## Joint Biotechnology Master Program

Palestine Polytechnic University
Deanship of Graduate Studies and
Scientific Research

Bethlehem University
Faculty of Science

# *In vitro* shoot regeneration and genetic differentiation of Baladi cauliflower (*Brassica oleracea var. botrytis*) in Hebron.

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*In Partial Fulfillment of the Requirements for the Degree*
*Master of Science in Biotechnology*

**September, 2021**

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***In vitro* shoot regeneration and genetic differentiation of Baladi cauliflower (*Brassica oleracea var. botrytis*) in Hebron.**

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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# Abstract

Cauliflower exhibits a transition from vegetative to flowering growth triggered by low temperature (vernalization) which controls late or early flowering. FLC gene homologs in *Brassica* species controls flowering response. In this study, integrated molecular and tissue culture approaches were used to explore genetic variation in the flowering genes and to improved cauliflower crop. In the molecular approach, part of the *BoFLC.HP* gene was sequenced in hybrid and local "Baladi" cauliflower using the F7R7 primer to characterize early or late FLC gene in cauliflower. Sequenced *BoFLC.HP* region showed a point mutation at position 91 and an indel at position 212 to 219 in intron number 2 between Baladi and F1- hybrid cauliflower which controls flowering time. In the tissue culture approach, hypocotyl and cotyledon explants from four-day-old seedlings were cultured on Murashige and Skoog (MS) salts supplemented with BA, KIN and TDZ alone or in combination with IBA or NAA. The highest shoot regeneration response (80%) was observed in hypocotyl explants on full strength MS media supplemented 0.5 mg/L BA and 0.5 mg/L IBA. Cotyledon explants, showed 26% shoot regeneration on full strength MS media supplemented 2.0 mg/L BA and 0.1 mg/L NAA. One of the most important tools in crop improvement is the process of genetic transformation with the *Agrobacterium*, which requires an efficient *in vitro* shoot regeneration protocol from explants. These two aspects will be a prerequisite in cauliflower crop development in the future.



**Keywords:** *Brassica oleracea var. botrytis*, flowering time, vernalization, *BoFLC.HP* gene, *in vitro* shoot regeneration, Hypocotyl, Cotyledon.

## ملخص بالعربي

يعتبر الانتقال من المرحلة الخضرية إلى مرحلة الإزهار مرحلة حرجة في القرنبيط والتي تحتاج إلى درجة حرارة باردة (vernalization) للتحكم في الإزهار المتأخر أو المبكر. تتحكم المتجانسات الجينية *FLC* في *Brassica* بالاستجابة المزهرة. في هذه الدراسة، تم استخدام أساليب زراعة الأنسجة والجزيئية لاستكشاف التباين الوراثي في الجينات المزهرة ولتحسين محصول القرنبيط. في النهج الجزيئي، تم ترتيب تسلسل جين *BoFLC.HP*في القرنبيط الهجين والبلدي باستخدام برايمر F7R7 لتمييز بين الازهار المبكر والمتأخر. في *BoFLC.HP* أظهر برايمر "F7R7" طفرات استبدال في الموقع ٩١و طفرة اضافة / حذف من الموقع ٢١٢ ل ٢١٩ في إنترون رقم ٢ بين القرنبيط البلدي والهجين تتحكم في وقت الازهار. في زراعة الأنسجة، تمت زراعة نباتات hypocotyl وcotyledon من شتلات عمرها ٤ ايام على وسائط مختلفة لتجديد البراعم تعتمد على أملاح Murashige and Skoog (MS) المضاف اليها (BA) او (TDZ) (KIN) بمفرده أو مع (IBA) أو (NAA). أعلي نسبة تجدد براعم hypocotyl كانت في الوسط الذي يحتوي علي MS كاملة القوة تحتوي على ٠,٥ ملجم / لتر BA مع ٠,٥ ملجم / لتر IBA بقيمه ٨٠٪. في cotyledon لوحظ أعلي نسبة تجدد براعم في وسط MS يحتوي على ٢,٠ مجم / لتر BA مع ٠,١ مجم / لتر NAA بقيمه ٢٦٪. في تجدد البراعم داخل المختبر، أعطت نتائج hypocotyl أفضل معدل تجدد للبراعم من cotyledon في نبات القرنبيط، وهذا يشير إلى أن النمط الجيني يلعب دورًا مهمًا في تجديد النبتة. من أهم الأدوات في تحسين المحاصيل هي عملية التحول الجيني باستخدام البكتريا *Agrobacterium* الأمر الذي يتطلب بروتوكولًا فعالًا لتجديد الفروع في المختبر من النباتات المستأصلة.

# DEDICATION

When my feet stood on the path of the beginning that was long then we could not see the range, it was no more than my small steps Now it is almost over we feel nostalgic to the first day of this project.

But for the end road I will still carry the burden of the longing that will accompany us for life, we have given it everything in it to stay, her fragrance between our breath and digging in our memory.

I dedicate this project to Everyone who contributed to my arrival at the final route, to everyone who taught we something new, to all who stood beside me and helped me in all difficulties, to my professors and doctors at the university, I especially mention Dr. Rami Arafeh and Zaid Al-Taradeh and my families.

And also dedicate to whom God made them my brothers in deen and who loved them in for the sake of God my friends in the university, to those who did not know us and will not know them. To whom I wish to keep their photos in my eyes, to the souls that inhabited my hearts.

# ACKNOWLEDGMENT

Our first and greatest thanks are to Allah. the Almighty for providing our source of inspiration, wisdom, knowledge and understanding. He has been the source of my strength throughout this project and on His wings only have we soared.

Next, I would like to express my deep gratitude to my supervisor Dr. Rami Arafeh for his effort and encouragement throughout the preparation of this project and I would like to express thanks to the lab technicians Mr. Zaid Al-Tarada, Mr. Husan Al-Tarada and Asmaa Tamimi. Their knowledge and endless support were a great asset from which I learned plenty.

I wish to express thanks my families for material and moral support and I would like to thank a special friend who did their best whenever we needed or asked for anything.

It is difficult to acknowledge everyone who was involved in preparation of this project by name. Nevertheless, we appreciate their help no matter how simple it might have been .

Finally, we owe our colleagues great thanks for supporting us through our college years and for making those years the best years of our life.

# ABBREVIATIONS

| Abbreviation | Word or Sentence |
|---|---|
| °C | Degree Centigrade Celsius |
| g | Gram |
| ANOVA | Analysis Of Variance |
| % | Percent |
| Mg | Milligram |
| L | Liter |
| Ml | Milliliter |
| µl | Microliter |
| *et al* | And Others |
| sec. | Second |
| min. | Minutes |
| $m^2$ | Square Meter |
| µmol | Micromoles |
| PPFD | Photosynthetic Photon Flux Density |
| kpa | Kilopascal |
| rpm | Round Per Minute |
| MS | Murashige And Skoog |
| X MS½ | Half Strength MS |
| BA | Benzylaminopurine |
| TDZ | Thidiazuron |
| KIN | Kinetin |
| NAA | Naphthalene Acetic Acid |
| IBA | Indole-3-Butyric Acid |
| PGRs | Plant Growth Regulators |
| pH | Potential Hydrogen |
| SDW | Sterile Distilled Water |
| UV | Ultraviolet |
| p | p-Value |

# List of Tables


| Table | Description | Page |
|---|---|---|
| **Table 3.1** | The primer pair sequence used in detecting of the mutation of the *BoFLC.HP* gene**.** | **9** |
| **Table 3.2** | Components and volumes of master mix for a single PCR reaction. | **10** |
| **Table 4.1** | The effect of BA with IBA on shoot regeneration after four weeks of culture. | **17** |
| **Table 4.2** | The effect of 1.0 mg/l KIN with different concentrations of IBA on shoot regeneration after four weeks of culture. | **19** |
| **Table 4.3** | The effect of 0.5 mg/l KIN with different concentrations of IBA on shoot regeneration after four weeks of culture | **21** |
| **Table 4.4** | The effect of 2.0 mg/l BA with different concentration of NAA on shoot regeneration after four weeks of culture. | **23** |
| **Table 4.5** | Effect of TDZ with and without NAA on shoot regeneration after four weeks of culture. | **25** |
| **Table 5.1** | Percentage of shoot regeneration for KIN alone or with IBA between Pavlovj *et al* study and this study | **36** |
| **Appendix Table 1** | Area and production of cauliflower in Palestinian territory. | **44** |
| **Appendix Table 2** | Analysis of variance (ANOVA) for effect of TDZ hormone on shoot regeneration at different TDZ concentration after four weeks of the *in vitro* shoot regeneration | **46** |
| **Appendix Table 3** | Analysis of variance (ANOVA) for effect of IBA with BA hormone on shoot regeneration at different concentration after four weeks of the *in vitro* shoot regeneration. | **47** |
| **Appendix Table 4** | Analysis of variance (ANOVA) for effect of 1 mg/ml KIN with different concentration of IBA hormone on shoot regeneration after four weeks of the *in vitro* shoot regeneration. | **48** |
| **Appendix Table 5** | Analysis of variance (ANOVA) for effect of 0.5 mg/ml KIN with different concentration of IBA hormone on shoot regeneration after four weeks of the *in vitro* shoot regeneration. | **49** |

# List of Figures


| Figure | Description | Page |
|---|---|---|
| **Figure 1.1** | Baladi and hybrid cauliflower. | **3** |
| **Figure 4.1** | Gel image of *BoFLC.HP* gene in Baladi and hybrid cauliflowers. | **13** |
| **Figure 4.2** | BLAST result for the *BoFLC.HP* gene from first band sequence | **15** |
| **Figure 4.3** | Sequence Alignment for the *BoFLC.HP* gene between Baladi and hybrids cauliflowers and reference sequence. | **16** |
| **Figure 4.4** | Point Mutation of the *BoFLC.HP* gene between Baladi and hybrids cauliflowers. | **16** |
| **Figure 4.5** | *In vitro* shoot regeneration from hypocotyl and cotyledon explants in full -strength MS media with BA and IBA. | **17** |
| **Figure 4.6** | *In vitro* shoot regeneration from hypocotyl and cotyledon explants in full-strength MS media with 1mg/l KIN and different concentrations of IBA. | **20** |
| **Figure 4.7** | The effect of 0.5 mg/l KIN with different concentrations of IBA on *in vitro* shoot regeneration from hypocotyl explants | **22** |
| **Figure 4.8** | The effect of 2.0 mg/L BA with different concentrations of NAA on *in vitro* shoot regeneration from different explants. | **25** |
| **Figure 4.9** | *In vitro* shoot regeneration from hypocotyl and cotyledon explants in full MS media | **26** |
| **Figure 4.10** | *In vitro* shoot regeneration from callus explants. | **27** |
| **Figure 4.11** | *In vitro* shoot regeneration from leaf explants. | **28** |
| **Figure 4.12** | Cauliflower plant from hypocotyl explants regeneration *in vitro* | **29** |
| **Figure 4.13** | Statistical analysis of the effect of PGRs on *in vitro* shoots regeneration from hypocotyl explants. | **31** |
| **Figure 4.14** | Statistical analysis of the effect of 2.0 mg/L BA with different concentrations of NAA on shoots regeneration from cotyledon explants | **32** |

# Table of contents

## Contents

Abstract ... iii
ملخص بالعربي ... iv
DEDICATION ... v
ACKNOWLEDGMENT ... vi
ABBREVIATIONS ... vii
List of Tables ... viii
List of Figures ... ix
Table of contents ... i
Chapter One: Introduction ... 1
1.1 Cauliflower (*Brassica oleracea L. var. botrytis*) ... 1
1.3 Origin of Cauliflower ... 1
1.2 Importance of cauliflower: nutritional and medicinal value ... 2
1.4 Cauliflower in Palestine ... 2
1.5 Development and flowering time of cauliflower ... 3
1.6 Vernalization requirement of cauliflower and FLC genes ... 6
1.7 Plant tissue culture ... 7
1.8 *In vitro shoot* regeneration in *B. oleracea* ... 8
Chapter Two: Objectives ... 10
Chapter Three: Materials and Methods ... 11
Plant Material ... 11
3.1 Genetic material isolation and purification ... 11
3.1.1 Oligonucleotide Primers ... 11
3.1.2 DNA extraction ... 11
3.1.3 PCR reaction ... 12
3.1.4 Gel electrophoresis and documentation ... 13
3.1.5 DNA purification and sequencing ... 13
3.2 *In vitro* culture of cauliflower ... 13
3.2.1 Seed surface sterilization: ... 13
3.2.2 Media Preparation and Sterilization ... 14
3.2.3 Growth conditions ... 14
3.3. *In vitro* shoot regeneration ... 14
3.4 Experimental design and statistical analysis ... 14

Chapter four: Results ..........15
4.1. Molecular analysis of the flowering gene in *B. oleracea var. botrytis*. ..........15
4.1.1 PCR amplification of the *BoFLC.HP* gene..........15
4.1.2. Genetic variation between Baladi and hybrid varieties in *BoFLC.HP* gene..........16
4.1.3. Sequences Alignment for the *BoFLC.HP* gene between Baladi and two type of hybrid..........17
4.2. *In vitro* shoot regeneration of *B. oleracea var. botrytis*. ..........19
4.2.1. The effect of BA with IBA on *in vitro shoot* regeneration..........19
4.2.2 The effect of kinetin and IBA on *in vitro* shoot regeneration. ..........22
4.2.4 The Effect of BA with different concentrations of NAA on *in vitro* shoot regeneration. ..........26
4.4.5. Effect of different concentrations of TDZ with and without NAA on *in vitro* shoot regeneration. ..........28
4.2.6. *In vitro* shoot regeneration from callus explants. ..........30
4.2.7. *In vitro* shoot regeneration from leaf explants..........31
4.3. Transfer plant regeneration from hypocotyl explants to soil in greenhouse (acclimatization) ..........32
4.4. Statistical analysis for the effect of PGRs on shoot regeneration from hypocotyl explants. ..........32
4.5. Statistical analysis for the effect of PGRs on shoot regeneration from cotyledon explants. ..........35
Chapter Five: Discussion ..........36
Part one: Molecular analysis of flowering gene in *B. oleracea var. botrytis*. ..........36
Part two: *In vitro* shoot regeneration of *B. oleracea var. botrytis* ..........37
Chapter Six: Conclusion and future works ..........41
Chapter Seven: References ..........42
Chapter Eight: Appendix ..........47

# Chapter One: Introduction

## 1.1 Cauliflower (*Brassica oleracea L. var. botrytis*)

*Brassica oleracea var. botrytis*, the cauliflower, is a type of several *Brassica oleracea* varieties in the genus *Brassica* belonging to the family *Brassicaceae*. *Brassica oleracea* is a species commonly known for its polymorphism, which also includes other important vegetables for human consumption such as broccoli, cabbage, brussels sprouts, kohlrabi and kale. The name of cauliflower comes from the Latin word *caulis*, for cabbage, and *floris*, for flower. Cauliflower is an important vegetable crop grown in about 80 countries worldwide, in which the production of 3.5 million tons makes it an economically important vegetable (Hodgkin, 1995).

Cauliflower is an annual crop grown from seeds to produce a large tight heads of aborted white inflorescence meristem called curds. Typically, only the head (the white curd) is eaten, while the stalk and the surrounding thick green leaves are used in vegetable soups or discarded. Although the curd is usually white, cauliflower also comes in other colors such as purple, yellow, and orange.

The weather is a limiting factor for producing cauliflower, it grows best in mild to cold temperatures (10-21°C), with plentiful sun and moist soil, rich in organic matter. High temperatures produce poor quality curds and the very low temperature delays maturity and produce small unmarketable curds may be formed.

## 1.3 Origin of cauliflower

Cauliflower is thought to have been domesticated in the Mediterranean region since the greatest range of variability in the wild types of *B. oleracea* is found there. In the Middle Ages, cauliflower was associated with the island of Cyprus and the Arab botanists Ibn al-'Awwam and Ibn al-Baitar claiming its origin to be in Cyprus. This association continued into Western Europe, where cauliflower was sometimes known as Cyprus colewort (Fenwick *et al.*, 1983). Moreover, there was extensive

trade in Western Europe in cauliflower seeds from Cyprus and this lead to move it to other areas like Syria, Turkey, Egypt, Italy, Spain and northwestern Europe.

