.0 mg/l BAP and 0.1 mg/l NAA; **B.** 1.0 mg/l BAP and 0.5 mg/l NAA; **C.** 2.0 mg/l BAP; **D.** 3.0 mg/l BAP. **E.** Short shoots observed with 1.0 mg/l BAP and 0.5 mg/l NAA. **F.** Non-regenerative callus with 3.0 mg/l BAP. **G-H:** Top dying disease affecting plants grown in 2.0 mg/l BAP.

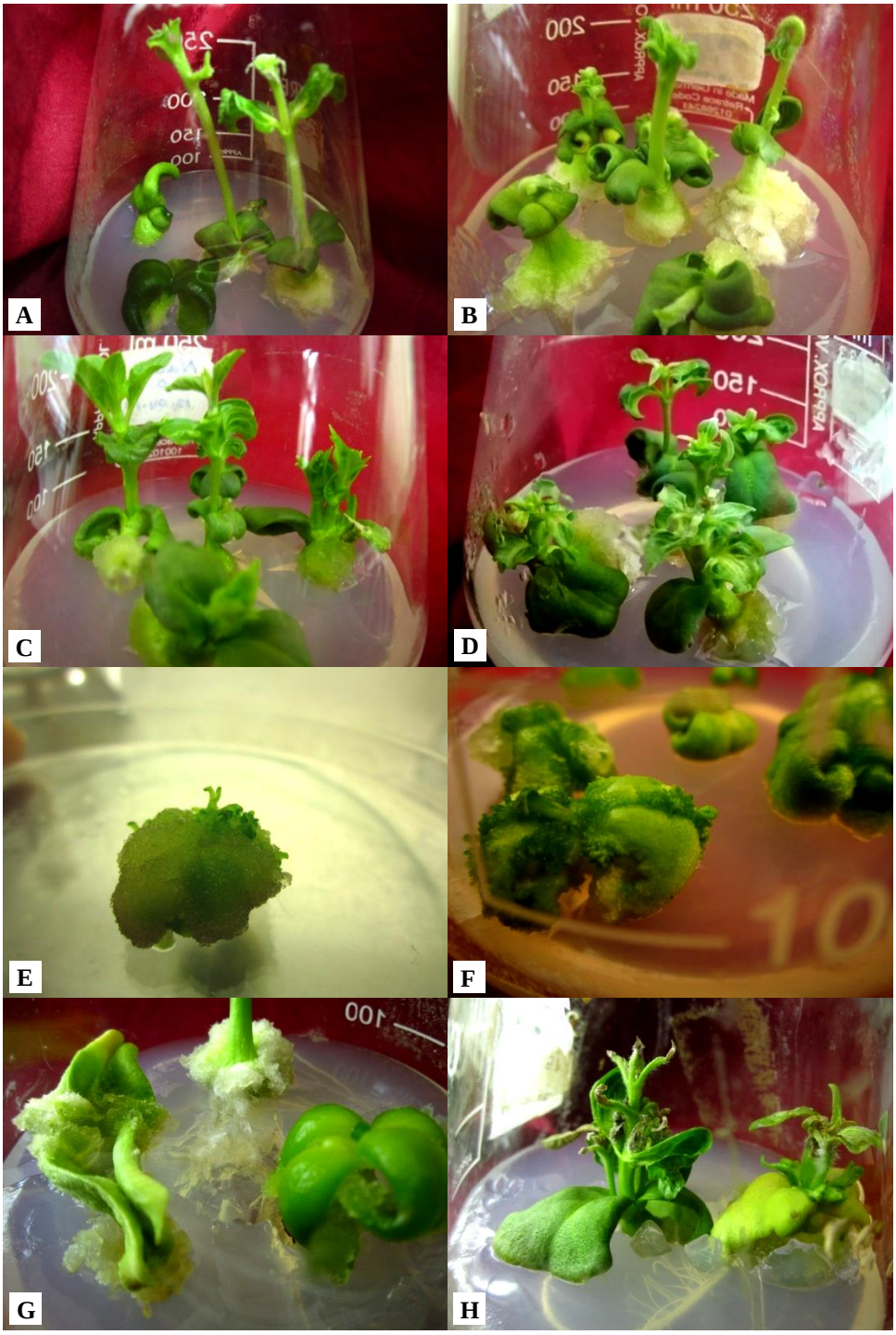

Figure 3.2: Shoots grown from embryonic explant of BARI Surjomukhi-2 variety on different hormone supplemented MS media after 25 days. **A-D:** Shoots obtained with **A.** 1.0 mg/l BAP and 0.1 mg/l NAA; **B.** 1.0 mg/l BAP and 0.5 mg/l NAA; **C.** 2.0 mg/l BAP; **D.** 3.0 mg/l BAP. **E-F:** Bud-like structures observed in calli in media supplemented with 1.0 mg/l BAP and **E.** 0.1 mg/l NAA; **F.** 0.5 mg/l NAA. **G.** Non regenerative calli with 1.0 mg/l BAP and 0.1 mg/l NAA. **H:** Top dying disease affecting plants grown in 2.0 mg/l BAP.

3.2 Analysis of rhizogenesis observed from induced shoots

Well developed shoots were divided in two groups and placed in two different rooting media: ½MS fortified with either 0.4 mg/l IBA or 1.0 mg/l IBA.

For both the varieties, percentage of root initiation did not change much between the two concentrations (tables 3.5 and 3.9). Single factor ANOVAs were conducted to compare the effects of the different hormonal treatments on root formation for each variety. For BRAC Hysun 33 (table 3.6), there was no significant effect of IBA concentrations on the percentage of root formation at the $p<0.05$ level for the three conditions [$F(1, 6) = 0.43$, $p = 0.537$]. Similarly for BARI Surjomukhi-2 (table 3.10), there was no significant effect of IBA concentrations on the percentage of root formation at the $p<0.05$ level for the three conditions [$F(1, 6) = 1.8$, $p = 0.228$].

