PLANT GROWTH REGULATORS FOR ENHANCING *IN VITRO* CORMLET PRODUCTION IN SAFFRON

(*Crocus sativus* L.)

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(2019-09-014)

B. Sc. – M. Sc. (INTEGRATED) BIOTECHNOLOGY

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THESIS

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KERALA, INDIA

2024

DECLARATION

I, hereby declare that this thesis entitled **"Plant growth regulators for enhancing *in vitro* cormlet production in saffron (*Crocus sativus* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled "**Plant growth regulators for enhancing *in vitro* cormlet production in saffron (*Crocus sativus* L.)**" is a record of research work done independently by **Ms. Neethu R. S. (2019-09-014)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the advisory committee of **Ms. Neethu R. S. (2019-09-014)**, a candidate for the degree of **B. Sc. - M. Sc. (Integrated) Biotechnology**, agree that the thesis entitled **"Plant growth regulators for enhancing *in vitro* cormlet production in saffron (*Crocus sativus* L.)"** may be submitted by Ms. Neethu R. S., in partial fulfilment of the requirement for the degree.

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NEETHU R. S.

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LIST OF ABBREVIATIONS

%	Percent
µL	Microlitre
µM	Micromolar
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
BA	Benzyl adenine
BAP	6-Benzylaminopurine
℃	Degree Celsius
CCC	Chlorocholine chloride
CD	Conserved domain
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
CMC	Chlormequat chloride
Ct	Cycle threshold
CTAB	Cetyltrimethylammonium bromide
DAT	Days after treatment
DEPC	Diethyl pyro carbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
et al	And others

Fig.	Figure
g	Gram
g/L	Gram per litre
GA	Gibberellin
GA3OX	Gibberellin 3 oxidase
HCl	Hydrochloric acid
HgCl2	Mercuric chloride
hr	Hour
IAA	Indole acetic acid
Kn	Kinetin
L.	Linnaeus
m	Metres
M	Molarity
m amsl	Meters above mean sea level
mg	Milligram
min.	Minute
mL	Millilitre
mM	Millimolar
MPQ	Mepiquat chloride
MS	Murashige and Skoog
N	Normality
NAA	Naphthalene acetic acid

NaOCl	Sodium hypochlorite
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
PBZ	Paclobutrazol
PCR	Polymerase chain reaction
PGM	Phosphoglucomutase
PGR	Plant growth regulators
ppm	Parts per million
Psi	Pounds per square inch
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-qPCR	Reverse transcription - quantitative PCR
s	Second
Sl. No	Serial number
spp.	Several species
SS	Starch synthase
SUSY	Sucrose synthase
TDZ	Thidiazuron
UV	Ultra violet
V	Volt
viz.	Namely

w/v	Weight by volume
β	Beta

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Introduction

1. INTRODUCTION

Saffron (*Crocus sativus* L.) commonly known as 'Red Gold' is a bulbous perennial plant belonging to Iridaceae family produces the most expensive flower in the world which cost around 2.5 to 3.5 lakhs per kilogram. The dried stigmas are the spice of commerce. Due to its color, bitterness, and potent aroma, saffron is used in food preparation, perfumes, and cosmetic products (Serghini *et al*., 2017). Saffron, particularly its primary compounds *viz.,* crocins, crocetin, picrocrocin, and safranal, demonstrate positive impacts on neuropsychiatric conditions such as depression, anxiety, as well as age-related ailments like cardiovascular issues, vision problems, neurodegenerative diseases, and sarcopenia (El-Midaoui *et al*., 2022).

Saffron is a sterile geophyte propagated vegetatively by corms and does not yield viable seeds due to irregularities in meiosis and gamete formation (Mushtaq *et al*., 2014). Corms survive for only one season and divide into cormlets that eventually give rise to new plants. The conventional method for propagating saffron has numerous drawbacks, including seasonality and the requirement of peculiar climatic conditions. Plant tissue culture has emerged as a well established technology for generating high quality corms (Devi *et al*., 2011; Salwee *et al*., 2014; Mir *et al*., 2014).

Plant growth regulators (PGRs) are naturally occurring organic compounds found in higher plants which regulate growth and other physiological processes at a location different from their source of synthesis. A combination of PGRs *viz.*, thidiazuron (TDZ) and indole acetic acid (IAA) is reported for the production of *in vitro* cormlets in saffron (Parray *et al*., 2012). According to Li *et al* (2021) a higher concentration of GA can inhibit cormlet formation in *Gladiolus hybridus*, a crop belonging to the same family of saffron. Hence the present study was taken up to understand whether anti-gibberellin could enhance *in-vitro* cormlet formation in saffron.