## 1.2 Importance of cauliflower: nutritional and medicinal value

Cauliflower is a healthy vegetable that contains a significant source of nutrients, tasty and easy to prepare, very low-calorie value and contains some of almost every vitamin and mineral such as vitamins C, K, A and B9, potassium, manganese, phosphorus, and magnesium. Additionally, it contains fibers, sulforaphane and flavonoids which possess antioxidant and anti-inflammatory properties and anticancer activity such as colon, prostate, breast, leukemia, pancreatic and melanoma (Clarke *et al*., 2008) (Metwali & Al-Maghrabi, 2012). Moreover, it reduces and prevents the risk of several diseases, including cardiovascular diseases (Gorinstein *et al*., 2006) and diabetes (Slavin & Lloyd, 2012). Cauliflower contains high amount of fibers which is important for digestive health and obesity prevention due to its ability to promote fullness and reduce overall calorie intake (Slavin, 2005). Moreover, cauliflower is high in fibers and water but low calorific value, which may help with weight loss.

Cauliflower also is a good source of choline a nutrient that plays a major role in maintaining the integrity of cell membranes, synthesizing DNA and supporting metabolism(Sanchez-Lopez et al., 2019). It is also involved in brain development and the production of neurotransmitters that are necessary for a healthy nervous system(Bekdash, 2018). Moreover, it helps to prevent cholesterol from accumulating in the liver(Sherriff *et al*., 2016).

## 1.4 Cauliflower in Palestine

Cauliflower is an important vegetable crop grown in about 80 countries worldwide on approximately 9 million Ha worldwide with a production of around 27 million tons in 2020 combined with broccoli (Sherriff et al., 2016). In 2019, the Palestinian production of cauliflower was about 34502 tons on an area of 11294 $m^2$ (PCBS, 2009) Appendix Table 1.

There are two types of cauliflower grown in Palestine: local cauliflower which is called "Zahara Baladi" with yellow curd, and hybrid cauliflower (white curd) Figure 1.1. Local cauliflower is grown in the cold regions of the mountain areas in the West Bank in April and May as a rainfed crop and continues to grow for 6-7 months, on the other hand, hybrid cauliflower matures in 70 days and depends on irrigation. Due to the short time the hybrid cauliflower needs to grow compared to local cauliflower and the abundance of water in Palestine, its use increased until the local cauliflower disappeared at least 40 years ago in the north. Moreover, the quality and curd size of Baladi cauliflower usually are larger than the hybrid and the price is much higher (1.0 kg of local cauliflower equal 5.0-7.0 NIS but 1.0 kg of hybrid equal 2.0-4.0 NIS) on average.

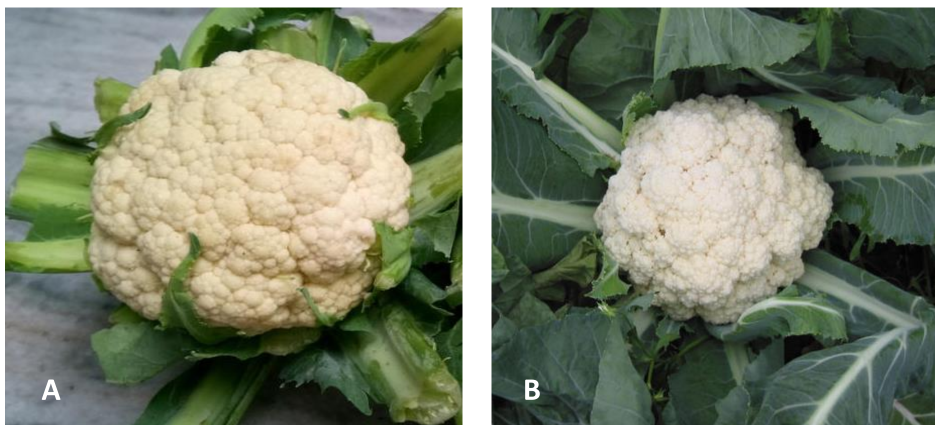



**Figure 1.1**: **A**: Baladi and **B**: hybrid cauliflower.

## 1.5 Development and flowering time of cauliflower

The transition from vegetative to flowering growth is a critical phase in the life cycle in cauliflowers. Timing of transfer to flowering depends on integration of endogenous factors such as leaf number and gibberellin (GA) biosynthesis(Bernier & Périlleux, 2005; Mutasa-Göttgens & Hedden, 2009) and exogenous factor such as photoperiod, vernalization, and stress(Amasino, 2005; Yaish et al., 2011). In cauliflower, curd development in cauliflower is regulated by temperature by the vernalization process, which is the induction of a plant's flowering by exposure to the prolonged cold temperature in winter. Moreover, vernalization is obligatory to transfer to the flowering phase and formation of the edible and good quality curd as marketable plant and bolting (elongation of inflorescence stem). High temperature

can lead to prolong of vegetative development phase by delayed vernalization and weakens the harvest traits. Moreover, exposure to more heat often results in bracting (Grevsen *et al*., 2003), whereas more low temperatures can lead to premature flower bud development that causes riciness (Grevsen *et al*., 2003). Both factors are responsible for low curd quality. In addition to, flowering time is important in cauliflower because early flowering leads to reduction of yield and commercial values but late flowering produces high quality and economically valuable (Mao *et al*., 2014; Wang *et al*., 2014). Considering the value of early and late flowering cauliflower, it is important to plan flowering time before planting.

## 1.6 Background on genetics of the flowering in cauliflower

Although much research has been done on the phenotypic effects of temperature regulated curd induction, underlying genetic pathways remain largely unknown. Up until now, most research on the underlying genetic mechanisms pathways to temperature regulated floral timing has been previously elucidated in *Arabidopsis thaliana* (Fornara *et al*., 2010). Like cauliflower, the flowering time of *A. thaliana* can be heavily influenced by temperature. While high temperatures can delay curd initiation in cauliflower, flowering in most *A. thaliana* accessions is known to be accelerated by higher temperatures (Verhage et al., 2014).

General flowering pathways and main flowering time genes seem to be conserved in several agronomically important crops, among them the closely related *Brassica* species, including cauliflower (Lagercrantz *et al*., 1996; Schranz *et al*., 2006).

## 1.7 Flowering timing by temperature in *A. thaliana*

There are multiple mechanisms that control the ambient temperature dependent flowering response in *A. thaliana*, which could give insight in the temperature dependent flowering curd initiation response mechanisms in *B. oleracea*. Some of these mechanisms can be directly linked to flowering time control, while other mechanisms are indirect and are also dependent upon other environmental signals (e.g., day-length and age) (Verhage et al., 2014). Figure 1.2 shows a small overview of the main floral inducing pathways and key genes.

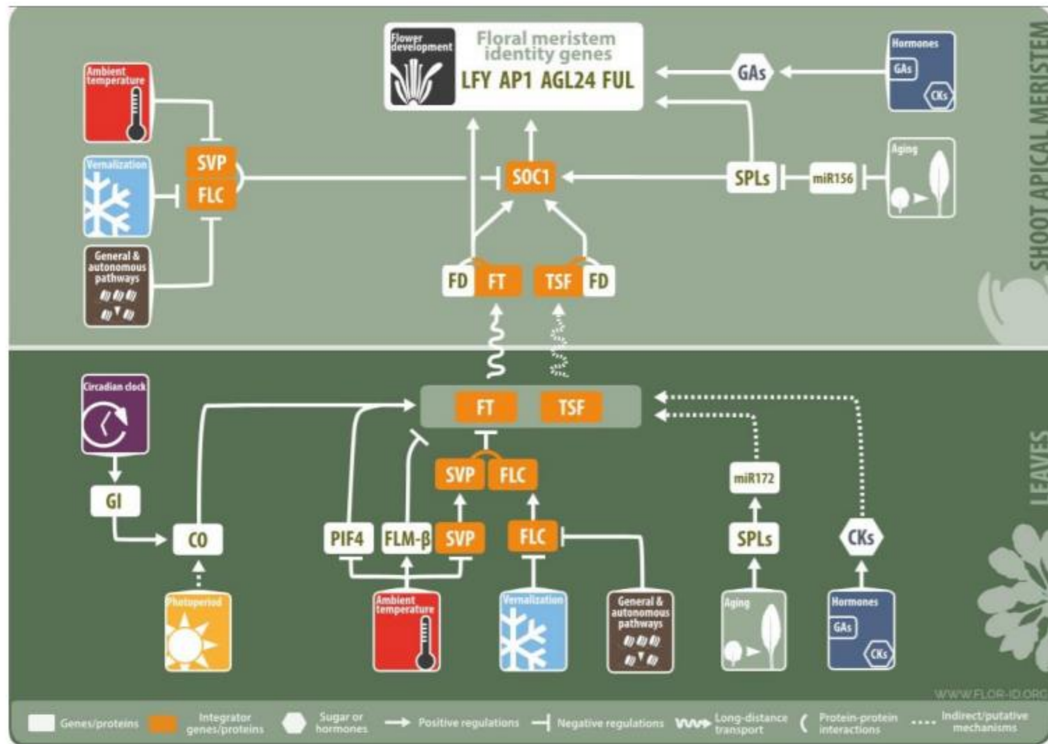



**Figure 1.2 Floral inducing pathways in *A. thaliana*.** Multiple endogenous and exogenous cues regulate flowering time through various floral inducing or repressing pathways(Bouché et al., 2016).

## 1.8 MADS-box genes

MADS-box genes play a crucial role in plant development, especially in flower development. The term 'MADS' was derived from four members of the MADS family in fungi, plants and animals: MCM1 in yeast, AGAMOUS in *Arabidopsis*, DEFICIENS in snapdragon, and SERUM RESPONSE FACTOR in human (Jack, 2001; Yanofsky *et al.*, 1990). MADS-box genes possess a highly conserved MADS domain that consists of roughly 60 amino acids at the amino-terminal end of the protein, followed by the I domain, the K domain and the C region from N-termini to C-termini (Xu *et al.*, 2014). K domain is also highly conserved, while I domain and C region are quite variable. MADS domain encodes a DNA binding and dimerization function. The MADS-domain transcription factor *FLOWERING LOCUS C* (FLC) plays a central role in conferring a response to vernalization (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). The transcriptional and post-transcriptional regulation of *FLC* and how these contribute

to environmental responses have been reviewed in detail (Costa & Dean, 2019; Whittaker & Dean, 2017).

## 1.9 Vernalization requirement of cauliflower and FLC genes

In vernalization pathway, *FLOWERING LOCUS C* (*FLC*) and *VERNALIZATION 2 (VRN2*) genes play a key regulatory role in the transition from vegetative to flowering phase (Schmitz & Amasino, 2007). *FLC* encodes a MADS-box protein that binds directly to a region of DNA in the first intron and prevents floral transition by directly repressing floral integrators, among them *FLOWERING TIME (FT), FLOWERING LOCUS D (FD), and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*(Deng *et al*., 2011; Michaels & Amasino, 1999). At vernalizing temperature, expression of *(VRN2)*, together with *VERNALIZATION 1 (VRN1)* and *VERNALIZATION INSENSITIVE 3 (VIN3*) is induced, which mediates suppression of *FLC* mRNA transcription and translation, thereby releases flowering genes *FT, FD* and *SOC1* from *FLC* repression (He & Amasino, 2005; Jung & Müller, 2009). This in turn leads to the subsequent activation of meristem identity genes, for example, *LEAFY (LFY)* and *APETALA 2 (AP2)* that enhance flower induction (Jung & Müller, 2009; Sung & Amasino, 2004).

In *Brassica* crop species, the comparative studies have enabled the isolation of multiple copies of *Arabidopsis* FLC homologs. Four FLC homologs have been identified (*BrFLC1, BrFLC2, BrFLC3 and BrFLC5*) in *B. rapa*, four (*BoFLC1, BoFLC3, BoFLC4 and BoFLC5)* in *B. oleracea* (Lin *et al*., 2005; Okazaki *et al*., 2007; Schranz *et al*., 2002) and five (*BnFLC1, BnFLC2, BnFLC3, BnFLC4 and BnFLC5*) in *B. napus* (Tadege *et al*., 2001). In a study of genetic mapping analysis of *B. rapa*, Schranz *et al*. (2002) demonstrated that *BrFLC1, BrFLC2 and BrFLC5* are playing an important role in vernalization and controls flowering response. It is not known which of the four *BoFLCs* play an important role for flowering in *Brassica* species.

In *Brassica oleracea*, (Okazaki *et al*., 2007) reported a frameshift mutation in QTL and genomic analyses in exon number 4 of *BoFLC2* in non-vernalization broccoli because of a single base deletion, suggesting that *BoFLC2* contributes to the control of flowering time. A loss-of-function mutation at *BoFLC.C2* in cauliflower has been associated with an early flowering phenotype (Ridge *et al*., 2015). These results

indicate that the *BoFLC2* function may support important differences between annual and biennial *Brassica* varieties, particularly in the flowering time.

Another study by Abuyusuf *et al*. (2019) reported the sequence variation in the *BoFLC1.C9* gene for early and late flowering cabbage lines. They identified sequence variation of 67 bp insertions in intron 2, which contributed to the flowering time variation of the *BoFL1.C9* gene in the early-flowering line compared to the late-flowering one upon vernalization by one set of primer ‘F7R7’ proposed as a marker.

Sun *et al*. (2019) reported the high-quality genome sequence of cauliflower. The assembled cauliflower genome was 584.60 Mb in size, contained 47,772 genes. 56.65% of the genome was composed of repetitive sequences. The genome of cauliflower was sequenced by PacBio and Illumina sequencing technologies. Our knowledge of genome variation and agriculturally important trait formation in cauliflower is still lacking.

Nowadays, there is a strong demand for improving cauliflower breeding with tissue culture technique and genetic engineering. Efficient and reliable tissue culture regeneration protocol is a prerequisite for efficient cauliflower breeding for using genetic transformation with *Agrobacterium*. Moreover, the development of a suitable regeneration protocol for each genotype is necessary due to the very high genotypic specificity for regeneration.

## 1.7 Plant tissue culture

Plant tissue culture (PTC) is a collection of techniques used to maintain or grow plant cells, tissues or organs on synthetic nutrient media under aseptic condition and controlled light, temperature and humidity. The most important contribution made through PTC is the demonstration of the unique capacity of plant cells to regenerate full plants, via organogenesis or embryogenesis, irrespective of their source. Shoot regeneration can be achieved from various tissues and organs including hypocotyls, cotyledons, roots, leaves, peduncle segments, callus and cell cultures, thin cell layers and protoplasts (Vinitha & Stewart, 2004) can often be used to generate a new or whole plant (totipotency) on culture media contain required nutrients and plant hormones and growth regulators (PGRs). For example, high auxin promotes roots

proliferation, while cytokinins yield shoots. A balance of both auxin and cytokinin will often produce an unorganized growth of cells or callus.

## 1.8 *In vitro shoot* regeneration in *B. oleracea*

Tissue culture and genetic engineering technology are two integrative techniques to achieve *Brassica* crops improvement. The successful application of these approaches requires an efficient and reliable tissue culture shoot regeneration protocol. *In vitro* shoot regeneration from explants of *Brassica* is attractive because of an abundant and convenient supply of explants. Regeneration in *B. oleracea* has been reported from leaf and root segments (Lazzeri & Dunwell, 1986), cotyledons (Narasimhulu & Chopra, 1988) and hypocotyl (Poulsen & Nielsen, 1989). Tissue regeneration and transformation frequency in *Brassica* is highly genotype dependent (Boszoradova *et al.*, 2011; Poulsen, 1996).

The progress of plant regeneration in cauliflower is challenging. Pareek and Chandra (1978) reported somatic embryogenesis but direct shoot regeneration failure in cauliflower.  A study by Smith and Bhalla (1998) compared shoot regeneration potential from seedling explants of Australian cauliflower using root explants obtained high frequencies of regeneration in variety. Arctic Bhalla and de Weerd (1999) reported the best results were obtained with curd explants on MS medium with BA and gibberellic acid.

Qin *et al*. (2006) reported the optimal media for cauliflower shoot differentiation and rooting were modified MS medium supplemented with NAA at 0.5 mg/L, TDZ at 0.25 mg/L, BA at 3.0 mg/L, $AgNO_3$ at 2.0 mg/L and MS supplemented with IBA at 0.4 mg/L, respectively. Another study by Pavlovj  *et al*. (2010) tested ability of cauliflower to regenerate shoots *in vitro*. Cotyledon, hypocotyl and root explants of seven-day-old seedlings were incubated on Murashige and Skoog's (MS) medium supplemented with 1.0 mg /L BA or KIN in combination with 0, 0.1, and 0.2 mg/L IBA.