However, roots were longer in plantlets grown in media with 0.4 mg/l IBA than those grown in 1.0 mg/l IBA (figure 3.3), and also initiated faster. Tap roots were more common at 1.0 mg/l IBA, while 0.4 mg/l IBA gave both tap and fibrous roots. In both media combinations and both varieties, callusing was seen at the base of some shoots within 2-3 days of inoculation. These shoots also gave thin roots, and the plants did not survive when placed in soil. For both varieties, slight shoot elongation and leaf emergence had occurred for all plantlets.

The concentration of hormones in the media the plants had been grown in seemed to have an effect on the root formation. For BRAC Hysun 33, root formation was best in shoots that had been grown in media containing 1.0 mg/l BAP and 0.5 mg/l NAA (95.79%), while least root formation occurred in plants grown in 3.0 mg/l BAP (79.76%). For BARI Surjomukhi-2, highest root formation was observed in shoots that were grown in 3.0 mg/l BAP (94.29%), but lowest root formation was seen with 2.0 mg/l BAP (80.00%). To determine if these results were significant, single-factor ANOVA tests were carried out.

For the BRAC Hysun 33 plantlets (tables 3.7 and 3.8), there was a significant effect of shooting hormone concentration on the percentage of root formation at the $p<0.05$ level for the three conditions [$F(3,12) = 13.26$, $p = 0.000$] in case of plantlets that rooted in 0.4 mg/l IBA, and for the conditions [$F(3,12) = 6.53$, $p = 0.007$] in case of plantlets that

rooted in 1.0 mg/l IBA. Post hoc comparisons using the Tukey HSD test indicated that, the mean root formation in shoots that were grown in the treatment containing 1.0 mg/l BAP and 0.5 mg/l NAA was significantly higher than the mean root formation in shoots grown in either only 2.0 mg/l BAP or only 3.0 mg/l BAP, but not significantly higher than the mean root formation in shoots grown in 1.0 mg/l BAP and 0.1 mg/l NAA. The mean root formation in shoots grown in the two treatments containing only BAP were also not significantly different from each other. Also, for only the plantlets rooted in 0.4 mg/l IBA, the mean root formation in the shoots grown in 1.0 mg/l BAP and 0.1 mg/l NAA was significantly higher than the mean root formation in shoots grown in only 3.0 mg/l BAP.