Plant growth retardants are compounds utilized to curb plant growth without altering developmental patterns or inducing phytotoxic effects. Anti-gibberellins represent a category of plant growth regulators that impede the synthesis of gibberellic acid within plants (Wiens *et al.,* 2023). The present study used anti-gibberellins *viz.,* chlorocholine

chloride (CCC), paclobutrazol (PBZ), chlormeqaut chloride (CMC) and mepiquat chloride (MPQ) to assess its impact on cormlet initiation *in vitro*.

Elucidating the molecular basis of cormlet formation is crucial for enhancing *in vitro* cormlet production. As there are no reports at present in this regard, studies were also attempted to understand the molecular crosstalk in the biosynthesis pathway of gibberellin and starch during *in vitro* cormlet formation by studying the expression profile of key genes *viz*., *gibberellin3oxidase (GA3OX), starch synthase (SS), sucrose synthase (SUSY) and phosphoglucomutase (PGM)*.

Review of literature

2. REVIEW OF LITERATURE

2.1 Saffron (*Crocus sativus* L.)

Saffron (*Crocus sativus* L.) commonly known as 'Red Gold" is regarded as the most expensive spice in the world overall. It is a sterile, triploid male monocotyledon plant, which is a member of the *Iridaceae* family, reproduces only vegetatively by corms. The dried stigmas of the *Crocus sativus* plant and the spice derived from them are referred to as saffron. Due to their color, bitterness, and potent aroma, dried saffron stigmas are used in food preparation, perfumes, and cosmetic products (Serghini *et al*., 2017).

2.1.1 Origin and distribution

Although its wild species is unknown, saffron is one of the oldest plants known to have been cultivated. Saffron's origins can be traced back at least 5,000 years to Mesopotamian cave paintings. Although the exact origin of its cultivation is unknown, it was discovered to have been practised in Crete and Thera during the Minoan era. According to folklore, saffron originated in Asia Minor, Persia, and Ancient Greece. Eventually, saffron production made its way to China and India. At the moment, saffron is grown in several countries, including Afghanistan, Iran, Spain, Italy, and Azerbaijan. (Fujii *et al*., 2022).

2.1.2 Morphology

This herbaceous perennial reaches a height of 10 to 25 cm from its bulbs. The sub-ovoid bulb can be found in a variety of forms and sizes. The corms, characterized by their tuberous-bulb structure, are compressed and flattened at the base, measuring approximately 4.5–5.5 cm in diameter, and are enveloped by multiple reticulated fibrous layers. Typically, corms exhibit one or two primary buds at the apex and around 4–5 or more secondary buds, arranged in an irregular spiral pattern depending on their size. Corms originating from secondary buds tend to be smaller in size compared to those produced by apical buds. Each parent corm generates 1–3 medium to large

daughter corms from apical buds and several smaller corms from lateral buds, the quantity depending on the size of the parent corm (Gresta *et al*., 2008).

Two distinct kinds of roots are found in saffron: thin, fibrous roots at the base of the mother bulb and contractile roots formed at the base of lateral buds (Kalesi *et al*., 2004). On each bud, there are five to eleven dark-green leaves that are erect, narrow and grass-like. They have an inner white band and an outside rib, and their length varies from 20 to 60 cm (Srivastava *et al.,* 2010).

2.1.3 Floral biology of crop

Early in the autumn, towards the end of September, *Crocus sativus* flowers start to emerge. They are purple in colour and have six tepals, three of which are internal and the other three are exterior. These tepals meet at a long tube that emerges from the upper part of the ovary. The flowers are covered in white, membrane-like bracts when they first emerge. A narrow style that is 9 to 10 cm long emerges from the inferior ovary that makes up the pistil. A single stigma made up of three filaments that are an intense red colour forms the style's terminal (Mzabri *et al*., 2019).