Callus and shoots induction is a crucial step to apply genetic engineering in plant improvement programs. Previous studies have shown that several key factors affecting transformation are highly dependent on genotype. Among these key factors are genetic susceptibility to *Agrobacterium* and *in vitro* tissue culture

response regarded as important ones (Sparrow *et al.*, 2004). Therefore, it is necessary to investigate the shoot regeneration ability in *B. oleracea var. botrytis* may be a promising material for further transformation and crop improvement.

# Chapter Two: Objectives

The overall objective of this study is to outline the essential procedure needed in developing local "Baladi' cauliflower in Palestine towards early flowering.

1- To evaluate the genetic variation (at the DNA level) between the local "Baladi' and hybrid cauliflower regarding flowering gene *BoFLC.HP*.

2- To establish and optimize an efficient and stable protocol for high frequency *in vitro* direct shoot regeneration in local "Baladi" cauliflower from different explant origins.

# Chapter Three: Materials and Methods

The experimental work was conducted in the Plant Tissue Culture and Molecular Laboratory at the Palestine-Korea Biotechnology Center, Palestine Polytechnic University، Hebron- Palestine.

## Plant Material

The Baladi variety was grown from seeds that were obtained from a mature plant grown in Hebron. Plantlets were used for both tissue culture experiments and DNA isolation and molecular study. Leaf material for the molecular study were obtained from the two varieties White Corona and Coroner.

## 3.1 Genetic material isolation and purification

### 3.1.1 Oligonucleotide Primers

The primer set used to amplify *the BoFLC.HP* gene was taken from the study of Abuyusuf et al. (2019) table (3.1).

**Table 3.1:** The primer pair sequence used in detecting the mutations in the *BoFLC.HP* gene**.**

| **Gene name** | **Primer's direction** | **Primer code** | **Sequences (5'→3')** | **Product size (bp)** |
|---|---|---|---|---|
| *BoFLC.HP* | Forward | F7: | GGAAAGCAACATGGTGATGA | 438 |
| | Reverse | R7: | CATGGTGTGAACCAGAGTCC | |

### 3.1.2 DNA extraction

To isolate DNA from the cauliflower, leaves were cut and grounded with sterile sea sand and mixed with 500.0 µl of lysis buffer in mortar and pestle. According to EZ-DNA kit (Cat# 20-600-50, Biological Industries), 600.0 µl of the mixture was transferred to a marked sterile microfuge tube. Secondly, 7.0 µl of RNase–A was

added to the mixture and then the mixture was incubated for 30 min at 56°C in a heating block. To separate cell debris from the aqueous phase, the tube was spin at 12000 rpm for 5 min. Then the supernatant was transferred to a clean microfuge tube. Thirdly, 250.0 µl of chloroform: isoamyl alcohol (24:1) was added to the tube and mixed a few times by inverting the microfuge tube. After mixing, the tube was spin at 12000 rpm for 5 min. And the upper aqueous phase was transferred to a clean microfuge tube. Fourthly, 50.0 µl of Ammonium Acetate (7.5M) was added followed by 500.0 µl of ice-cold absolute ethanol to each tube. For DNA precipitation, tubes were spinned at 12000 rpm for 1 min to form a pellet then the supernatant was removed and DNA in the pellet was washed by adding 500.0 µl of ice-cold 70% ethanol. Finally, the DNA was re-suspension by dissolving in 200.0 µl of sterile 1X TE buffer.

### 3.1.3 PCR reaction

The targeted fragment was amplified using the primers of the *BoFLC.HP* gene (Table 3.1) and the reaction mixture components and volumes that are listed in table 3.2. All master mix components, primers, and DNA samples were mixed in PCR reaction tubes.

**Table 3.2**: Components and volumes of master mix for a single PCR reaction.

| **No.** | **Components** | **Volumes 26 µl** |
|---|---|---|
| 1- | SDW | 16.4 µl |
| 2- | 10X Taq reaction buffer ($Mg^{2+}$ free) | 2.5 µl |
| 3- | $MgSO_4$ | 2.5 µl |
| 4- | dNTPs | 0.5 µl |
| 5- | Primer forward (100 pmol/µl in TE buffer) | 1.0 µl |
| 6- | Primer reverse (100 pmol/µl in TE buffer) | 1.0 µl |
| 7- | Taq DNA polymerase | 0.1 µl |
| 8- | Sample DNA | 1.0 µl |

| 9 | DMSO | 1.0 µl |
|---|---|---|
| Total reaction volume | | 26.0 µl |

Thermocycler conditions were set at 96°C (hot start) for 6 minutes to ensure initial DNA denaturation, followed by 35 cycles of 1) denaturation at 95°C for 40 seconds, 2) annealing at 56°C for 40 seconds, and 3) extension at 72°C for 1 minute. After completion of the last cycle, a final extension was included at 72°C for 10 minutes.

### 3.1.4 Gel electrophoresis and documentation

Agarose gel (2.0% w/v) was prepared with an appropriate number of wells using the materials mentioned in the gel reagents section then 3.0 µl of ethidium bromide (EtBr) was added. 7.0 µl of each sample of PCR products mixed with 3.0 µl of loading buffer and then applied to the wells in addition to 3.0 µl of 100 bp. Ladder (GeneDirex®) in boundary wells and electrophoresed by the electrophoresis system at 120V for 50-90 minutes as required to complete separation of ladder bands. Fragments were visualized by a UV screen and then moved to a gel documentation system to take a photograph of the DNA bands.

### 3.1.5 DNA purification and sequencing

Targeted DNA bands of both PCR amplifications were cut out of the gel and purified according to the gel purification kit (NucleooSpin® Gel and PCR clean-up kit) to get the amplified targeted DNA purified and get rid of any other unwanted components.
Purified DNA was sequenced by Big Dye® Xterminator™ purification kit using both forward and reverse primers of the *BoFLC.HP* gene. Then the samples were analyzed in DNA sequencher.

## 3.2 *In vitro* culture of cauliflower

### 3.2.1 Seed surface sterilization:

Seeds were surface-sterilized in 1% (v/v) sodium hypochlorite (Chlorox®) by vigorous shaking for 15 min in the laminar air-flow cabinet. Then, seeds were washed twice with sterile deionized water (SDW) for 1 min. Finally, seeds were dipped in 70%

ethanol solution for 30 seconds then washed twice with SDW for 1 min each. After the seeds were sterile, they were cultured on a germination medium.

### 3.2.2 Media Preparation and Sterilization

According to the purpose of each experiment, MS medium (Murashige and Skoog,1962) was used as a basal medium for *in vitro* culture. The medium was prepared by dissolving 4.4 g/L together with sucrose 30.0 g/L in deionized water. Suitable plant growth regulators (PGRs) were added according to each experiment's purpose. After the mixture was completed to its final volume, the pH of the medium was adjusted to 5.8 with 1.0 M KOH or 1.0 M HCl. Finally, the media was solidified with agar at 7.0 g/L. The media was sterilized by autoclaving at 121°C and 15 Psi for 20 min and then transferred to the media storage bottles and kept until use. For the seed germination step, a half-strength hormone/PGRs free MS medium was used.

### 3.2.3 Growth conditions

All cultures were incubated in the growth room at 23±1°C in a 16:8 light: dark photoperiod under cool-white fluorescent illumination of 40-45 $\mu mol.m^{-2}.sec^{-1}$ photosynthetic photon flux density (PPFD).

## 3.3. *In vitro* shoot regeneration

To evaluate the effect of PGRs on shoot regeneration, full MS media was used with different types of PGRs: the first media contained 0.5 mg/L BA with different concentrations of IBA (0.5, 0.25, 0.1 mg/L), the second media contained TDZ alone in different concentrations (0.5, 1.5, 2.5 mg/L) or TDZ at 1.0 mg/L with NAA at 0.5 mg/L. Finally, the media contained (0.5, 1.0 mg/L) KIN alone or with different concentrations of IBA (0.5, 0.25, 0.1 mg/L) was used.

## 3.4 Experimental design and statistical analysis.

All experiments were arranged in CRD (Completely Randomized Design). A significant difference between means was tested by the analysis of variance (ANOVA). Means were separated by Fisher LDS at $p \leq 0.05$.

# Chapter four: Results

## 4.1. Molecular analysis of the flowering gene in *B. oleracea var. botrytis*.

### 4.1.1 PCR amplification of the *BoFLC.HP* gene.

PCR reaction was amplified of *BoFLC.HP* gene in Baladi and hybrid cauliflowers and it shows two different sizes of bands in one sample. The first band for late flowering with expected size was between 480-500 bp and the second band for early flowering with expected size was between 420-450 bp. Figure 4.1 shows all bands in the *BoFLC.HP* gene in Baladi and hybrid cauliflower.

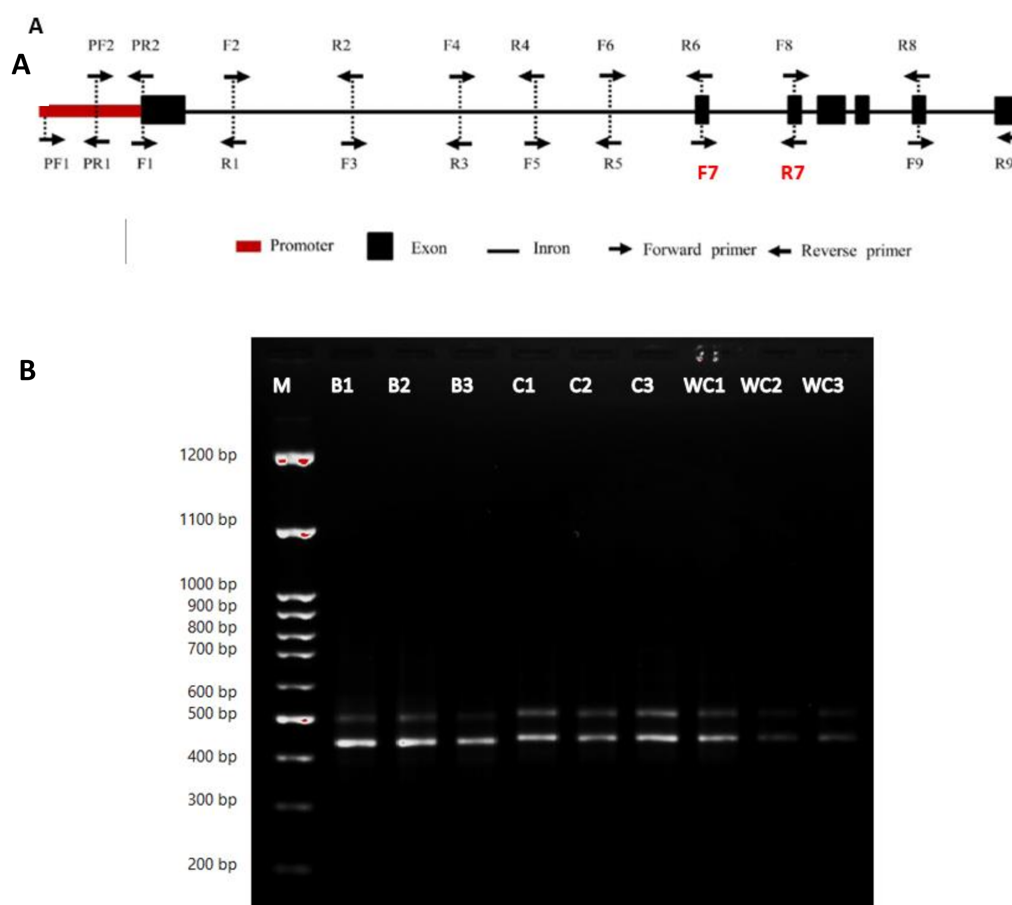



**Figure 4.1** Gel image of the *BoFLC.HP* gene in Baladi and hybrid cauliflowers. **(A)** a Schematic representation of the positions of primers used to detect polymorphism covering the promoter to stop codon region. Red box, promoter; black box, exon; black line, intron; forward arrow, forward primer; reverse arrow, reverse primer. ***(B)*** *BoFLC.HP* gene was amplified by PCR and loaded on 2.0% agarose gel, stained with EtBr and visualized using a gel documentation system. Line M contains 100 bp DNA

ladder (Promega/G5711), Line (**B**) Baladi (**C**) Coroner (**W**) White Corona cauliflower and line.

### 4.1.2. Genetic variation between Baladi and hybrid varieties in *BoFLC.HP* gene.

Two variations at the genetic level were revealed. First an indel mutation between the two hybrids and the Baladi at position (212-219). Second, a point mutation was observed also in position (91).

The band size (420 - 450 bp) from each line for three samples was purified and sequenced. A full sequence of bands was obtained by sequencing. The total length of the band was blasted using the NCBI Blastn nucleotides website . Baladi cauliflower, one of 32 blast results was related to the *BoFLC1* gene in *Brassica oleracea var. botrytis* from a sequence for the first band, which was used as a reference sequence with 96.9% identities, the score was: 433 with no gaps and 7e-117 E value. For hybrid cauliflower, one of the 49 blast results was related to the *BoFLC1* gene in *Brassica oleracea var. botrytis* from a sequence for the first band, which was used as a reference sequence with 94.79% Identities, the score was: 438 with no gaps and 2e-118 E value. Figure 4.2 shows blast results from Baladi and hybrid cauliflower sequence.

select all 32 sequences selected GenBank Graphics Distance tree of results New MSA Viewer

| A Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|
| Brassica oleracea var. alboglabra flowering time protein (FLC1) gene, FLC1-b allele, partial cds | Brassica olerace... | 662 | 662 | 98% | 0.0 | 96.96% | 700 | EF158120.1 |
| Brassica oleracea var. alboglabra clone BAC JBo032J18 flowering time protein (FLC1) gene, FLC1-b allele, p... | Brassica olerace... | 638 | 638 | 97% | 1e-178 | 96.40% | 707 | EF158118.1 |
| Brassica oleracea HDEM genome, scaffold: C9 | Brassica oleracea | 634 | 1964 | 98% | 2e-177 | 95.53% | 63239600 | LR031875.1 |
| Brassica oleracea var. italica flowering time protein (FLC1) gene, FLC1-f allele, partial cds | Brassica olerace... | 494 | 494 | 75% | 3e-135 | 96.09% | 521 | EF158121.1 |
| Brassica oleracea flowering time protein (FLC1) gene, FLC1-f allele, partial cds | Brassica oleracea | 475 | 475 | 72% | 1e-129 | 95.96% | 508 | EF158122.1 |
| Brassica napus genome assembly, chromosome: C09 | Brassica napus | 470 | 2015 | 98% | 5e-128 | 98.15% | 66465249 | HG994373.1 |
| Brassica oleracea flowering time protein (FLC1) gene, FLC1-a allele, partial cds | Brassica oleracea | 464 | 464 | 67% | 2e-126 | 97.78% | 502 | EF158127.1 |
| Brassica napus cultivar Tapidor flowering locus C (FLC.C9-b) gene, partial cds | Brassica napus | 453 | 663 | 98% | 5e-123 | 97.04% | 3811 | JQ255383.1 |
| Brassica napus cultivar Tapidor flowering locus C (FLC.C9-a) gene, partial cds | Brassica napus | 453 | 663 | 98% | 5e-123 | 97.04% | 6378 | JQ255382.1 |
| Brassica oleracea var. alboglabra flowering time protein (FLC1) gene, FLC1-a allele, partial cds | Brassica olerace... | 453 | 663 | 96% | 5e-123 | 97.04% | 742 | EF158124.1 |
| Brassica oleracea var. alboglabra clone BAC JBo032J18 flowering time protein (FLC1) gene, FLC1-a allele, p... | Brassica olerace... | 453 | 663 | 96% | 5e-123 | 97.04% | 744 | EF158117.1 |
| Brassica oleracea var. alboglabra flc1 gene for flowering protein, exons 1-7 | Brassica olerace... | 449 | 659 | 98% | 7e-122 | 96.68% | 6426 | AM231517.1 |
| Brassica oleracea FLC1 (FLC1) gene, exons 2 through 7 and partial cds | Brassica oleracea | 448 | 657 | 98% | 2e-121 | 96.67% | 1395 | AY115674.1 |
| Brassica oleracea var. italica flowering time protein (FLC1) gene, FLC1-d allele, partial cds | Brassica olerace... | 436 | 436 | 67% | 5e-118 | 95.93% | 506 | EF158125.1 |
| Brassica oleracea var. botrytis flowering time protein (FLC1) gene, FLC1-a allele, partial cds | Brassica olerace... | 433 | 433 | 64% | 7e-117 | 96.90% | 474 | EF158123.1 |
| Brassica oleracea var. italica flowering time protein (FLC1) gene, FLC1-e allele, partial cds | Brassica olerace... | 409 | 409 | 67% | 1e-109 | 94.40% | 501 | EF158126.1 |
| Brassica oleracea var. italica partial flc1 gene for flowering protein exons 2-7 | Brassica olerace... | 409 | 619 | 98% | 1e-109 | 94.40% | 1666 | AM231521.1 |
| Brassica napus cultivar Westar disrupted FLC.A10 gene, partial sequence | Brassica napus | 340 | 510 | 98% | 4e-89 | 90.37% | 13900 | MN105089.1 |
| Brassica rapa clone 22_FLC1 MADS-box transcription factor FLC1 gene, complete cds | Brassica rapa | 340 | 504 | 98% | 4e-89 | 90.37% | 4314 | MH880314.1 |
| Brassica juncea clone 19_FLC1 MADS-box transcription factor FLC1 gene, complete cds | Brassica juncea | 340 | 511 | 98% | 4e-89 | 90.37% | 4931 | MH880313.1 |
| Brassica napus genome assembly, chromosome: A10 | Brassica napus | 340 | 510 | 98% | 4e-89 | 90.37% | 20778245 | HG994364.1 |

select all *49 sequences selected* GenBank Graphics Distance tree of results New MSA Viewer