Similarly, for the BARI Surjomukhi-2 plantlets (tables 3.11 and 3.12), there was a significant effect of shooting hormone concentration on the percentage of root formation at the $p<0.05$ level for the three conditions [$F(3,12) = 19.09$, $p = 0.000$] in case of plantlets that rooted in 0.4 mg/l IBA, and for the conditions [$F(3,12) = 19.95$, $p = 0.000$] in case of plantlets that rooted in 1.0 mg/l IBA. Post hoc comparisons using the Tukey HSD test showed that, the mean root formation in shoots that were grown in 3.0 mg/l BAP was significantly higher than the mean root formation in shoots grown in all other shooting hormone concentrations. The root formation in the shoots grown in the two treatments having 1.0 mg/l BAP but varying NAA concentrations were not significantly different from each other. Also, for only the plantlets rooted in 1.0 mg/l IBA, the mean root formation in the shoots grown in 1.0 mg/l BAP and 0.5 mg/l NAA was significantly higher than the mean root formation in shoots grown in only 2.0 mg/l BAP.

Table 3.5: Effect of different combinations of hormonal supplementations on the *in vitro* root regeneration from shoots of BRAC Hysun 33.

Rooting hormone	Shooting hormone		Responsive shoot (%)	Time taken to initiate roots (days) ± SD	Average length in 25 days (cm) ± SD
IBA (mg/l)	BAP (mg/l)	NAA (mg/l)			
0.4	1.0	0.1	91.11	5.02 ± 0.88	3.21 ± 1.54
	1.0	0.5	95.92	4.98 ± 0.94	2.94 ± 1.12
	2.0	0.0	85.71	7.25 ± 4.22	3.41 ± 1.01
	3.0	0.0	76.19	6.59 ± 3.31	2.85 ± 1.21
1.0	1.0	0.1	88.64	5.41 ± 1.27	2.39 ± 1.08
	1.0	0.5	95.65	6.02 ± 1.47	3.29 ± 1.99
	2.0	0.0	85.71	6.22 ± 3.51	2.60 ± 1.41
	3.0	0.0	83.33	7.06 ± 3.63	2.15 ± 0.65

values obtain from quadruplicate trials

Table 3.6: ANOVA results for root formation in BRAC Hysun 33 variety, based on rooting hormone concentrations.

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	0.5	1	0.5	0.428571	0.536963	5.987378
Within Groups	7	6	1.166667			
Total	7.5	7				

Table 3.7: ANOVA results for root formation in BRAC Hysun 33 variety, based on shooting hormone concentrations. The shoots underwent rooting in 0.4 mg/l IBA.

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	31.5	3	10.5	13.26316	0.000406	3.490295
Within Groups	9.5	12	0.791667			
Total	41	15				

Table 3.8: ANOVA results for root formation from BRAC Hysun 33 variety, based on shooting hormone concentrations. The shoots underwent rooting in 0.1.0 mg/l IBA.

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	12.25	3	4.083333	6.533333	0.007219	3.490295
Within Groups	7.5	12	0.625			
Total	19.75	15				

Table 3.9: Effect of different combinations of hormonal supplementations on the *in vitro* root regeneration from shoots of BARI Surjomukhi-2.

Rooting hormone	Shooting hormone		Responsive shoot (%)	Time taken to initiate roots (days) ± SD	Average length in 25 days (cm) ± SD
IBA (mg/l)	BAP (mg/l)	NAA (mg/l)			
0.4	1.0	0.1	88.00	5.32 ± 1.39	3.08 ± 0.94
	1.0	0.5	89.29	4.44 ± 1.29	2.89 ± 1.02
	2.0	0.0	77.78	6.48 ± 2.04	3.02 ± 0.91
	3.0	0.0	94.44	6.56 ± 2.20	3.08 ± 0.85
1.0	1.0	0.1	81.48	5.50 ± 1.37	2.14 ± 1.11
	1.0	0.5	92.86	5.77 ± 1.58	2.75 ± 1.03
	2.0	0.0	82.61	6.63 ± 1.98	3.24 ± 0.98
	3.0	0.0	94.12	6.59 ± 2.67	2.03 ± 0.89

values obtain from quadruplicate trials

Table 3.10: ANOVA results for root formation from BARI Surjomukhi-2 variety, based on rooting hormone concentrations.

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	1.125	1	1.125	1.8	0.228258	5.987378
Within Groups	3.75	6	0.625			
Total	4.875	7				

Table 3.11: ANOVA results for root formation from BARI Surjomukhi-2 variety, based on shooting hormone concentrations. The shoots underwent rooting in 0.4 mg/l IBA.