2.2 Composition of saffron

The complex chemistry of saffron includes primary metabolites such carbohydrates, minerals, lipids, vitamins, amino acids, and proteins that are ubiquitous in nature. Many substances, such as carotenoids, monoterpenes, and flavonoids, which mostly consist of anthocyanins, are classified as secondary metabolites. These substances are not universally present in the body but are crucial for the growth and reproduction of the organism (Bathaie *et al.*, 2014). Saffron is highly valued for its flavor, aroma, and color. Its quality is determined by the components that give it these characteristics. The primary chemical components of saffron are crocin, picrocrocin, and saffranal, which give it its characteristic color, flavor, and aroma, respectively. Its distinctive color is caused by crocetin glycosyl esters (Sampathu *et al*., 1984).

2.3 USES OF SAFFRON

Saffron is the most intriguing and enticing species because of the coloring, bitterness, and strong fragrance of its dried stigmas. This is a geophyte herbaceous plant whose stigmas have long been utilized for culinary coloring, medicinal reasons, and the creation of cosmetics and scents. Almost all of it uses these days are for food coloring and taste, despite the fact that new research has increased interest in its potential medical benefits (Khazdair *et al*., 2015)

2.3.1 Uses of saffron in cosmetics and fragrances

Since ancient times, saffron has been utilized for cosmetic purposes because of its properties that support eternal youth, it can be administered topically, taken as an infusion, mixed with fat, or macerated in donkey milk. In traditional Greek medicine, it can be used to cure acne, wounds, and skin disorders. Additionally, it can be applied to rejuvenate the skin on the face. It is also possible for the physical look to seem younger and more vibrant. Hindu women in a different group used saffron to create the bindi, or yellow dot, that is worn on the forehead. When the spice dries, it releases an exquisite aroma that originates from safranal, the main fragrant ingredient of saffron. In ancient Greece, saffron was used as a royal dye and fragrance in salons, courts, theaters, and lavatories (Mzabri *et al*., 2019).

2.3.2 Uses of saffron in medicine

Saffron and its constituents have been shown to have a wide range of positive benefits on gynaecological problems as well as a number of ailments, such as depression and Alzheimer's disease. Because of this phytomedicine's exceptional safety level, the majority of its effects can be employed in clinical trials. Some applications of saffron, such as its antidepressant and anti-Alzheimer properties, are acceptable for the next stage of clinical investigation or medication development. *C. sativus* extract was tested against placebo or specific antidepressant drugs in antidepressant clinical studies; saffron shown efficacy as an antidepressant drug in these trials. In therapeutic trials involving women, saffron alleviated premenstrual syndrome symptoms more effectively than placebo and on par with existing medications (Moshiri *et al*., 2015)

2.4 AGRO TECHNOLOGY

2.4.1 Geographic distribution

Saffron is widely cultivated in Mediterranean countries, East Asia and many countries of the world (latitude 30-50°N and longitudes 10°E to 80°W) in areas of low annual rainfall, cold winters and hot summers (Arslan *et al*., 2007). The plant grows from altitude of sea level to almost 2000 m, although it is more acclimatized to hill sides, plateaus and mountain valleys ranging in altitudes between 600 and 1700 m (Yasmin *et al*., 2013). In Morocco, it is cultivated at an altitude between 1200 and 1400 m amsl. In India, *Crocus spp*. is grown well in a temperate climate with sunny days and grow best at an altitude of 2140 m amsl. This crop can be cultivated in temperate, semi-arid, and arid areas in the range of 1500–2800 m above sea level (Kothari *et al*., 2021).

2.4.2 Climatic requirement

According to Kumar *et al*. (2009), temperature is the primary environmental element influencing the growth and flowering of *Crocus spp.* The ideal climate for high safron yields is one with mild winters, moderate summers, and rainy autumns. Anthesis is preferred in Kashmir's temperate climate when the average maximum air temperature falls below 20°C. For vernalization, the plant needs 1100 cooling hours during the vegetative period. The crop in Kashmir, India experiences a highest average temperature of 11.4 °C, a lowest average temperature of − 0.33 °C, and 474 mm of precipitation during the vegetative period. Temperatures between 23 and 25 °C are ideal for vegetative growth in the early phases of growth, whereas temperatures below 16 °C are ideal for increasing the number of daughter corms that can be produced. The typical air temperature in Palampur, Himachal Pradesh, is between 19 and 23 °C in September and October between 8 and 13 °C (average of 30 years) in November and December, making it a perfect location for saffron growing (Kumar *et al*., 2009).