**B**

| Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|
| Brassica oleracea HDEM genome, scaffold: C9 | Brassica oleracea | 769 | 2142 | 99% | 0.0 | 100.00% | 63239600 | LR031875.1 |
| Brassica oleracea var. alboglabra flowering time protein (FLC1) gene, FLC1-b allele, partial cds | Brassica olerac... | 675 | 675 | 99% | 0.0 | 96.15% | 700 | EF158120.1 |
| Brassica oleracea var. alboglabra clone BAC JBo032J18 flowering time protein (FLC1) gene, FLC1-b all... | Brassica olerac... | 652 | 652 | 99% | 0.0 | 95.22% | 707 | EF158118.1 |
| Brassica oleracea var. italica flowering time protein (FLC1) gene, FLC1-f allele, partial cds | Brassica olerac... | 604 | 604 | 77% | 1e-168 | 100.00% | 521 | EF158121.1 |
| Brassica oleracea flowering time protein (FLC1) gene, FLC1-f allele, partial cds | Brassica oleracea | 586 | 586 | 75% | 5e-163 | 100.00% | 508 | EF158122.1 |
| Brassica napus genome assembly, chromosome: C09 | Brassica napus | 481 | 2082 | 99% | 3e-131 | 96.31% | 66465249 | HG994373.1 |
| Brassica oleracea flowering time protein (FLC1) gene, FLC1-a allele, partial cds | Brassica oleracea | 475 | 475 | 70% | 1e-129 | 95.97% | 502 | EF158127.1 |
| Brassica napus cultivar Tapidor flowering locus C (FLC.C9-b) gene, partial cds | Brassica napus | 464 | 687 | 99% | 3e-126 | 95.30% | 3811 | JQ255383.1 |
| Brassica napus cultivar Tapidor flowering locus C (FLC.C9-a) gene, partial cds | Brassica napus | 459 | 681 | 99% | 1e-124 | 94.97% | 6378 | JQ255382.1 |
| Brassica oleracea var. alboglabra flowering time protein (FLC1) gene, FLC1-a allele, partial cds | Brassica olerac... | 459 | 679 | 98% | 1e-124 | 94.97% | 742 | EF158124.1 |
| Brassica oleracea var. alboglabra clone BAC JBo032J18 flowering time protein (FLC1) gene, FLC1-a all... | Brassica olerac... | 459 | 681 | 99% | 1e-124 | 94.97% | 744 | EF158117.1 |
| Brassica oleracea var. alboglabra flc1 gene for flowering protein, exons 1-7 | Brassica olerac... | 455 | 678 | 99% | 2e-123 | 94.65% | 6426 | AM231517.1 |
| Brassica oleracea FLC1 (FLC1) gene, exons 2 through 7 and partial cds | Brassica oleracea | 453 | 676 | 99% | 5e-123 | 94.63% | 1395 | AY115674.1 |
| Brassica oleracea var. italica flowering time protein (FLC1) gene, FLC1-d allele, partial cds | Brassica olerac... | 448 | 448 | 70% | 3e-121 | 94.30% | 506 | EF158125.1 |
| Brassica oleracea var. botrytis flowering time protein (FLC1) gene, FLC1-a allele, partial cds | Brassica olerac... | 438 | 438 | 68% | 2e-118 | 94.76% | 474 | EF158123.1 |
| Brassica oleracea var. italica flowering time protein (FLC1) gene, FLC1-e allele, partial cds | Brassica olerac... | 403 | 403 | 70% | 6e-108 | 91.95% | 501 | EF158126.1 |
| Brassica oleracea var. italica partial flc1 gene for flowering protein exons 2-7 | Brassica olerac... | 403 | 626 | 99% | 6e-108 | 91.95% | 1666 | AM231521.1 |
| Brassica napus cultivar Westar disrupted FLC.A10 gene, partial sequence | Brassica napus | 340 | 522 | 99% | 4e-89 | 88.59% | 13900 | MN105089.1 |
| Brassica rapa clone 22_FLC1 MADS-box transcription factor FLC1 gene, complete cds | Brassica rapa | 340 | 517 | 99% | 4e-89 | 88.59% | 4314 | MH880314.1 |
| Brassica juncea clone 19_FLC1 MADS-box transcription factor FLC1 gene, complete cds | Brassica juncea | 340 | 524 | 99% | 4e-89 | 88.59% | 4931 | MH880313.1 |
| Brassica napus genome assembly, chromosome: A10 | Brassica napus | 340 | 522 | 99% | 4e-89 | 88.59% | 20778245 | HG994364.1 |
| Brassica napus cultivar Tapidor MITE Tourist-like, complete sequence; and FLC (FLC.A10) gene, compl... | Brassica napus | 340 | 522 | 99% | 4e-89 | 88.59% | 6705 | JX901142.1 |

**Figure 4.2**: BLAST result for the *BoFLC.HP* gene from first band sequence. BLAST done using the NCBI Blastn nucleotides website (http://www.ncbi.nlm.nih.gov/). *botrytis.* **(A)** Baladi cauliflower band sequence with 96.9% identities with the *BoFLC1* gene in *brassica*. **(B)** hybrid cauliflower band sequence with 94.79% identities with the *BoFLC1* gene in *brassica oleracea var. botrytis*.

### 4.1.3. Sequences Alignment for the *BoFLC.HP* gene between Baladi and two type of hybrid.

Sequence alignment for *BoFLC.HP* gene between hybrid and Baladi cauliflower bands with reference sequence from the blast (EF158123.1) by using MAGA-X program. Sequence analysis was done by using GeneStudio software. The first identical mutation was an Indel between the two hybrids and Baladi at position (212-219) and other point mutations were observed between reference sequence (EF158123.1) with Baladi and hybrid at position 231 and 252. Figure 4.3 shows sequence alignment for the *BoFLC.HP* gene between Baladi and hybrids type with the reference sequence. Secondly, The Point mutation between the sequence from hybrid and Baladi cauliflower from the late flowering band and this mutation substitution Cytosine (C) to Thymine (T) at position (91). Figure 4.4 shows the mutation of the *BoFLC.HP* gene between Baladi and hybrids cauliflower.

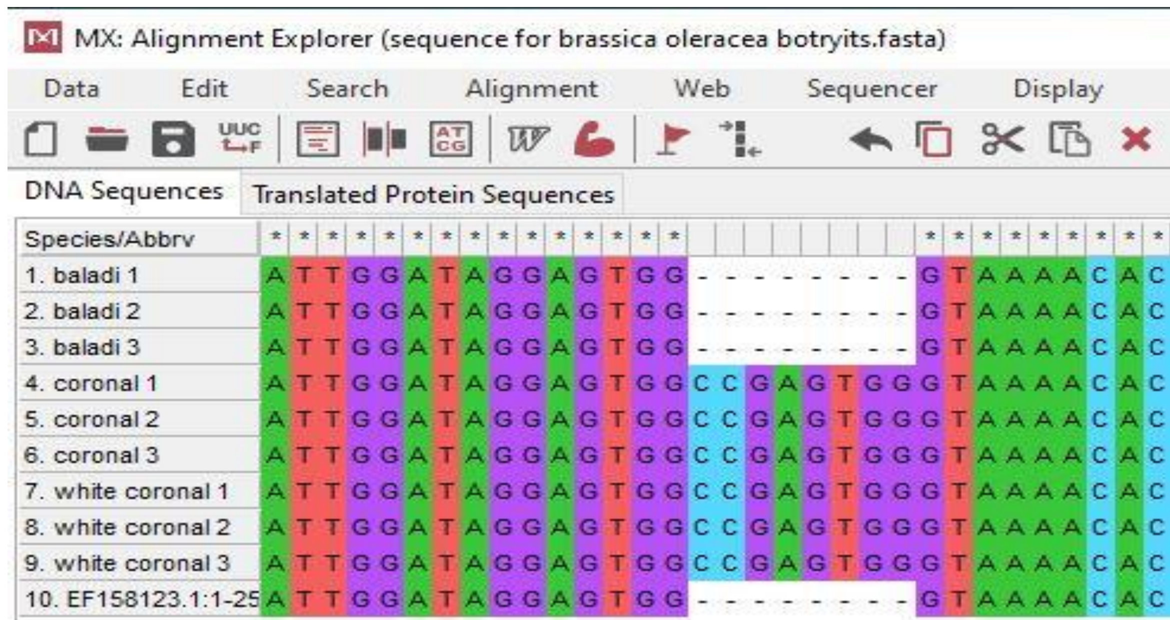
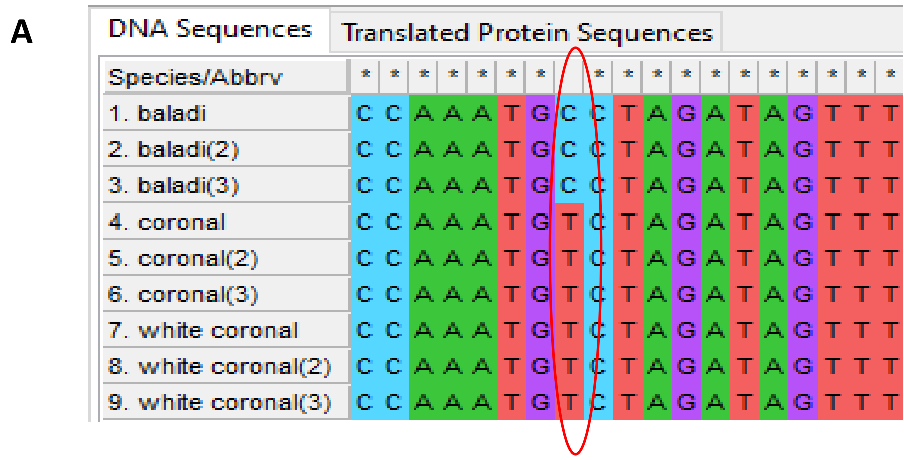



**Figure 4.3** Sequence Alignment of the *BoFLC.HP* gene between Baladi and hybrids cauliflowers and the reference sequence. DNA Sequence Alignment for hybrid and Baladi cauliflower for first band using the MAGA-X program. Indel mutation between Baladi, hybrid and reference sequence in 8 bp and different point mutation between reference sequence with Baladi and hybrids cauliflower.

**A**



**B**

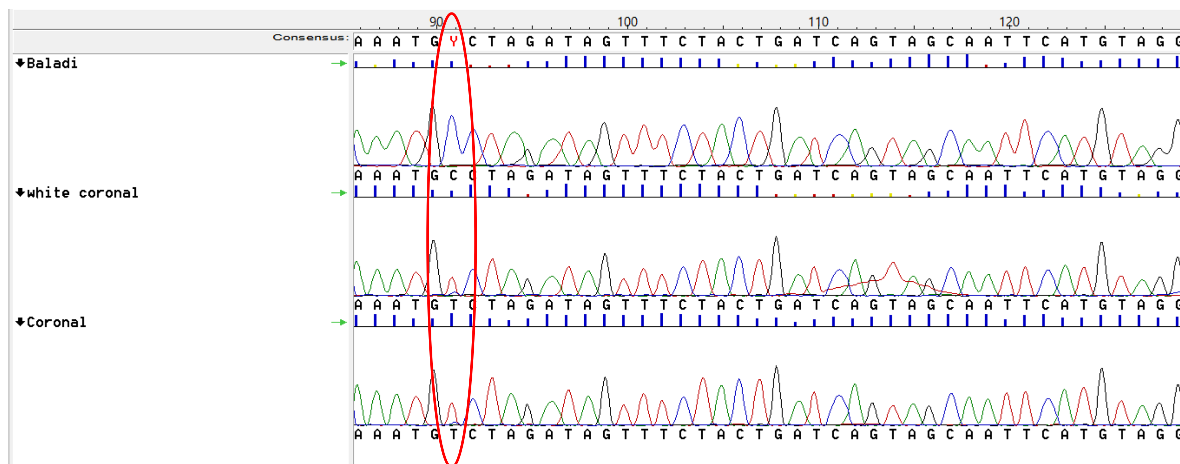



**Figure 4.4**: Point Mutation in the *BoFLC.HP* gene between Baladi and hybrids cauliflowers. **(A)** DNA Sequence alignment using MAGA-X program. **(B)** Sequence analysis for hybrid and Baladi cauliflower for the late flowering band using Gene Studio program. Point mutation between Baladi and hybrid for (C) substitution (T).

## 4.2. *In vitro* shoot regeneration of *B. oleracea var. botrytis*.

Full seed germination (100%) was obtained in all experiments. Different cytokinins and auxins were combined to examine their effect on *in vitro* shoot regeneration in the different explants from cauliflower.

### 4.2.1. The effect of BA and IBA on *in vitro shoot* regeneration.

The combination of BA and different concentrations of IBA resulted in different responses of shoot regeneration from hypocotyl and cotyledon explants. The results for the percentage of shoot regeneration, average number of shoot regeneration and rooting were recorded after four weeks of culture and presented in table 4.1.

**Table 4.1**: The effect of BA and IBA on shoot regeneration after four weeks of culture.

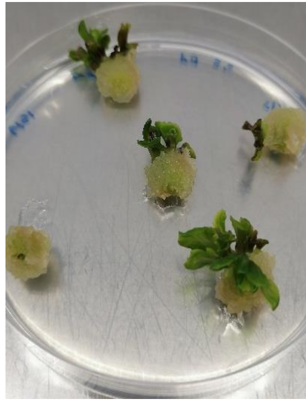
| | Shoot regeneration (%) | | Average No. of regenerated shoots | | Rooting (%) | |
|---|---|---|---|---|---|---|
| **MS media contains** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** |
| 0.5 mg/L BA + 0.5 mg/L IBA | 80% | 0% | 3.4 **(A)** | 0.0 | 0% | 0% |
| 0.5 mg/L BA+ 0.25 mg/L IBA | 40% | 0% | 0.5 **(B)** | 0.0 | 0% | 0% |
| 0.5 mg/L BA + 0.1 mg/L IBA | 20% | 0% | 0.2 **(B)** | 0.0 | 0% | 0% |

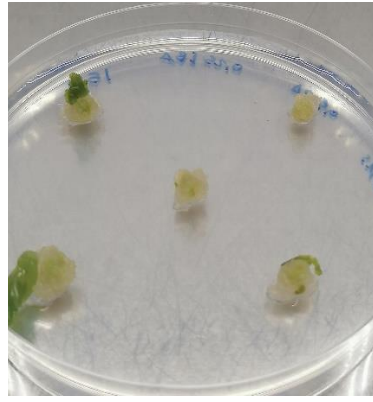
Means were separated according to Fisher LSD test at $p \leq 0.05$. Similar letters in the same column indicate no significant difference.