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	26.25	3	8.75	19.09091	0.000073	3.490295
Within Groups	5.5	12	0.458333			
Total	31.75	15				

Table 3.12: ANOVA results for root formation from BARI Surjomukhi-2 variety, based on shooting hormone concentrations. The shoots underwent rooting in 1.0 mg/l IBA.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	23.688	3	7.895833	19.9474	0.000059	3.490295
Within Groups	4.75	12	0.395833			
Total	28.435	15				

Figure 3.3: A-E: Root formation in BRAC Hysun 33 variety. **A.** Tap root formation in ½MS + 0.4 mg/l IBA; **B.** Fibrous roots formation from in ½MS + 0.4 mg/l IBA; **C.** Multiple tap roots in ½MS + 1.0 mg/l IBA; **D.** Fibrous root formation in ½MS + 1.0 mg/l IBA; **E.** No root formation in ½MS + 0.4 mg/l IBA. **F-J**: Root formed from BARI Surjomukhi-2 variety. **F.** Tap roots formation from in ½MS + 0.4 mg/l IBA; **G.** Fibrous root formation in ½MS + 0.4 mg/l IBA; **H**. Tap root formation in ½MS + 1.0 mg/l IBA; **I.** Fibrous root formation in ½MS + 1.0 mg/l IBA; **J.** Callus formation and formation of thin roots in ½MS + 0.4 mg/l IBA.

3.3 Analysis of transplantation and acclimatization in natural environment

For both varieties, plants that produced roots were transferred to soil for hardening. 281 plants were hardened for BRAC Hysun 33, while for BARI Surjomukhi-2 variety, 200 plants were taken.

For BRAC Hysun 33 (table 3.13 and figure 3.4), plants that underwent shooting in media containing 0.1 mg/l BAP and 0.5 mg/l NAA had the highest percentage of survivors in terms of hardening (47.44%), with 37 surviving plants. Of these, 24 were rooted in 0.4 mg/l IBA, which is 65%. Remaining 14 plants were rooted in 1.0 mg/l IBA. While this hormone combination gave the lowest number of flowers (14), 93% of them gave seeds. Plants also reached the maximum average height for this variety. Media containing 2.0 mg/l BAP gave the lowest survival percentage (21.21%), the shortest plants, and 17 flowers with only 53% of them giving seeds.

For BARI Surjomukhi-2 (table 3.14 and figure 3.5), plants grown in media with 0.1 mg/l BAP and 0.1 mg/l NAA had the highest survivor percentage in terms of hardening (54.54%), with a total of 24 surviving plants. Of these, 14 were rooted in 0.4 mg/l IBA, which is 58%. Remaining 10 plants were rooted in 1.0 mg/l IBA. For this combination, only 1 plant gave a single flower, which did produce seeds. Media containing 3.0 mg/l BAP gave the lowest survival percentage (21.54%).

As for rooting hormone concentration, it was seen that when plants were transferred to soil for hardening, survival rate was significantly higher in the plantlets grown in media containing 0.4 mg/l IBA (42.07% for BRAC Hysun 33 and 42.16% for BARI Surjomukhi-2) than the plantlets grown in media with 1.0 mg/l IBA (30.15% for BRAC Hysun 33 and 30.61% for BARI Surjomukhi-2).

Table 3.13: Effect of different combinations of hormonal supplementations on hardening of BRAC Hysun 33.

Shooting hormone		Rooting hormone	Plantlets moved to soil	Surviving plantlets	Mature height (cm) ± SD	No. of flowers	flowers producing seeds
BAP (mg/l)	NAA (mg/l)	IBA (mg/l)					
1.0	0.1	0.4	38	20	36.90 ± 8.09	17	12
		1.0	36	14	38.60 ± 12.03	7	4
1.0	0.5	0.4	44	24	43.42 ± 6.59	10	9
		1.0	34	13	35.50 ± 8.39	4	4
2.0	0.0	0.4	33	8	18.72 ± 12.29	14	7
		1.0	33	6	5.75 ± 3.57	3	2
3.0	0.0	0.4	30	9	21.44 ± 8.73	14	8
		1.0	33	8	33.06 ± 10.59	14	5

Table 3.14: Effect of different combinations of hormonal supplementations on hardening of BARI Surjomukhi-2.