2.5 CORM DEVELOPMENT IN SAFFRON

In saffron, the daughter corm begins to develop not only as it forms vascular connections with the mother corm, which facilitates the transfer of nutrients and water, but also when the mother corm becomes established as a source organ. In the early stages of development, the daughter corm increases the size and number of cells, reaches its maximum concentration of starch, and experiences a decline in the very high content of soluble sugars at the start of its development. Even if they are still developing, the leaves can still serve as a source organ at this point. Due to an increase in cell size, the corm will acquire weight in the second stage while keeping the high starch concentration attained in the first. Leaves also serve as the source organs, where there is a consistent amount of soluble sugar even while the reserve of starch has decreased. A notable reduction in root biomass may be the cause of the corm's development halt, which would restrict water availability and cause leaf aging. Changes in sugar levels resulting from these mechanisms may indicate the termination of corm development and leaf senescence (Pallotti *et al*., 2024).

2.6 PLANT GROWTH REGULATORS

Plant growth regulators (PGRs) are naturally occurring organic compounds found in higher plants, distinct from nutrients, which regulate growth and physiological functions at sites distant from their production. Since these hormones are created in plants, Thimann *et al*. (2003) referred to them as "phytohormones" to differentiate them from animal hormones. According to Bisht Tejpal Singh phytohormones are organic substances that higher plants naturally create, which regulates growth or other physiological processes at a location different from its source of synthesis and is active in trace levels. Operating in small quantities, they modulate various plant processes and are often referred to as biostimulants or bioinhibitors. These regulators exert their effects by interacting with specific enzymes or enzyme systems within plant cells, thereby influencing plant metabolism. Typical examples of PGRs encompass auxins, gibberellins, cytokinins, ethylene, growth retardants, and growth inhibitors. Auxins were the first hormones discovered in plants, followed by gibberellins and cytokinins. Bhattacharjee *et al*. (1983) reported that plant growth regulators are crucial for inducing dormancy breakage and enhancing the production of high-quality corms in gladiolus.

2.6.1 Plant growth retardants

Plant growth retardants refer to synthetic organic compounds that, upon application to responsive plants, effectively decrease the rate of stem elongation, typically without inducing significant developmental abnormalities (Cathey *et al*., 1964). Plant growth retardants are compounds utilized to curb plant growth without altering developmental patterns or inducing phytotoxic effects. Their primary mechanism involves reducing cell elongation and suppressing the rate of cell division. These compounds exert their effects by antagonizing gibberellins (GAs) and auxins, the plant hormones primarily responsible for shoot elongation. Commercially utilized inhibitors of GA biosynthesis include onium-type compounds, N-heterocycle compounds (triazole-type), structural mimics of 2-oxoglutaric acid, and 16,17-dihydroGAs (Rademacher, 2000). Kozak *et al*. (2006) demonstrated that the addition of plant growth retardants to an *in vitro* medium could effectively enhance tuberization in various plant species.

2.6.2 Chlorocholine chloride

Chlorocholine chloride, also known as (2-chloroethyl) trimethylammonium chloride (CCC), serves as an anti-gibberellin growth retardant and has been extensively utilized to regulate plant growth (Menhenett *et al.*, 1984; Jiao *et al*., 1986; Berova *et al*., 2000). CCC functions as an inhibitor of gibberellic acid biosynthesis by blocking the conversion of geranylgeranyl pyrophosphate (GGPP) to ent-kaurene, an early step in the process (Wang *et al*., 2009). It was observed that gibberellic acid (GA) induces a significant reduction in the activity of adenosine diphosphate-glucose pyrophosphorylase (AGPase), a pivotal enzyme involved in starch synthesis. Treatment with CCC may counteract this decrease in starch synthesis by inhibiting GA production, potentially leading to long-term alterations in plant growth through changes in endogenous hormone levels (Zheng *et al*., 2012). Moreover, research by Sharma *et al*. (2008) demonstrated that CCC effectively promotes tuberization and enhances starch content in potato tubers.

2.6.3 Paclobutrazol

Paclobutrazol (PBZ), a member of the triazole family, exhibits growth-regulating properties and is predominantly utilized as a growth retardant and stress protectant. PBZ achieves growth retardation by disrupting gibberellin biosynthesis, specifically by inhibiting the oxidation of ent-kaurene to ent-kauronoic acid through the inactivation of cytochrome P450-dependent oxygenase. Remarkably, PBZ demonstrates superior effectiveness compared to other plant growth regulators, even at relatively low application rate (Desta and Amare, 2021). Research conducted by Jabir *et al*. confirmed the efficacy of paclobutrazol in modulating the growth of the 'Nau-yh' cultivar of radish. PBZ treatment resulted in a notable enhancement of taproot growth coupled with a reduction in plant height. Additionally, PBZ treatment significantly increased primary root growth while decreasing shoot development. Foliar application of PBZ effectively controlled excessive vegetative growth, improved photosynthetic capacity, and maintained water balance in leaves, thereby redirecting more photoassimilates towards sink organs such as bulbs (Zheng *et al*., 2012).