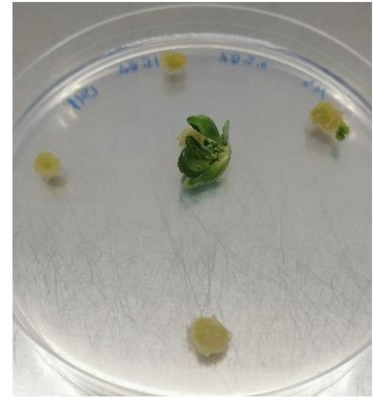
The results revealed differences in the number of shoot regeneration from callus between the three media types. No rooting was observed from hypocotyl or cotyledon explants. The highest percentage of shoot regeneration from callus was observed in hypocotyl explants on full-strength MS media contained 0.5 mg/L BA and 0.5 mg/L IBA within 80% followed by full-strength MS media contained 0.5 mg/L BA and 0.25 mg/L IBA then full-strength MS media contained 0.5 mg/L BA and 0.1 mg/L IBA (40%, 20%) respectively. The highest mean for the number of shoot regeneration was observed in full-strength MS medium with 0.5 mg/L BA and 0.5 mg/L IBA with a mean shoot regeneration of 3.4, followed by media contained 0.5 mg/L BA and 0.25 mg/L IBA with a mean shoot regeneration of 0.5. The lowest mean of shoot regeneration was observed in media containing 0.5 mg/L BA and 0.1 mg/L IBA with mean shoot regeneration of 0.2. For cotyledon explants, no shoot regeneration was observed in any media and only callus formation was observed. Figure 4.5 shows the results effect of BA and IBA on *in vitro* shoot regeneration.

**A** **B** **C**

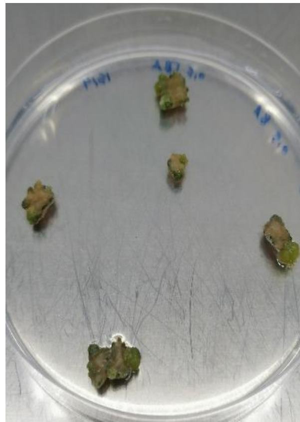
**Hypocotyl**

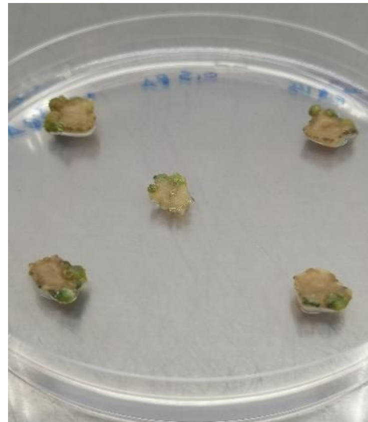






**Cotyledon**





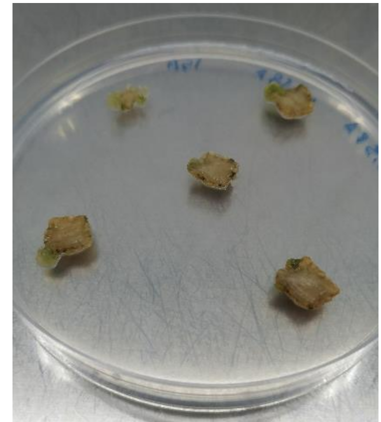


**Figure 4.5**: *In vitro* shoot regeneration from hypocotyl and cotyledon explants in full -strength MS media with BA and IBA. (**A)** Media Contained 0.5 mg/L BA and 0.5 mg/L IBA for, **(B)** 0.5 mg/L BA and 0.25 mg/L IBA and **(C)** 0.5 mg/L BA and 0.1 mg/L IBA. No *in vitro* adventitious roots were grown in any type of the media.

### 4.2.2 The effect of kinetin and IBA on *in vitro* shoot regeneration.

The different concentrations of IBA and 1.0 mg/L KIN resulted in different shoot regeneration results from hypocotyl and cotyledon explants. The results for the percentage of shoot regeneration, average number of shoots and rooting were recorded after four weeks of culturing and presented in table 4.2.

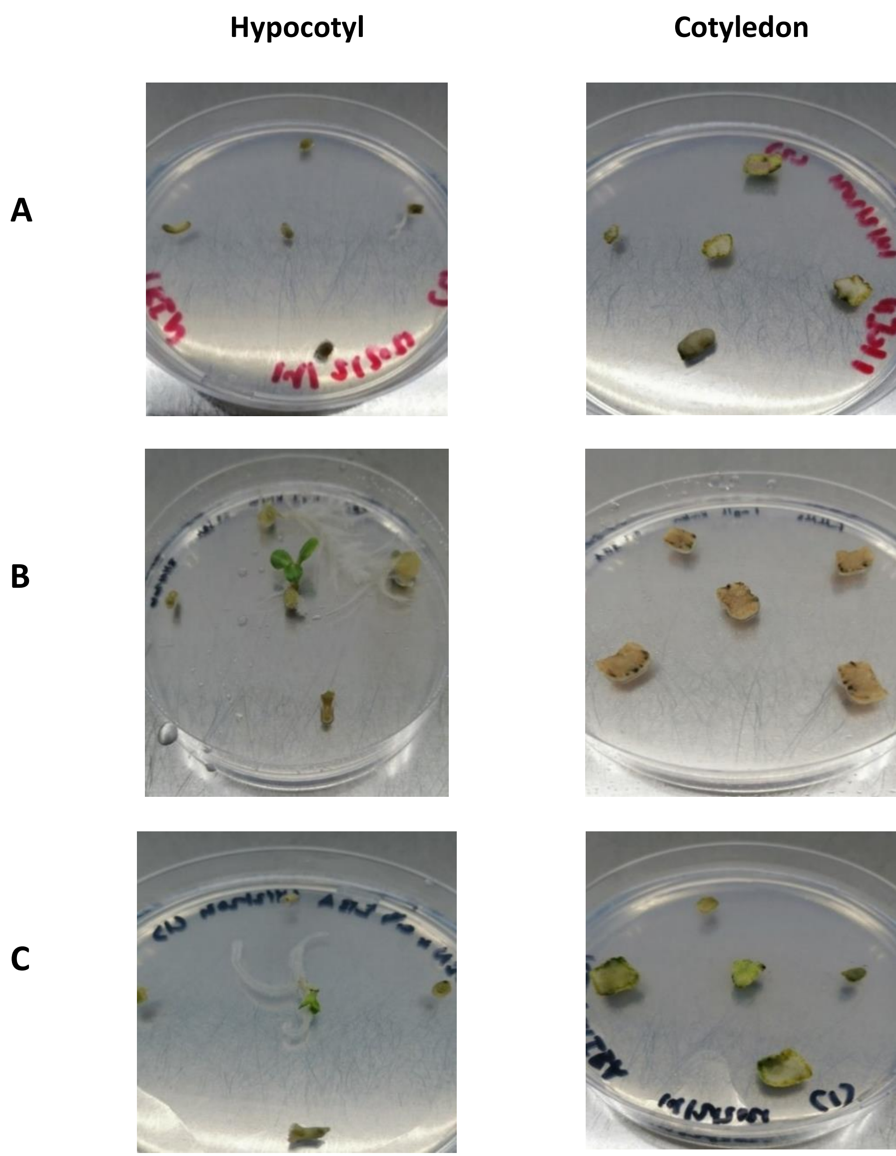
**Table 4.2**: The effect of 1.0 mg/L KIN and different concentrations of IBA on shoot regeneration after four weeks of culture.

| | **Shoot regeneration (%)** | | **Average No. of regenerated shoots** | | **Rooting (%)** | |
|---|---|---|---|---|---|---|
| **MS media contains** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** |
| 1.0 mg/L KIN | 0% | 0% | 0.0 **(D)** | 0.0 | 20% | 6% |
| 1.0 mg/L KIN+0.2 mg/L IBA | 28% | 0% | 0.76 **(A)** | 0.0 | 44% | 0% |
| 1.0 mg/L KIN + 0.1 mg/L IBA | 20% | 0% | 0.2 **(C)** | 0.0 | 33.3% | 0% |
| 1.0 mg/L KIN + 1.0 mg/L IBA | 20% | 0% | 0.4 **(B)** | 0.0 | 60% | 30% |

Means were separated according to Fisher LSD test at $p \leq 0.05$. Similar letters in the same column indicate no significant difference.

The results showed differences in the number of shoot regeneration from callus between the four media types. Rooting was observed from hypocotyl and cotyledon explants. The highest percentage of shoot was observed from hypocotyl explants in full-strength MS media contained 1.0 mg/L KIN and 0.2 mg/L IBA its values 28% followed by MS media contained 1.0 mg/L KIN and 0.1 mg/L IBA followed by MS media contained 1.0 mg/L KIN and 1.0 mg/L IBA then MS media contained 1.0 mg/L KIN alone (20%, 20%, 0%) respectively. The highest mean number of shoots was observed in full-strength MS medium with 1.0 mg/L KIN and 0.2 mg/L IBA with a mean shoot regeneration of 0.76 followed by media contained 1.0 mg/L KIN and 1.0

mg/L IBA with a mean shoot regeneration of 0.4 then media contained 1.0 mg/L KIN and 0.1 mg/L IBA with a mean shoot regeneration of 0.2. The lowest mean shoot regeneration was observed in media contained 1.0 mg/L KIN within 0.0. For the cotyledon explants, no shoots regeneration were observed in any media and only callus formation was observed. Figure 4.6 shows the effect of 1.0 mg/L KIN and different concentrations of IBA on *in vitro* shoots regeneration from hypocotyl and cotyledon explants.



**D**

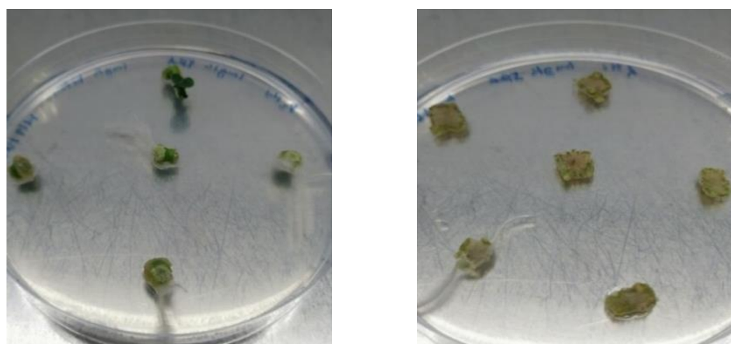


**Figure 4.6**: *In vitro* shoot regeneration from hypocotyl and cotyledon explants in full-strength MS media with 1.0 mg/L KIN and different concentrations of IBA. (**A)** Media Contained 1.0 mg/L KIN (**B**) 1.0 mg/L KIN and 0.2 mg/L IBA (**C)** 1.0 mg/L KIN and 0.1 mg/L IBA and **(D)** 1.0 mg/L KIN and 1.0 mg/L IBA.

The different concentrations of IBA and 0.5 mg/L KIN resulted in different shoot regeneration results from hypocotyl and cotyledon explants. The results for the percentage of shoot regeneration, average number of shoot regeneration and rooting were recorded after four weeks of culturing and presented in table 4.3.

**Table 4.3**: The effect of 0.5 mg/L KIN and different concentrations of IBA on shoot regeneration after four weeks of culture.

| | **Shoot regeneration (%)** | | **Average No. of regenerated shoots** | | **Rooting** | |
|---|---|---|---|---|---|---|
| **MS media contains** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** |
| 0.5 mg/L KIN | 26% | 0% | 0.3 **(B)** | 0.0 | 46% | 0% |
| 0.5 mg/L KIN + 0.2 mg/L IBA | 12% | 0% | 0.2 **(C)** | 0.0 | 44% | 0% |
| 0.5 mg/L KIN + 0.1 mg/L IBA | 33.3% | 0% | 0.5 **(A)** | 0.0 | 77.7% | 0% |
| 0.5 mg/L KIN + 1.0 mg/L IBA | 0% | 0% | 0.0 **(D)** | 0.0 | 60% | 53.3% |

Means were separated according to Fisher LSD test at $p \leq 0.05$. Similar letters in the same column indicate no significant difference.

The results showed differences in the number of shoots regeneration from callus between the four media types. Rooting was observed from hypocotyl and same

cotyledon explants. The highest percentage of shoot regeneration was observed in hypocotyl explants in full-strength MS contained 0.5 mg/L KIN and 0.1 mg/L IBA its values 23.3% followed by 0.5 mg/L KIN followed by 0.5mg/L KIN and 0.5 mg/L IBA then 0.5 mg/L KIN and 1.0 mg/L IBA (26%, 12%, 0%) respectively. The highest mean number of shoot regeneration was observed in full-strength MS medium contained 0.5 mg/L KIN and 0.1 mg/L IBA with a mean shoot regeneration of 0.5 followed by media contained 0.5 mg/L KIN with a mean shoot regeneration of 0.3 then media contained 0.5 mg/L KIN and 0.2 mg/L IBA with a mean shoot regeneration of 0.2. The lowest mean shoot regeneration was observed in media contained 0.5 mg/L KIN and 1.0 mg/L IBA with a mean shoot regeneration of 0.0. For rooting the highest rooting was observed in media contained 0.5 mg/L KIN and 0.1 mg/L IBA from hypocotyl explants within 77.7%. For the cotyledon explants, no shoot regeneration was observed in any media and only callus formation was observed. Figure 4.4 shows the results effect 0.5 mg/L KIN and different concentrations of IBA on *in vitro* shoot regeneration from hypocotyl explants.

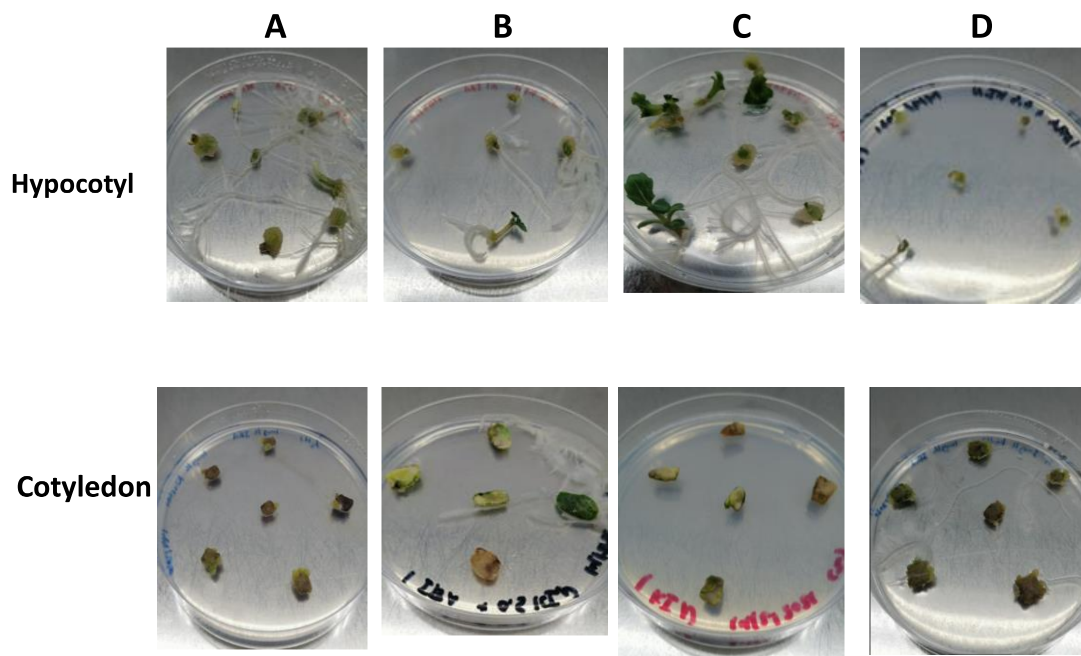



**Figure 4.7**: The effect of 0.5 mg/L KIN and different concentrations of IBA on *in vitro* shoot regeneration from hypocotyl and cotyledon explants. **(A)** media contained 0.5 mg/L KIN alone, **(B)** 0.5 mg/L KIN and 0.2 mg/L IBA, **(C)** 0.5 mg/L KIN and 0.1 mg/L IBA and **(D)** 0.5 mg/L KIN and 1.0 mg/L IBA.