Shooting hormone		Rooting hormone	Plantlets moved to soil	Surviving plantlets	Mature height (cm) ± SD	No. of flowers	flowers producing seeds
BAP (mg/l)	NAA (mg/l)	IBA (mg/l)					
1.0	0.1	0.4	22	14	31.50 ± 6.02	1	1
		1.0	22	10	29.50 ± 7.98	0	0
1.0	0.5	0.4	25	14	21.00 ± 9.45	1	1
		1.0	26	11	21.00 ± 8.17	3	0
2.0	0.0	0.4	21	7	14.50 ± 10.54	3	1
		1.0	19	3	13.50 ± 9.10	0	0
3.0	0.0	0.4	34	8	32.50 ± 13.37	11	3
		1.0	31	6	30.83 ± 17.33	9	3

Figure 3.4: Hardening in BRAC Hysun 33 variety. **A.** Bud formation in plant; **B-D**: Flower formation. Petals are brightly coloured; **E-G.** Seed production. Petals dry out and fall off at this stage; **H.** Top dying disease caused plant to die; **I.** Plantlet remained this size for more than 3 months and no buds were formed.

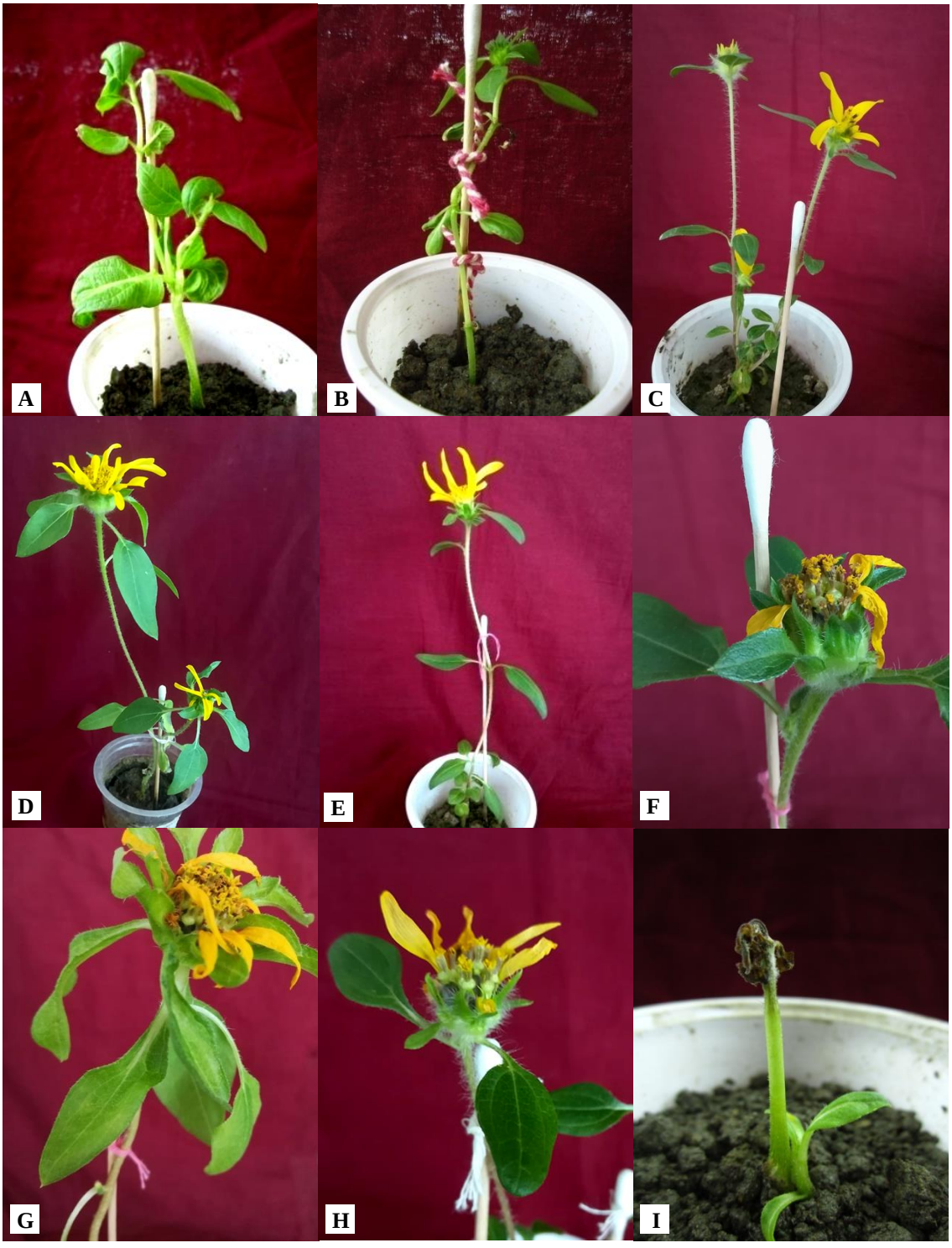

Figure 3.5: Hardening in BARI Surjomukhi-2 variety. **A.** Plantlet grew in size and gave well formed leaves, but no buds were formed; **B.** Plant gave buds but no flowers; **C-E**: Flower formation. Petals are brightly coloured; **F-H.** Seed production. Petals dry out and fall off at this stage; **I.** Top dying disease caused plant to die.

3.4 Reproducibility of the tissue culture products

Seeds from BRAC Hysun 33 gave far more viable seeds than BARI Surjomukhi-2 (table 3.15), thought the average time taken for germination remained the same.

Table 3.15: Reproducibility of both varieties.

Variety	Total number of seeds	Number of seeds germinated	Percentage of seeds germinated (%)	Avg. time taken to germinate
BARI Surjomukhi-2	105	8	7.62	4 days
BRAC Hysun 33	322	289	89.75	4 days

3.5 Comparative analysis of regeneration efficiency of two sunflower varieties

In this experiment, it was observed that BRAC Hysun 33 is the better variety of the two (table 3.16). BRAC Hysun 33 gave a much higher amount of shoot initiation compared to BARI Surjomukhi-2, with negligible difference in shoot length. For rhizogenesis, there was barely any difference between the two varieties; percentage initiation, time taken to form roots and root lengths were very similar. The hardening process, however, seemed to show no