2.6.4 Chlormequat chloride

Chlormequat chloride (CMC) is a synthetic plant growth regulator renowned for its ability to impede gibberellin biosynthesis, consequently leading to the shortening and strengthening of plant stems, as well as a reduction in branching and foliage, particularly in certain species of shrubs and trees. CMC molecules feature positively charged groups like ammonium, phosphonium, or sulphonium, which directly interfere with gibberellin synthesis just prior to ent-kaurene formation. When applied through spraying, CMC has been observed to induce notable effects on potato plants, including stem growth reduction, internode length reduction, increased leaf number and size, decreased stolon length, and accelerated tuberization. Furthermore, CMC exhibits high efficacy in suppressing the growth of microplants in *in vitro* potato culture, thereby extending the interval between passages (Nistor *et al*., 2010).

2.6.5 Mepiquat chloride

Mepiquat chloride (MPQ) functions as a plant growth regulator, specifically as a gibberellin synthetic growth retardant. It operates by inhibiting ent-copalyl

diphosphate synthase (CPS) and ent-kaurene synthase (KS) in the early stages of gibberellic acid (GA) metabolism. Being water-soluble, MPQ can effectively regulate plant growth when applied via seed soaking or leaf spraying methods (Wu *et al*., 2019). Widely utilized in cotton cultivation worldwide, MPQ plays a crucial role in managing plant height and enhancing production. Its foliar application impedes gibberellic acid biosynthesis, facilitated by its easy absorption through leaves and subsequent diffusion throughout the plant, leading to morphological changes such as reduced internodal distance, plant height, node count, and height-to-node ratio (Murtza *et al*., 2022).

2.7 *IN VITRO* PROPAGATION

Since the flowers of saffron are sterile and do not yield viable seeds, corms are used to propagate the plant. A corm can produce up to ten cormlets, which eventually sprout into new plants, throughout its one-season lifespan. Reproduction is therefore dependent on humans; the corms need to be physically dug up, split apart, and replanted. Conventional methods of propagating saffron have numerous drawbacks, including seasonality and the need for particular environmental conditions (Karaoğlu *et al*., 2007).

In spite of its triploid origin, saffron cannot be genetically improved through traditional breeding methods. The only way for corms to multiply in large quantities is by vegetative propagation. Application of micro propagation techniques such as tissue culture is one viable solution to create high-quality corms and to address the issues of corm rot (caused by infection by many fungal infections) and poor rate of multiplication.

Saffron may be propagated quickly and widely through the use of *in vitro* culture methods, which may be extremely beneficial (Small *et al*., 2016). Regeneration methods involving somatic embryogenesis and organogenesis (direct or indirect) have demonstrated the feasibility of using tissue culture to grow corms and saffron plants. However, saffron's reaction to *in vitro* changes depends on a variety of factors, including carbon sources, plant growth regulators (PGRs), baseline culture media formulation, explant size and type, and genotype (Moshtaghi *et al*., 2020). The saffron

was first successfully cultured *in vitro* by using Murashige and Skoog media supplemented with indole-3-acetic acid (IAA) and/or 2,4-dichlorophenoxyacetic acid (2,4-D) (Ding *et al*.,1981).

2.7.1 Explant disinfection

Micropropagation is a technology that produces a large number of high-quality seedlings in a short amount of time, but its biggest drawback is contamination. Numerous microorganisms, including viruses, viroids, and micro-arthropods like mites, thrips, and their vectors, have been found to be contaminants in plant tissue cultures (Altan *et al*., 2010). Thus, one of the primary requirements that must be fulfilled for the establishment and upkeep of plant *in vitro* cultures is the asepsis of both tissues and other materials.