### 4.2.4 The Effect of BA and NAA on *in vitro* shoot regeneration.

The different concentrations of 2.0 mg/L BA with NAA resulted in different responses of shoot regeneration from hypocotyl, cotyledon and root explants. The results for the percentage of shoot regeneration, average number of shoot regeneration and rooting were recorded after four weeks of culturing and presented in table 4.4.

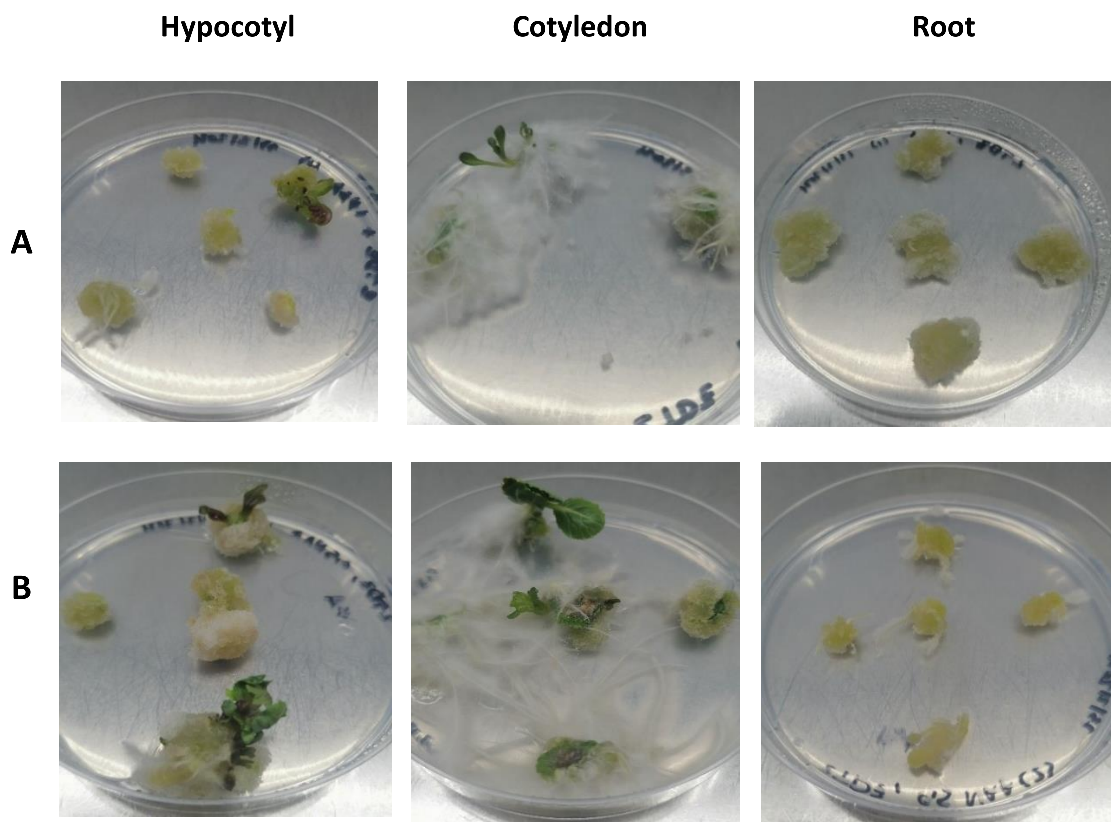
**Table 4.4**: The effect of 2.0 mg/L BA and different concentrations of NAA on shoot regeneration after four weeks of culture.

| | **Shoot regeneration (%)** | | | **Average No. of regenerated shoots** | | | **Rooting %** | | |
|---|---|---|---|---|---|---|---|---|---|
| **MS media contains** | **Hypocotyl** | **Cotyledon** | **Root** | **Hypocotyl** | **Cotyledon** | **Root** | **Hypocotyl** | **Cotyledon** | **Root** |
| 2.0 mg/L BA+1.0 mg/LNAA | 33.3% | 6% | 0% | 0.8 **A** | 0.1 **C** | 0 | 66% | 80% | 0% |
| 2.0 mg/L BA+0.5 mg/LNAA | 13.3% | 20% | 0% | 0.4 **B** | 0.2 **B** | 0 | 40% | 100% | 100% |
| 2.0 mg/L BA+0.1 mg/LNAA | 6% | 26.6% | 0% | 0.06 **C** | 0.33 **A** | 0 | 6% | 66% | 100% |

Means were separated according to Fisher LSD test at $p \leq 0.05$. Similar letters in the same column indicate no significant difference.

The results revealed differences in the number of shoots regeneration between three different m edia types. Rooting was observed from hypocotyl, cotyledon and root explants. The highest percentage of shoot regeneration was observed from hypocotyl explants in full MS contained 2.0 mg/L BA and 1.0 mg/L NAA its values 33.3% followed by 2.0 mg/L BA and 0.5 mg/L NAA then 2.0 mg/L BA and 0.1 mg/L NAA (13%, 6%) respectively. The highest mean number of shoot regeneration for hypocotyl was observed in full-strength MS medium contained 2.0 mg/L BA and 1.0 mg/L NAA with a mean shoot regeneration of 0.8 followed by 2.0 mg/L BA and 0.5 mg/L NAA with a mean shoot regeneration of 0.4 then 2.0 mg/L BA and 0.1 mg/L NAA with a mean shoot regeneration of 0.06. For the cotyledon explants, the highest

percentage of shoot regeneration was observed in full-strength MS contained 2.0 mg/L BA and 0.1 mg/L NAA its values 26.6% followed by media contained 2.0 mg/L BA and 0.5 mg/L NAA then media contained 2.0 mg/L BA and 1.0 mg/L NAA (20%,6%) respectively. The highest mean number of shoots regeneration for cotyledon was observed in full-strength MS medium contained 2.0 mg/L BA and 0.1 mg/L NAA with a mean shoot regeneration of 0.33 followed by media contain 2.0 mg/L BA with 0.5 mg/L NAA with a mean shoot regeneration of 0.2 then media contained 2.0 mg/L BA with 1.0 mg/L NAA with a mean shoot regeneration of 0.1. For the root explants, no shoot regeneration was observed in a media type only the callus and roots formation were observed. Figure 4.8 shows the effect of 2.0 mg/L BA and different concentrations of NAA on *in vitro* shoot regeneration from different explants.



**C**

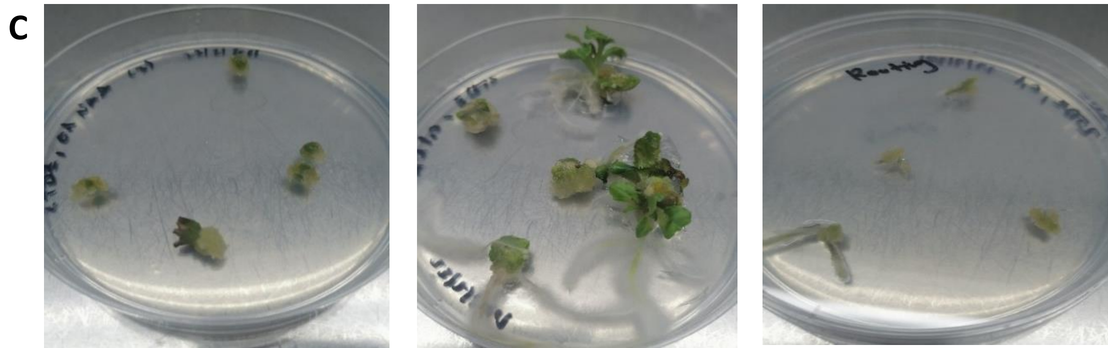


**Figure 4.8**: The effect of 2.0 mg/L BA and different concentrations of NAA on *in vitro* shoot regeneration from different explants. **(A)** Full-strength MS media contained 2.0 mg/L BA and 1.0 mg/L NAA **(B)** 2.0 mg/L BA and 0.5 mg/L NAA and **(C)** 2.0 mg/L BA and 0.1 mg/L NAA for hypocotyl, cotyledon and root explants.

### 4.4.5. Effect of different concentrations of TDZ and NAA on *in vitro* shoot regeneration.

The different concentrations of TDZ with and without NAA resulted in different shoot regeneration results from hypocotyl and cotyledon explants. The results for the percentage of shoot regeneration, average number of shoot regeneration and rooting were recorded after four weeks of culturing and presented in table 4.5.

**Table 4.5**: Effect of TDZ and NAA on shoot regeneration after four weeks of culture.

| | **Shoot regeneration (%)** | | | **Average No. of regenerated shoots** | | | **Rooting (%)** | | |
|---|---|---|---|---|---|---|---|---|---|
| **MS media contains** | **Hypocotyl** | **Cotyledon** | **leaf** | **Hypocotyl** | **Cotyledon** | **Leaf** | **Hypocotyl** | **Cotyledon** | **Leaf** |
| **TDZ + NAA** | | | | | | | | | |
| 1.0 mg/L TDZ with 0.5 mg/L NAA | 16% | 0% | ------ | 0.38 | 0.0 | ----- | 55.5% | 86.6% | ------- |
| **TDZ without NAA** | | | | | | | | | |
| 0.5 mg/L TDZ | 72% | 0% | 0% | 5.5 **A** | 0.0 | 0.0 | 0% | 0% | 0% |
| 1.5 mg/L TDZ | 40 % | 0% | 0% | 2.0 **B** | 0.0 | 0.0 | 0% | 0% | 0% |

| 2.5 mg/L TDZ | 60% | 0% | 0% | 2.9 **AB** | 0.0 | 0.0 | 0% | 0% | 0% |
|---|---|---|---|---|---|---|---|---|---|

Means were separated according to Fisher LSD test at p≤ 0.05. Similar letters in the same column indicate no significant difference.

The results revealed differences in the number of shoots regeneration between the three media types. No rooting was observed on TDZ without NAA hormone from hypocotyl or cotyledon explants only rooting formation was observed on TDZ with NAA. The highest percentage of shoot regeneration from callus was observed from hypocotyl explants on MS contained 0.5 mg/L TDZ within 72% followed by MS contained 2.5 mg/L TDZ followed by MS contained 1.5 mg/L TDZ then MS contained 1.0 mg/L TDZ and 0.5 mg/L NAA (60%,40%, 16%) respectively. The highest mean for number of shoots regeneration from callus was observed in hypocotyl explants in full-strength MS medium contained 0.5 mg/L TDZ with a mean shoot regeneration of 5.5, followed by media contained 2.5 mg/L TDZ with a mean shoot regeneration of 2.9 then media contained 1.5 mg/L TDZ with a mean shoot regeneration of two. The lowest mean shoot regeneration was observed for media contained 1.0 mg/L TDZ with 0.5 NAA with a mean shoot regeneration of 0.38. For the cotyledon explants, no shoot regeneration was observed in any media and only the callus formation was observed. Figure 4.9 shows the results effect of different concentrations of TDZ on *in vitro* shoot regeneration for hypocotyl and cotyledon explants.

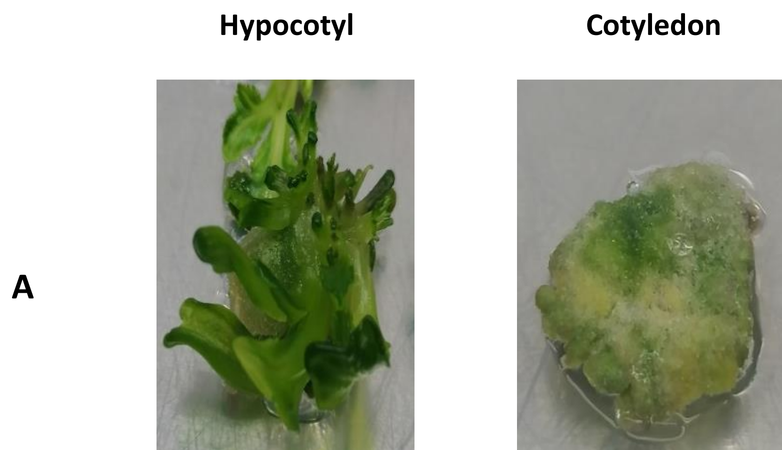


**Figure 4.9**. *In vitro* shoot regeneration from hypocotyl and cotyledon explants in full MS media. **(A)** Media contained 0.5 mg/L TDZ, (**B)** 2.5 mg/L TDZ and (**C**) 1.5 mg/L TDZ. No *in vitro* adventitious roots were grown in any type of the media. **(D)** Media contained 1.0 mg/L TDZ and 0.5 mg/L NAA and rooting was observed in and cotyledon explants.

## 4.2.6. *In vitro* shoot regeneration from callus explants.

The callus explants were grown in full strength MS media contained 2.0 mg/L TDZ and 0.5 mg/L NAA. Callus was taken after the hypocotyl explants gave callus tissue in full-strength MS media contained 0.5 mg/L KIN. No shoot regeneration or rooting

was observed for callus explant after four weeks of culturing and only the callus formation was observed. Figure 4.10 shows *in vitro* shoot regeneration from callus explants.

**Figure 4.10:** *In vitro* shoot regeneration from callus explants.

### 4.2.7. *In vitro* shoot regeneration from leaf explants.

The leaf explant was grown in full strength MS media contained 0.5, 1.5 and 2.5 mg/L TDZ. Leaf was taken after seed germination in half-strength MS media without hormone. No shoot regeneration or rooting was observed for leaf explants after four weeks of culturing and only the callus formation was observed. Figure 4.11 shows *in vitro* shoot regeneration from leaf explants.

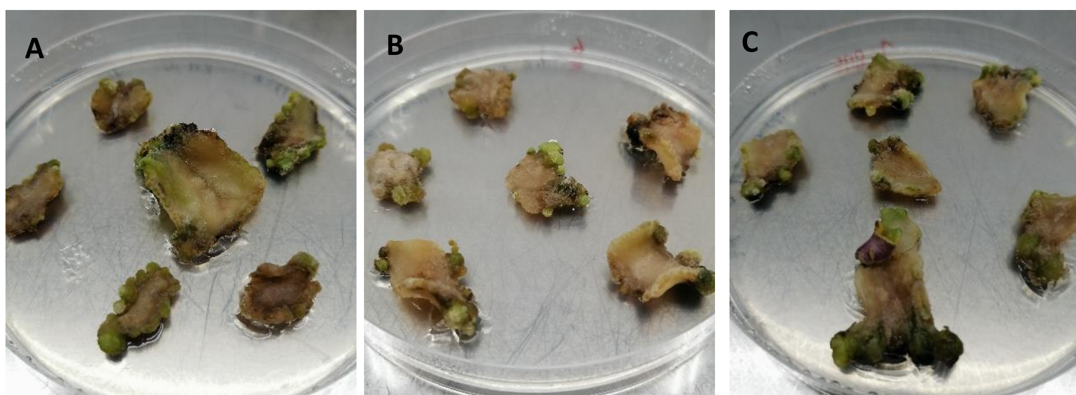


**Figure 4.11:** *In vitro* shoot regeneration from leaf explants. **(A)** Leaf explants from full strength MS media contained 0.5 TDZ, **(B)** 0.5 mg/L TDZ and **(C)** 2.5 mg/L TDZ.

## 4.3. Transfer regenerated plants to soil in the greenhouse (acclimatization)

After plant regeneration from hypocotyl explants in different media, the regenerated plantlets were placed in seed germination media without any hormone/PGRs to form roots then to be transferred to soil. Figure 4.12 shows the cauliflower plant from hypocotyl explants regeneration in tissue culture.