differences between the varieties. While survival rate of both varieties were similar, BRAC Hysun 33 had more total surviving plants and this variety also gave much more flowers and seeds compared to BARI-Surjomukhi-2, and on average BRAC Hysun 33 plants also were taller. BRAC Hysun 33 also produced far more viable seeds. For these reasons, it can be concluded that BRAC Hysun 33 is the better variety for *in vitro* regeneration and the protocol developed here is reproducible.

Table 3.16: A comparison of the regeneration efficiency of the two varieties.

Factors compared	BRAC Hysun 33	BARI Surjomukhi-2
Shoot induction (%)	91.09	59.5
Average shoot height (cm)	3.20 ± 1.10	3.36 ± 0.98
Percentage root initiation (%)	88.07	88.16
Time taken for root induction (days)	6.00 ± 2.72	5.95 ± 2.04
Average root length (cm)	2.87 ± 1.38	2.75 ± 1.04
Survival percentage at hardening (%)	36.30	36.50
Total Surviving plants	102	73
Final height (cm)	29.95 ± 13.78	25.32 ± 11.49
Number of flowers	83	28
Seed producing flowers	51	9
Viability of seeds (%)	89.75%	7.62%
Time taken to germinate (days)	4	4
Best media composition for shooting	MS + 1.0 mg/l BAP + 0.5 mg/l NAA	MS + 3.0 mg/l BAP
Best media composition for rooting	½MS + 0.4 mg/l IBA	½MS + 0.4 mg/l IBA

CHAPTER 4

— DISCUSSION —

4. Discussion

For successful *in vitro* regeneration, researchers have tried to use an assortment of explants, such as, stems, roots, leaves, hypocotyl, cotyledons, root segments, shoot tips, cotyledonary petioles, and embryonic axes (Inoka and Dahanayake, 2015; Gürel and Kazan, 1998; Ozyigit *et al.*, 2007; Abd Elaleem *et al.*, 2015; Chowdhury, 2015; Oshin, 2015; Shormee, 2016; Abd Elaleem *et al*, 2015; Gürel and Kazan, 1998; Ozyigit *et al.*, 2006; Aurori, 2011; Chowdhury, 2015; Shormee, 2016). Out of all these, stems, shoots tips, hypocotyls and embryonic axes have shown the best results, with embryonic axis ranking first. While other explants have to undergo dedifferentiation and again redifferentiation, embryonic axis do not, making it more prone to organogenesis than other explants. So, embryonic axis explants were chosen for this experiment.