The most often used disinfectants for saffron are often sodium hypochlorite (NaOCl) at 1 to 5% for 15 to 20 minutes and/or mercury II chloride (HgCl2) at 0.1% for 8 to 15 minutes (Hesami *et al*., 2019). Corm explant contamination can be effectively reduced by using a three-step sterilization technique that targets both bacteria and fungi. The explant is immersed in water with two to three drops of Tween-20 and then rinsed three times with triple distilled water.The explants are then dipped in a mixture of hypochlorite bleach at 50% for 10 minutes, systemic fungicides like carbendizime (0.1%) and mancozeb (0.2%), and then a final dip in 1.6% mercuric chloride. (Salwee *et al*., 2014)

Crocus sativus L. corms were carefully cleaned using tap water and the detergent Extran (0.5%) and surfactant Tween-20, then rinsed with double-distilled water. These were then surface disinfected for one minute using 70% ethanol, followed by six minutes using 0.5% HgCl2 (w/v), and finally five times with sterile double-distilled water. After being sterilized, the corms were sliced with a sharp scalpel and cultured on media (Parray *et al.*, 2012)

2.7.2 Factors affecting disinfection efficiency

Numerous variables can affect the disinfection's effectiveness, such as the stock plant's physiological condition and cultivation conditions, the explant's size, age, and type, the disinfectant's type and concentration, exposure time, and temperature (Teixeira da Silva *et al*., 2015). In conclusion, these variables will impact not only the explant's asepsis but also its capacity for survival and regeneration, all of which are necessary conditions for a successful *in vitro* culture establishment.

2.7.3 Multiple shooting

The most crucial stage in the commercial use of tissue culture technology is multiple shoot development. MS media enriched with BAP (6.5 mg/l) and NAA (0.2 mg/l) is to be prepared in order to induce multiple shooting. After undergoing 16/8 hours of light/dark photoperiodic treatment at 17±2°C, the shoot cultures with 2-3 clumps of enlarged base must be sub-cultured on MS supplemented with BAP (6.5 mg/L) and NAA (0.2 mg/L) (Yasmin *et al*., 2019).

Sub-culturing of shoot clumps on varying concentrations of auxin (NAA) and cytokinin (BAP) in November at 17 ± 2°C under 16/8h light/dark photoperiodic treatment showed direct emergence of multiple shoot primordia from the base of an activated buds in both BAP and NAA. Maximum bud sprouting in September was secured by MS Medium supplemented with 0.5 mg/l naphthalene acetic acid (NAA) and 1.5 mg/l 6-benzyl amino purine (BAP) and direct multiple shoot primordia initiation on 6.5 mg/l BAP in November. Maximum shoot proliferation occurred at 6.5 mg/l BAP + 0.2 mg/l NAA; however, the plant growth regulators were harmful in stopping the growth at higher concentrations (Yasmin *et al*., 2013).

2.7.4 Adventitious shoot induction

The most crucial variables that impact the *in vitro* production of saffron shoots are the type of explant, PGRs, and incubation temperature. Saffron shoots have been successfully regenerated both directly and indirectly from a variety of explants, including ovaries, corms, and apical and lateral buds (Vahedi *et al*., 2014. The study conducted by Homes *et al*. (1987), which employed corms as explants, was one of the first to describe the successful regeneration of saffron shoots directly from the explant.

Later, Plessner *et al.,* (1990) demonstrated direct shoot regeneration from apical buds, using zeatin or kinetin as cytokinin. Bhagyalakshmi (1999) assessed how ovarian explant regeneration was impacted by culture media, incubation conditions, and explant age. The longest average leaf length (4.3 cm) was obtained with a combination of 0.1 mg/L NAA and 0.5 mg/L BAP. Furthermore, it was shown that only a particular developmental stage may be achieved by inducing saffron shoots *in vitro* using ovaries.

2.7.5 Bud sprouting

Seasonal factors influence bud sprouting (Devi *et al*., 2011). Bud sprouting was found to be season-dependent when explants were introduced onto MS media supplemented with various growth regulators for four months (May to September). The maximum sprouting (95%) occurred from minicorms/vertical corm sections with apical and auxillary meristematic region in September to October, while the remaining 85% sprouted from November to December. In January and February, there were the fewest sprouts (10%). The buds that appeared between January and June were able to grow again only in the subsequent growing season, which ranged from September to November.