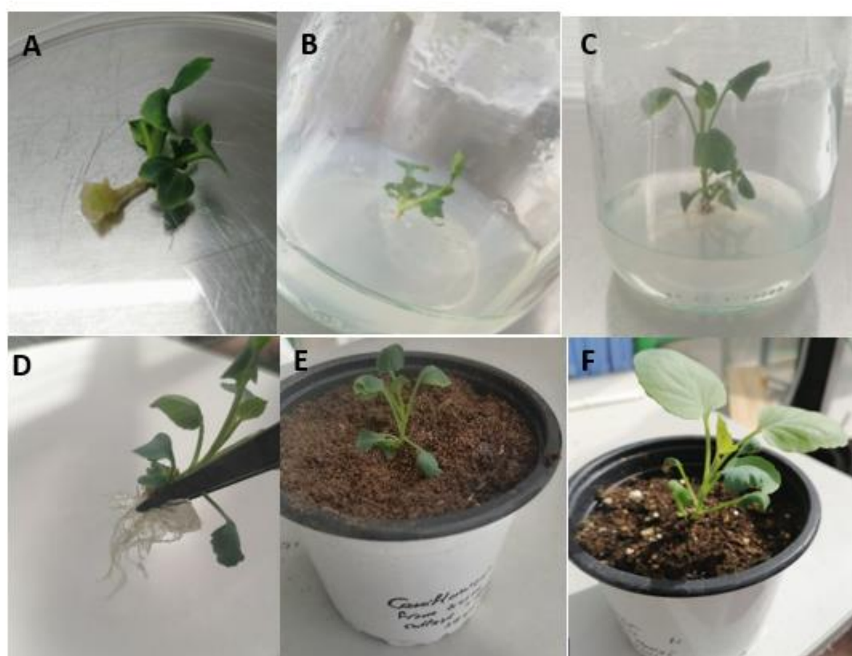
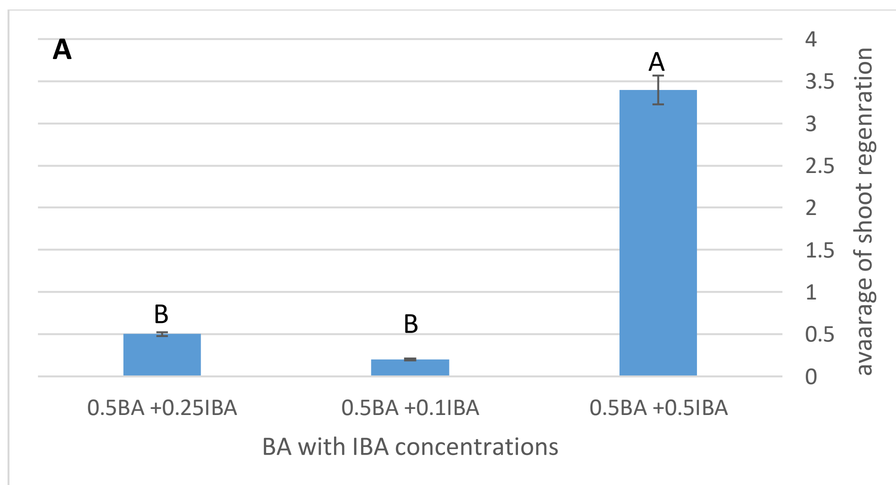


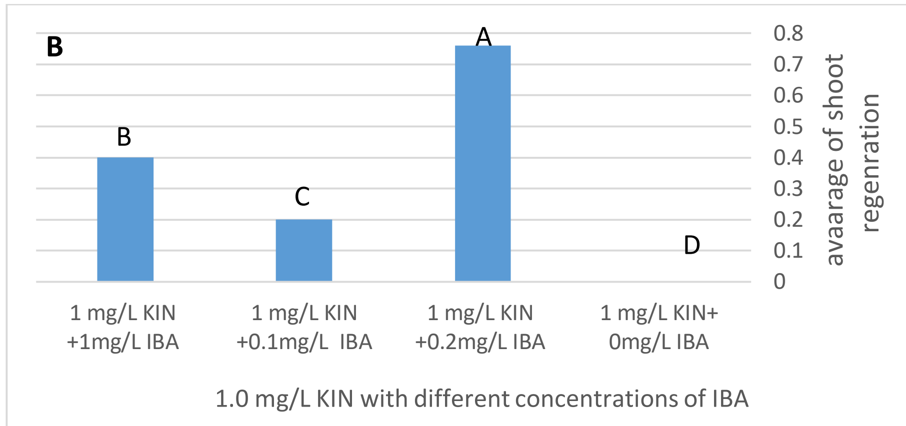

**Figure 4.12**: Cauliflower plant from hypocotyl explants regeneration *in vitro*. (**A**) Regeneration from hypocotyl explants, **(B**) zero day for plant in seed germination media, (**C** )10 day for plant in media (**D)** plant before agriculture, **(E)** zero day for plant after agriculture **(F)** 15 day for plant after agriculture.

## 4.4. Statistical analysis for the effect of PGRs on shoot regeneration from hypocotyl explants.

The differences between treatments were analyzed statistically using ANOVA, and means were separated using Fisher's LSD test. There was statistically significant difference ($p \leq 0.05$) in shoot regeneration between media contained 0.5 mg/L BA with 0.5 mg/L IBA and media contained 0.5 mg/L BA and 0.25 mg/L IBA, or between

media contained 0.5 mg/L BA with 0.5 mg/L IBA and media contained 0.5 mg/L BA with 0.1 mg/L IBA. However, there was no statistically significant difference ($p > 0.05$) in the number of shoots regeneration between media contained 0.5 mg/L BA and 0.25 mg/L IBA and media contained 0.5 mg/L BA and 0.1 mg/L IBA (Figure 4.13.A). For media contained 1.0 mg/L KIN with different concentrations of IBA, there was a statistically significant difference ($p < 0.05$) in the number of shoots regeneration between four types of media (0, 0.2, 0.1, 1) mg/L IBA (Figure 4.13.B). Moreover, there was statistically significant difference ($p < 0.05$) in the number of shoots regeneration in media contained 0.5 mg/L KIN with different concentrations of IBA (0, 0.2, 0.1, 1) mg/L (Figure 4.13.C). For media contained 2.0 mg/L BA with different concentrations of NAA, there was a statistically significant difference ($p < 0.05$) in the number of shoots regeneration between three types of media (1, 0.5, 0.1) mg/L NAA (Figure 4.13.D). However, there was no statistically significant difference ($p > 0.05$) in the number of shoots regeneration between media contained 0.5 mg/L TDZ with media contained 2.5 mg/L TDZ, or between media contained 1.5 mg/L TDZ and 2.5 mg/L TDZ. However, there was a statistically significant difference ($p \leq 0.05$) between media contained 1.5 mg/L TDZ media contained 0.5 mg/L TDZ (Figure 4.13.E). Figure 4.13 shows a statistical analysis of the effect of PGRs on *in vitro* shoots regeneration from hypocotyl explants.

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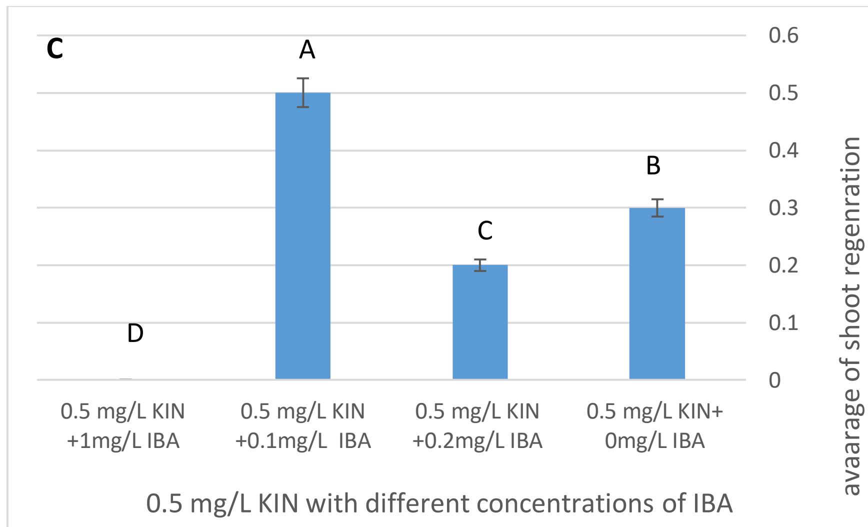
1.0 mg/L KIN with different concentrations of IBA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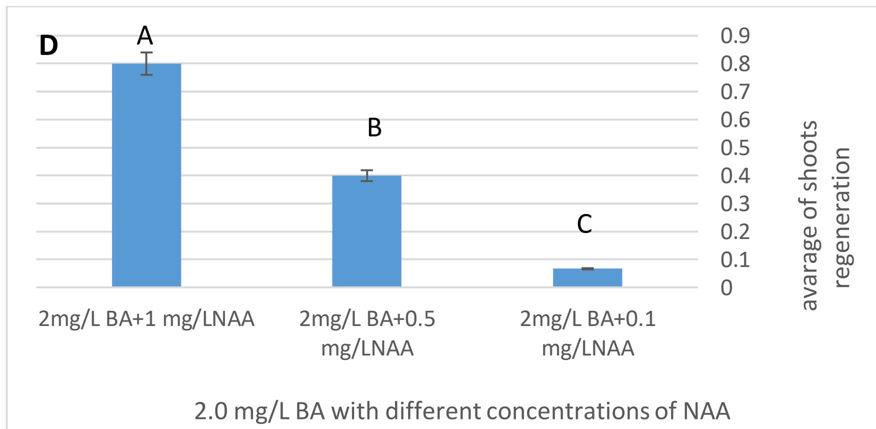
0.5 mg/L KIN with different concentrations of IBA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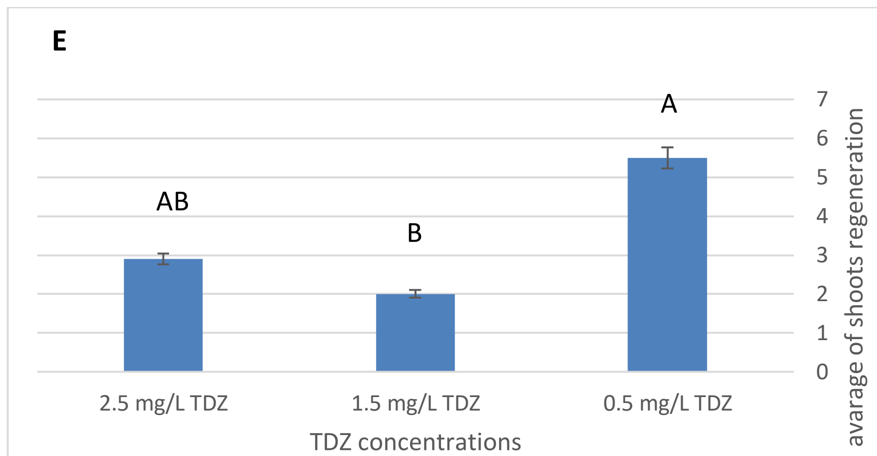


**Figure 4.13**: Statistical analysis of the effect of PGRs on *in vitro* shoots regeneration from hypocotyl explants. **(A)** The effect of BA and different concentrations of IBA, **(B)** effect of 1.0 mg/L KIN and different concentrations of IBA, **(C)** effect of 0.5 mg/L KIN and different concentrations of IBA, **(D)** effect of 2.0 mg/L BA and different concentrations of NAA and **(E)** effect of different concentrations of TDZ.

## 4.5. Statistical analysis for the effect of PGRs on shoot regeneration from cotyledon explants.

The difference between treatments was analyzed statistically using ANOVA, and means were separated using Fisher's LSD test. There was statistically significant difference ($p \leq 0.05$) in shoot regeneration from cotyledon explants between media contained 2.0 mg/L BA with different concentrations (1.0, 0.5, 0.1) mg/L of NAA. Figure 4.14 shows a statistical analysis of the effect of 2.0 mg/L BA with different concentrations of NAA on shoots regeneration from cotyledon explants.

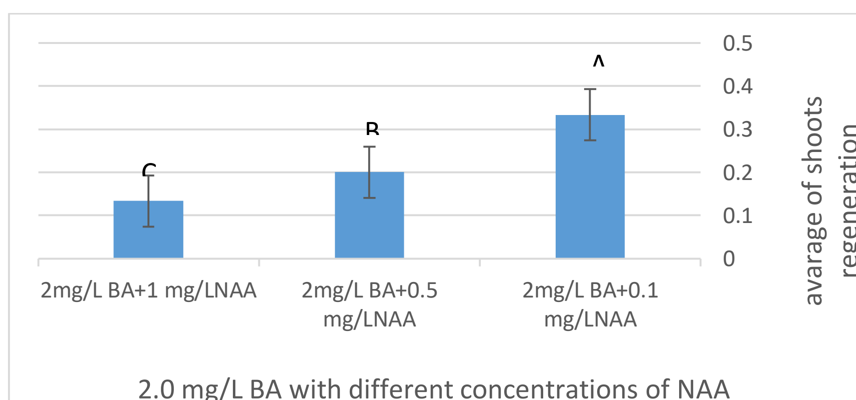


**Figure 4.14**: Statistical analysis of the effect of 2.0 mg/L BA with different concentrations of NAA on shoots regeneration from cotyledon explants. Statistically significant difference ($p \leq 0.05$) in shoot regeneration from cotyledon between three types of media (**A, B, C**).

# Chapter Five: Discussion

## Part one: Molecular analysis of flowering gene in *B. oleracea var. botrytis*.

Flowering is a major developmental transition in plant from vegetative to reproductive state. It is controlled by series of genetic (integrator and vernalization genes) and environmental factors. Therefore, it is important to study and analyze all molecular markers related to flowering to firstly distinguish early and late cauliflower varieties. The transition from vegetative to flowering phase is the result of the interactions between transducer proteins and integrator signals, which either enhance or inhibit the transition process (Jung & Müller, 2009). In *Arabidopsis*, most key flowering genes have been identified and functionally characterized (Fornara *et al*., 2010; Levy & Dean, 1998). Flowering genes are involved in a 'floral integrator' network, which consists of six regulatory gene pathways (Song *et al*., 2015). *FLC* is an important member of the floral integrator network and is affected by low temperatures. In the *B. oleracea*, four paralogous *BoFLC* genes (*BoFLC1, BoFLC2, BoFLC3* and *BoFLC5*) have been reported (Okazaki *et al*., 2007; Schranz *et al*., 2002). The *BoFLC2* has been associated with flowering time. In exon 4 of BoFLC2 non-vernalization type, a frameshift mutation is caused due to the 1 bp deletion (Okazaki *et al*., 2007; Ridge *et al*., 2014). These variations in the FLC2 gene were diverse in the *Brassica* crop, including SNP in the promoter affecting the expression of FLC2, and the insertion or deletion variation leading to loss of function of the FLC2, which all leads to early-flowering phenotype. In a segregating F2 population derived from a cross between late-flowering (*BoFLC2*) and early-flowering (*boflc2*) lines of cauliflower, the *BoFLC2 gene* behaves in a dosage-dependent manner and accounts for up to 65% of the variation in flowering time (Ridge *et al*., 2014). A loss-of - function mutation in *BoFLC2* confirmed its participation in earliness in annual cauliflower, reflecting a similar result for *AtFLC* in *A. thaliana* (Ridge *et al*., 2014). In *B.oleracea var. capitata*, insertions/deletions indel was revealed in intron number 2 of the *BoFLC.HP* gene account for approximately 80% of the variation in flowering time among F2 individuals and commercial lines (Abuyusuf *et al*., 2019).

The polymorphic *BoFLC.HP* gene contains MADS-box and K-box domain proteins, so it participates in changing the flowering time in cauliflower. In contrast, in the *Brassicaceae*, the suppression of flowering is mediated by vernalization, which mainly involves large changes in certain sites (Anderson *et al*., 2011; Salomé *et al*., 2011; Strange *et al*., 2011). In this study, indel and point mutation were found in the *BoFLC.HP* gene between early and late flowering lines by using F7R7 primers (Figure 4.3, 4.4). The polymorphic *BoFLC.HP* gene contains MADS-box domain proteins, thus it is involved in flowering time variation in cauliflower. In the *BoFLC.HP*, the 'F7R7' primer showed polymorphism across intron 2. This mutation is linked with flowering time variation in the Baladi (late-flowering) and hybrids (early-flowering) cauliflower. Abuyusuf et al. (2019) studies reported the sequence variations in the *BoFLC1.C9* gene for characterizing early and late flowering cabbage lines by 'F7R7' primer. In addition to, the reference sequence from NCBI is 96.9% identities with Baladi Cauliflower while 94.7% identities with hybrid Cauliflower (Figure 4.2). Reference sequence similar with Baladi sequence in different loci while different with the hybrid. However, the Baladi sequence is similar to a hybrid sequence in different loci while difference in reference sequences at positions 231 and 252 (Figure 4.3), this indicates genetic variation between sequences that affect flowering time as well.