According to experiments by Gürel and Kazan (1998), different genotypes give different results in *in vitro* regeneration. For this reason, two popular local varieties (BARI Surjomukhi-2 and BRAC Hysun 33) were chosen to determine the best Bangladeshi variety for increasing sunflower production. For embryonic axis explants of these two varieties, experiments have been conducted by Chowdhury (2015) and Shormee (2016) to find the best variety and the ideal hormone concentrations for *in vitro* regeneration.

MS media supplemented with two different hormones (NAA and BAP) were tested for shoot regeneration. Four treatments were used: 1.0 mg/l BAP + 0.1 mg/l NAA, 1.0 mg/l BAP + 0.5 mg/l NAA, 2.0 mg/l BAP, and 3.0 mg/l BAP. Chowdhury (2015) found that the best combination for BRAC Hysun 33 was 1.0 mg/l BAP with 0.1 mg/l NAA (58.33% regeneration), and the best treatment for BARI Surjomukhi-2 was 2.0 mg/l BAP (46.67% regeneration). Shormee (2016), on the other hand, found that 2.0 mg/l BAP gave highest shoot regeneration for both the varieties (66.67% and 46.67% respectively). In this study, however, the optimum shooting hormone concentrations were found to be different. BRAC Hysun 33 gave best result in 1.0 mg/l BAP with 0.5 mg/l NAA (97.03%), while BARI Surjomukhi-2's highest regeneration rate was with only 3.0 mg/l BAP (80.00%). BRAC Hysun 33's response is consistent with the findings of Ozyigit *et al*., (2006), reported with Turkish varieties. For BARI Surjomukhi-2, while the highest regeneration is achieved with 3.0 mg/l BAP, second highest regeneration was obtained

with 1.0 mg/l BAP and 0.5 mg/l NAA, but lowest regeneration was obtained with 2.0 mg/l BAP (ANOVA and Tukey post hoc tests were used to determine that these differences were due to changes in the hormone treatments, and not just by chance). This shows that regeneration efficiency does not increase with increasing concentration of BAP, as was determined by Chowdhury, (2015), but rather this variation could be a result of another factor, such as, the season and personal handlings.

In this experiment, it was found that seasonal variation does influence shoot formation. It was observed that, for BARI Surjomukhi-2, explants that had been inoculated during January to March gave less percentage regeneration than those inoculated in other months. Explants inoculated in the first 3 treatments were placed between January to June. Those placed in April to June gave higher response while those placed in January to March had given low response, thus bringing the overall percentage initiation down for the first 3 treatments. Such seasonal variations is absent in previous reports. Conversely, explants grown in 3.0 mg/l BAP had the better score as all explants had been placed in the October and November, when all explants had high response. For BRAC Hysun 33, explants were mostly inoculated between July and October, so seasonal impact could not be determined for this variety.

Shoot height seemed to be dependent on the hormonal concentration. Baker *et al*., (1999) stated that decreasing contact with high hormone concentrations may increase shoot growth. This phenomenon was observed in this experiment as shoot length did decrease with increase in hormone concentrations. Chowdhury (2015) and Shormee (2016), however, had found that the highest shoot length was obtained with the hormonal treatment that gave the maximum percentage of shoot regeneration.

As BRAC Hysun 33 performed much better than BARI Surjomukhi-2, it can be seen that shoot formation is genotype dependant.

One problem faced during shooting was top dying. In some of the plants, the tips of the plantlets became brown and rotten, probably due to the fact that enough nutrients could not be delivered to the tip of the plants, killing the cells from lack of nutrition. The dead region gradually increased in size until the plant died. If the top dying was observed later

on and the plant was still alive while transplantation to the soil, the plant did not survive the hardening process. Another problem faced was vitrification, which caused the plants to become translucent. These plants did not perform well in rooting. More studies need to be done to overcome these challenges.