According to Devi *et al*. (2011) low bud sprouting (14%) was seen in the months of May through August, whereas maximal bud sprouting (90%) was noted in November through December. In the medium containing 26.64 mM of BAP along with all the concentrations of 2,4-D (4.52, 9.05, and 18.10 µM) employed, bud sprouting from corm segments was initiated at 10^{o}C across all the factorial combinations of PGRs used. However, the shoots that sprouted in the medium containing 9.05 mM 2,4-D and 26.64 mM BAP continued to grow.

2.8 *In vitro* cormlet formation

Regenerated corms under natural conditions are rare as saffron is a sterile plant. As a result, the amount and rate of microcorms created during *in vitro* microcorm formation appear promising. According to Gui *et al.* (1998) and Sharma *et al.* (2008), it was discovered that during micropropagation, the *in vitro*-developed shoots had a tendency to expand at the base and generate microcorms. These microcorms are said to

be perfect for micropropagating saffron. After six months of cultivation, micro-corms were also formed in certain studies from flower, leaf segments, and bulbs grown on MS media supplemented with 2 mg/L BAP and 0.5 mg/L NAA (Karaoğlu *et al*., 2007).

The highest number of microcorms was seen in MS medium supplemented with 1.5 mg/L paclobutrazol, 0.5 mg/L NAA, and 2 mg/L BAP, or in ½ MS supplemented with TDZ (20 µM), IAA (10 µM), and 40 g/L sucrose. Thus, a comprehensive protocol for saffron corm generation *in vitro* was provided. It was demonstrated by the authors that the maximum amount of microcorms could be regenerated on ½ MS supplemented with TDZ (20 µM), IAA (10 µM), and sucrose (40 g/L) (Parray *et al*., 2012; Mir *et al*., 2014). Parray *et al*. (2012) reported that maximum germination (90%) of those microcorms could be obtained on MS containing 20 µM BAP and 15 µM NAA.

Direct organogenesis from meristematic corm areas may facilitate *in vitro* microcorm development and regeneration. The highest proportion of corm development (76.7%) was observed in MS supplemented with 1 mg/L IAA. The concentration of sucrose and carbohydrates is the primary determinant of cormogenesis in many cultivated species. Microcorm formation is aided by a higher sucrose concentration (6 to 9%). In the absence of sucrose, there are no microcorms development of any kind (Staikidou *et al*., 2005; Madubanya *et al*., 2006).

There is evidence that certain plant species use growth retardants to aid in the development of underground storage organs. Devi *et al.* (2011) first described the use of growth retardants to induce cormlet formation in saffron. The experiment on cormlet production was carried out from April through July. Cormlet development became visible as swelling at the base of the shoots after two to three weeks in culture. Following 13–16 weeks in culture, cormlets with fully formed tunics were visible, and the shoots eventually dried up. Remarkably, compared to other concentrations, 1.7 mM paclobutrazol generated 86.74 percent more cormlets on average; however there was no significant difference in the mean cormlet weight.

Potato microtuberization was achieved using paclobutrazol and chlorocholine chloride (Simco *et al*., 1993; Sharma *et al*., 2023). In gladiolus, paclobutrazol also

promoted corm initiation and development. According to Sharma *et al*. (2008) corm development was observed at high sucrose concentrations (8%), in conjunction with BAP (13.32 mM).

2.8.1 Germination of *in vitro* raised cormlets

The highest germination rate (90%) was seen on BAP (20 µM) and NAA (15 µM) for the *in vitro* grown cormlets sub-cultured on MS (1/2) strength media. BAP and NAA work well together to promote shoot development from corm buds and organogenesis in saffron. An 80% germination rate was observed on MS medium + BAP (5 µM) + NAA (3 µM) + 0.5 mg/l activated charcoal, which was the optimum medium when using activated charcoal. Additional combinations and doses that showed promise for cormlet germination were MS medium + 2,4-D (20 µM) + Kn (15 µM) (75% germination) and MS medium + TDZ (20 µM) + IAA (10 µM) (75% germination) (Parray *et al*., 2012).

2.9 GENES INVOLVED IN CORMLET FORMATION

2.9.2 *Gibberellin3Oxidase*

Gibberellic acid is a vital plant hormone at every stage of a plants growth and development. Reinecke *et al*., (2013) reports that GA3ox1 encodes a GA 3β-hydroxylase that catalyzes the conversion of GA20 into physiologically active GA1. The GA hormone is a member of the diterpenoid, tetracyclic phytohormone family. According to Claeys *et al*. (2012), GAs are positive regulators of germination, stem elongation, leaf expansion, flowering, and reproductive development. The terpenoid pathway in plastin is used for the production of GAs, which are further modified in the cytosol and endoplasmic reticulum before being converted into bioactive forms (Hou *et al*., 2013).