## Part two: *In vitro* shoot regeneration of *B. oleracea var. botrytis*

For regeneration cotyledon, hypocotyl and root explants were excised from seedlings and cultured on a full-strength MS medium contained either BA, TDZ or KIN alone or in combination with IBA or NAA. In most *Brassica* species, regeneration depends on the age of explants (Bhalla & Singh, 2008). Young explants give better responses than older explants in most *brassica* species (Chakrabarty *et al*., 2002; Sharma *et al*., 2014; Tomsone & Gertnere, 2003). Explants from 3- or 4-d-old seedlings gave the best regeneration rates in different *Brassica* species, which depended on this study.

The present study showed that hypocotyl explants taken from *B. oleracea var. botrytis* cultivars gave higher shoot regeneration more than cotyledon explants. The

genotype effect is one of the most important factors for *in vitro* regeneration of *B. oleracea* (Cardoza & Stewart, 2004; Zhang & Bhalla, 2004).

Early studies addressed shoot regeneration in cauliflower were challenging. Pareek and Chandra (1978) reported failure direct shoot regeneration but somatic embryogenesis from leaf callus of cauliflower on MS medium supplemented with IAA 1.0 mg/L and 0.5 mg /L KIN. Here, results showed hypocotyl explants not the cotyledons gave regeneration when using full-strength MS media supplement with 0.5 mg/L of KIN alone and 1.0 mg/L KIN with different concentrations of IBA (Table 4.2).

Pavlovj *et al*. (2010) used KIN alone and with IBA and gave a high rate of shoot formation from cotyledon and hypocotyl explants. In this study when using 1.0 mg/L KIN alone or with IBA (0.2, 0.1) mg/L no shoot regeneration was observed in cotyledon explants while poor shoot regeneration was observed from hypocotyl explants compared to Pavlovj *et al* study (Table 5.1). This result for high shoot regeneration in Pavlovj *et al* study is due to the use of hybrid cauliflower. The results show the BA with IBA and KIN with IBA make the best result for shoot regeneration (Table 4.1,4.2,4.3,4.4). A similar result was reported by (Pavlovj *et al*., 2010) in red cabbage where a combination of BA with IBA had a positive effect on shoot regeneration and 1.0 mg/L BA gave the highest number of shoots regeneration in broccoli.

**Table 5.1**: Percentage of shoots regeneration for KIN alone or with IBA between Pavlovj *et al* study and this study.

| | **Shoot regeneration (%)** | | **Shoot regeneration (%) from Pavlovj *et al* study** | |
|---|---|---|---|---|
| **Media contains** | **Hypocotyl** | **Cotyledon** | **Hypocotyl** | **Cotyledon** |
| 1.0 mg/L KIN | 0% | 0% | 51 % | 85% |
| 1.0 mg/L KIN+0.2 mg/L IBA | 28% | 0% | 56% | 75% |
| 1.0 mg/L KIN+ 0.1 mg/L IBA | 20% | 0% | 54% | 54% |

TDZ is a synthetic phenylurea, a cytokinin like compound effective in shoot regeneration in many plant species (Mithila *et al*., 2003; Pelah *et al*., 2002). In this project, results indicate that TDZ at low concentration increased shoot regeneration in Baladi cauliflower, whereas TDZ at 0.5 mg/L gave the highest direct shoot regeneration (table 4.5). Furthermore, the interaction between TDZ and NAA gave less regenerated shoots. Many published protocols for *Brassica* species regeneration are based on using TDZ (Christey *et al*., 1997; Henzi *et al*., 1999; Kumar *et al*., 2015; Yongen & Hanxia, 2003) and all literature reported that the TDZ supplemented media found to be very efficient for enhancing the frequency of shoot regeneration in *Brassica* species. Qin *et al*. (2006) reported 0.5 mg/L TDZ restrained shoot induction in hybrid cauliflower. Yu *et al*. (2010) reported 100% shoot regeneration from hypocotyl and cotyledon explants of cauliflower using TDZ at 0.1 mg/L and NAA at 0.01 mg/L, TDZ at 0.2 mg/L and NAA at 0.01 mg/L. Another study by Gaur and Srivastava (2017) reported 96% shoot regeneration from hypocotyl explants on MS medium supplemented with 0.44 mg/L TDZ with 0.08 mg/L IAA. The combination of two different cytokinins such as TDZ and BA improves the number and the quality of shoot regeneration more than using single cytokinin (Tomsone & Gertnere, 2003).

In this study, the media that contained BA with different concentrations of NAA enhanced the shoot regeneration for hypocotyl and cotyledon explants (Table 4.4). It was previously shown that BA alone or in combination with an auxin positively promoted shoot regeneration and production of various *Brassica* plants (Jin *et al*., 2000; Maheshwari *et al*., 2011; Metz *et al*., 1995; Sretenović-Rajičić *et al*., 2007). The presence of BAP in the medium increased the number of shoots produced in *B. oleracea in vitro* (Cheng *et al*., 2001). In addition, it has been shown that including NAA significantly improves shoot regeneration (Guo *et al*., 2005).

Successful *in vitro* rooting of cauliflower shoots was achieved after culturing plantlets in MS medium supplemented with different concentrations of NAA or IBA. Media contains BA with different concentrations of NAA increased the number of roots unlike media contains BA with different concentrations of IBA. The results showed that *Brassica oleracea* var. *botrytis* gave high rooting ability, but there were differences among them. Moreover, combining KIN with different concentrations of

IBA enhanced rooting in hypocotyl more than cotyledon explants. Overall, it is observed that the addition of any auxin improved rooting. Similar correlations were reported by Munshi *et al.* (2007) for cabbage and Ravanfar *et al*. (2009) for broccoli. Moreover, higher concentrations of NAA (0.5-1.0 mg/L) in the medium promoted callus formation at the base of the shoots (Figure 4.8 and Table 4.4) in hypocotyl, similar to the findings of (Munshi *et al*., 2007). On the other hand, callus induction for cotyledon explants was overall at a lower rate.

*Brassica* species are very rich sources of phenolic compounds, when explants remain in the medium for long period, the explants are vulnerable to chlorosis as well as necrosis. The cotyledon explants showed progressive chlorosis (they turned yellow), and they finally became brown and died. This result is due to the production of phenolic compounds from explants into the medium and subsequent oxidation to form toxic compounds (Figure 4.11). Similar effects during *in vitro* culture was observed as well in broccoli (Qin *et al*., 2007) and cabbage (Gerszberg *et al*., 2015) . On the other hand, it is observed that the media containing BA caused a high percentage of hyperhydricity (vitrification) in the shoot regeneration (Figure 4.5, A). In cauliflower, the replacement of BA with KIN was less favorable for shoot vitrification (Figure 4.6 (B) 4.7 (C)). A previous study by Pavlovj *et al*. (2010) reached similar conclusion in both cauliflower and cabbage.

# Chapter Six: Conclusion and future works

This study shows a clear differentiation between early and late flowering Baladi cauliflower in *BoFLC.HP* gene. It was clearly identified at the DNA sequencing the variation in *BoFLC.HP* gene between Baladi and hybrid cauliflower. The "F7R7" primer showed a point mutation at position 91 and indel at position 212 to 219 in intron number 2 between Baladi (early flowering) and hybrid cauliflower (late flowering) that controls flowering time. The F7R7 primer could be used as genetic marker to characterize flowering time variation and to select as well to develop early-and late-flowering cauliflower cultivars.

A clear protocol was established to produced direct shoots regeneration from different parts of cauliflower. The result for hypocotyl explants gave the highest regeneration results in more than cotyledon explants. The highest shoot regeneration from hypocotyl explants resulted in full strength MS media supplemented with 0.5 mg/L BA and 0.5mg/L IBA in 80%. In the cotyledon explants, the high shoot regeneration was observed in full strength MS media supplemented with 2.0 mg/L BA and 0.1mg/L NAA in 26%. This indicates that genotype plays an important role in shoot regeneration. These two results would contribute directly towards the development of Baladi cauliflower in Palestine, and facilitates the process of genetic transformation with *Agrobacterium*. Any further crop improvement efforts and any genetic transformation requires a detailed exome sequencing involves identification of genes (whole exome sequncing), gene expression and presence of copy number of genes.

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# Chapter Eight: Appendix

**Appendix Table 1**: Area and production of cauliflower in Palestinian territory.

| Agricultural year ▶ | 2016-2017 | | 2017-2018 | | 2018-2019 | |
|---|---|---|---|---|---|---|
| **Governorate ▼** | **Production** | **Area** | **Production** | **Area** | **Production** | **Area** |
| Palestinian Territory | **48061** | **16030** | **45872** | **14585** | **34502** | **11294** |
| West Bank | **42950** | **13931** | **42258** | **13099** | **30932** | **9808** |
| Jenin | 10450 | 4180 | 9875 | 3950 | 8850 | 2950 |
| Tubas | 9000 | 1500 | 8100 | 1350 | 452 | 753 |
| Tulkarm | 3846 | 1578 | 4335 | 1745 | 7088 | 2025 |
| Nablus | 1287 | 429 | 936 | 313 | 906 | 302 |
| Qalqiliya | 314 | 157 | 465 | 155 | 694 | 199 |
| Salfit | 120 | 60 | 56 | 28 | 54 | 27 |
| Ramallah and Al-Bireh | 494 | 160 | 620 | 180 | 383 | 160 |
| Jericho and Al-Aghwar | 2644 | 1322 | 2302 | 1151 | 2781 | 927 |
| Jerusalem | 81 | 81 | 31 | 39 | 31 | 39 |
| Bethlehem | 7912 | 1840 | 7031 | 1635 | 5031 | 1170 |
| Hebron | 6802 | 2624 | 8507 | 2553 | 4662 | 1256 |
| **قطاع غزة** | **5111** | **2099** | **3614** | **1486** | **3570** | **1486** |
| North Gaza | 534 | 203 | 236 | 139 | 236 | 139 |
| Gaza | 1625 | 700 | 1120 | 400 | 1120 | 400 |
| Deir Al-Balah | 2125 | 870 | 1946 | 740 | 1902 | 740 |
| Khan Yunis | 512 | 190 | 210 | 127 | 210 | 127 |
| Rafah | 315 | 136 | 102 | 80 | 102 | 80 |

## Tissue culture chemicals and reagents

1. The basal growth media; Murashige and Skoog (MS) medium including all vitamins (Duchefa Prod. No:M0255.0050).
2. The plant growth regulators (PGRs):
   - 6-Benzyleaminopurine (BA)
   - 1-Naphtalene acetic acid (NAA)
   - Indole-3-butyric acid (IBA)
   - Thidiazuron (TDZ)
   - Kinetin (KIN)

## DNA extraction reagents.

To isolation DNA from the cauliflower leaf it was used different reagent such as Lysis buffer, RNasa-A, chloroform: isoamyl Alcohol (24:1), Ammonium Acetate (7.5M), ice cold absolute ethanol. For DNA precipitation, it uses 70% ethanol. Finally, DNA resupesion with 1x TE buffer.

## Equipment and Reagents for PCR Reaction

1. Thermo cycler PCR machine () was used to perform PCR reactions.
2. Commercial master mix for reaction prepared with the following components:

- Distilled water
- 10X Taq reaction buffer -Cat. #:37A
- MgSO4 (mM) - Cat. #:37B
- dNTPs (mM) - Cat. #37C:
- Primer Forward (100 pmol/µl in TE buffer).
- Primer Reverse (100 pmol/µl in TE buffer).
- Taq DNA polymerase - Cat. #: HTD0078

## Gel Electrophoresis Reagents

Agarose from Sigma Aldrich (Cat#) was used to prepare 2.0 % (w/v) agarose gels with 1X TE buffer for running of PCR products.

## Statistical Analysis

**Appendix Table 2**: Analysis of variance (ANOVA) for effect of TDZ hormone on shoot regeneration at different TDZ concentrations after four weeks of the *in vitro* shoot regeneration.

| Summary | | | | |
|---|---|---|---|---|
| **Treatments** | **Sample size** | **Sum** | **Mean** | **Variance** |
| 0.5 TDZ | 10 | 55 | 5.5 | 12.5 |
| 1.5 TDZ | 10 | 20 | 2 | 11.55555556 |
| 2.5 TDZ | 10 | 29 | 2.9 | 14.54444444 |
| Total | 30 | 104 | | |

| ANOVA | | | | | |
|---|---|---|---|---|---|
| **Source of variation** | **SS** | **df** | **MS** | **F value** | **P-value** |
| Between Groups | 66.06666667 | 2 | 33.03333333 | 2.567357513 | 0.095335731 |
| Error | 347.4 | 27 | 12.86666667 | | |
| Total | 413.4667 | 29 | | | |

**Appendix Table 3**: Analysis of variance (ANOVA) for effect of IBA with BA hormone on shoot regeneration at different concentrations after four weeks of the *in vitro* shoot regeneration.

| Summary | | | | |
|---|---|---|---|---|
| **Treatments** | **Sample size** | **Sum** | **Mean** | **Variance** |
| 0.5BA +0.5IBA | 10 | 34 | 3.4 | 11.15555556 |
| 0.5BA +0.1IBA | 10 | 2 | 0.2 | 0.177777778 |
| 0.5BA +0.25IBA | 10 | 5 | 0.5 | 0.5 |
| Total | 30 | 41 | | |

| ANOVA | | | | | |
|---|---|---|---|---|---|
| **Source of variation** | **SS** | **df** | **MS** | **F value** | **P-value** |
| Between Groups | 62.46666667 | 2 | 31.23333333 | 7.918309859 | 0.001967459 |
| Error | 106.5 | 27 | 3.944444444 | | |
| Total | 413.4667 | 29 | | | |

**Appendix Table 4**: Analysis of variance (ANOVA) for effect of 1 mg/ml KIN with different concentrations of IBA hormone on shoot regeneration after four weeks of the *in vitro* shoot regeneration.

| Summary |
|---|

| Treatments | Sample size | Sum | Mean | Variance | Std.Dev |
|---|---|---|---|---|---|
| MS + I mg/L KIN | 15 | 0 | 0 | 0 | 0 |
| MS+1mg/L KIN+0.2 mg/L IBA | 25 | 19 | 0.76 | 4.273333 | 2.0672 |
| MS+1 mg/L KIN + 0.1 mg/L IBA | 15 | 3 | 0.2 | 0.171429 | 0.414 |
| MS+1 mg/L KIN + 1 mg/L IBA | 5 | 2 | 0.4 | 0.8 | 0.8944 |
| Total | 60 | 24 | 0.4 | 5.244762 | 1.3925 |

| **ANOVA** | | | | | |
|---|---|---|---|---|---|
| **Source of variation** | **SS** | **df** | **MS** | **F value** | **P-value** |
| Between Groups | 6.24 | 3 | 2.08 | 1.076923 | 0.366337 |
| Within-groups | 108.16 | 56 | 1.931429 | | |
| Total | 114.4 | 59 | | | |

**Appendix Table 5**: Analysis of variance (ANOVA) for effect of 0.5 mg/ml KIN with different concentrations of IBA hormone on shoot regeneration after four weeks of the *in vitro* shoot regeneration.

| **Summary** |
|---|

| Treatments | Sample size | Sum | Mean | Variance | Std.Dev |
|---|---|---|---|---|---|
| MS + 0.5 mg/L KIN | 15 | 4 | 0.266667 | 0.352381 | 0.5936 |
| MS+0.5mg/L KIN+0.1 mg/L IBA | 18 | 9 | 0.5 | 0.617647 | 0.7859 |
| MS+0.5 mg/L KIN + 0.2 mg/L IBA | 25 | 5 | 0.2 | 0.333333 | 0.5774 |
| MS+0.5 mg/L KIN + 1 mg/L IBA | 16 | 0 | 0 | 0 | 0 |
| Total | 74 | 18 | 0.9666 | 1.30 | 0.5924 |

| **ANOVA** | | | | | |
|---|---|---|---|---|---|
| **Source of variation** | **SS** | **df** | **MS** | **F value** | **P-value** |
| Between Groups | 2.188288 | 3 | 0.729429 | 2.17895 | 0.098175 |
| Within-groups | 23.43333 | 70 | 0.334762 | | |
| Total | 25.62162 | 73 | | | |

The End