In the next phase of the experiment, the effect of two different concentrations of the hormone IBA on the rooting efficiency was determined. The concentrations used in this experiment were 0.4 mg/l IBA and 1.0 mg/l IBA. Previously, Shormee, (2016), found that 1.0 mg/l IBA was the best concentration for root formation. This was also recognised by Inoka and Dahanake (2015) and Ozyigit *et al.* (2007). In this experiment, percentage of root formation was observed to be similar in both 0.4 mg/l IBA and 1.0 mg/l IBA for both BRAC Hysun 33 and BARI Surjomukhi-2 varieties. However, 0.4 mg/l IBA gave longer roots compared to 1.0 mg/l IBA, and roots also formed a little faster. There was no difference in rooting response between the two varieties, showing that root formation is not genotype dependent. This differed from Shormee (2016), who found that BRAC Hysun 33 variety gave roots faster than BARI Surjomukhi-2.

Interestingly, the shooting hormone concentration also had an effect on the root formation. For both varieties, best root formation took place with shoots that had been grown in the treatment that had given highest shooting. For BRAC Hysun 33 and BARI Surjomukhi-2, highest percentage of root initiation was obtained in shoots that had been grown in 1.0 mg/l BAP and 0.5 mg/l NAA and 3.0 mg/l BAP, respectively. Both ANOVA and Tukey post hoc tests supported that these differences in results were in fact caused by the different hormone treatments, and not by chance.

Since the roots have to be excised from the shoots at a very precise point (the node of the hypocotyl), there is a chance of cutting the shoots at a wrong location. In these cases, calli form at the position of the cut. These calli may or may not produce roots, but even if roots are formed they are not strong enough to survive during hardening. As a result, the cuts must be made very precisely to avoid such callusing.

When roots were well formed, the plants were transferred into soil in the next stage: acclimatisation. The soil used was a mixture of four parts autoclaved soil and one part

fertiliser. Shormee (2016) had used a mixture of soil and sand. While plant growth and survival had been observed, no flowering or seed formation was seen. In this experiment, plantlets from both varieties survived and gave flowers, which in turn formed seeds. In BRAC Hysun 33, the highest percentage of surviving plants were obtained from plantlets that had undergone shooting in 1.0 mg/l BAP and 0.5 mg/l NAA. Though this hormone treatment gave less flowers, almost all of these flowers gave seeds, and the plants had the tallest average height. Lowest survival rate was in plants grown in 2.0 mg/l BAP, and these plants were also the shortest. For BARI Surjomukhi-2 variety, rate of survival was highest in plantlets grown in 1.0 mg/l BAP and 0.1 mg/l NAA. 3.0 mg/l BAP gave the tallest plants with most flowers, though its rate of survival was lowest. Also, plants that had undergone root formation in 0.4 mg/l IBA survived more than those that formed roots in 1.0 mg/l IBA. This suggests that roots grown in 0.4 mg/l IBA were more adapted to survival compared to the roots formed in 1.0 mg/l IBA, even though the latter concentration gave slightly more roots compared to the other. Lastly, BRAC Hysun 33 gave far more viable seeds than BARI Surjomukhi-2, even though the average time for germination was the same for both varieties.

Overall, it seemed BRAC Hysun 33 was a better variety compared to BARI Surjomukhi-2, as it gave more shoot formation and many more surviving plantlets that gave more flowers and more viable seeds compared to BARI Surjomukhi-2. Previously, Chowdhury (2015) and Shormee (2016) had also determined that BRAC Hysun 33 had a better regenerative capacity than BARI Surjomukhi-2. This matches with the findings of Gürel and Kazan (1998), who said that hybrid varieties perform better than others, possibly due to greater hybrid vigour. BRAC Hysun 33, being a hybrid variety, therefore performed better than BARI Surjomuhki-2.

Therefore, the verdict of the experiment is that for *in vitro* regeneration of Bangladeshi sunflower, BRAC Hysun 33 variety should be used for micropropagation. ANOVA and Tukey post hoc tests determined that that the shooting hormone concentrations did produce significant differences in the results, and hence MS media with 1.0 mg/l BAP and 0.5 mg/l NAA is best for shooting, while ½MS media supplemented with 0.4 mg/l IBA is optimal for rooting in *in vitro* organogenesis and plantlet formation.

CHAPTER 5

— REFERENCES —

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