2.9.1 *Phosphoglucomutase*

The easily reversible interconversion of glucose-1-phosphate and glucose-6-phosphate is catalyzed by the widely distributed enzyme phosphoglucomutase (PGM, EC 2.7.5.1). According to Muhlbach *et al*., (1978), plants have two PGM isoforms, one

of which is found in the cytosol and the other in the plastids. The enzymes have been demonstrated to be phosphoproteins *in vivo*, and both isoforms require glucose-1,6-bisphosphate (Glc-1,6-bisP) as a cofactor (Salvucci *et al*., 1990). The cytosolic isoform participates in the metabolism of sucrose, supplying substrate for the synthesis of several cellular components as well as intermediates for glycolysis. According to Hattenbach *et al*., (1999), the plastidic PGM is crucial for starch synthesis because it stores net photosynthate in leaves during the day and is also crucial for the breakdown of assimilatory starch. Significant alterations in carbon partitioning occur when plastidial PGM activity is reduced, most notably a drop in the flux to starch synthesis and a decrease in starch content (Fernie *et al*., 2001).

2.9.2 *Sucrose synthase*

Sucrose, produced through photosynthesis, serves as the final outcome and acts as the main sugar conveyed through the phloem in the majority of plants. The glycosyl transferase enzyme sucrose synthase (SuSy) is essential for the metabolism of sugar, especially in sink tissues. The majority of SuSy proteins are homotetramers, with an average monomeric molecular weight of roughly 800 amino acids, or 90 kD. The reversible cleavage of sucrose into fructose and either uridine diphosphate glucose (UDP-G) or adenosine diphosphate glucose (ADP-G) is catalyzed by SuSy. The products of SuSy's cleavage of sucrose are utilized in numerous metabolic pathways, including the synthesis of complex carbohydrates, primary metabolite synthesis, and energy production (Stein *et al*., 2019).

2.9.3 *Starch synthase*

For green plants to store energy, they produce starch, a glucose polymer that is essential to the growth and reproduction of plants. The two glucose polymers amylopectin and amylose combine to generate insoluble, semi-crystalline starch granules, which are the basic building blocks of starch. Although the structure and biosynthesis of these polymers are different, they are composed of α-1,4-linked glucan chains joined by α-1,6-branch points. Starch is semi-crystalline because amylopectin provides its structural foundation. Amylose has fewer branches and is much smaller. It

is thought to fill up the gaps left by amylopectin in the semi-crystalline matrix, thus making the starch granule denser (Pfister *et al*., 2016).

Starch polysaccharide biosynthesis is carried out by members of the large starch synthase (SS) protein superfamily (He *et al*., 2022). The elongation of glucosyl chains during starch biosynthesis is mostly caused by the formation of alpha-1,4 glycosidic connections, which is primarily catalyzed by starch synthase (Yu *et al.*, 2022).

2.10 ROLE OF PHYTOHORMONES AND CARBOHYDRATES IN CORM DEVELOPMENT

Starch, the primary carbohydrate found in storage organs like tubers and seeds, plays a vital role in their development. It consists of amylopectin, amylose, and two glucose polymers, forming an insoluble glucan. Sucrose, sourced from photosynthetic organs like leaves, is transferred to non-photosynthetic organs (known as sink organs) where it undergoes conversion into starch for long-term storage in amyloplasts. Once in sink organs, sucrose is transported into sink cells either through transporters or plasmodesmata. Within the cytosol, sucrose undergoes two main pathways for breakdown. Cytosolic invertase (CIN) hydrolyzes sucrose into fructose and glucose, whereas sucrose synthase (SUS) cleaves it into fructose and uridine diphosphate glucose (UDP-G). In the latter pathway, UDP-G can be converted to adenosine diphosphate glucose (ADP-G) by enzymes like UGPase and AGPase20. ADP-G serves as the primary substrate for starch biosynthesis in flowering plants. Starch synthesis involves adding the glucosyl moiety to existing glucan chains by starch synthases. The process of starch synthesis is regulated by phytohormones. In maize and potato, gibberellic acid (GA) inhibits starch accumulation, whereas its inhibitor, chlorocholine chloride, significantly boosts starch content (Yu *et al*., 2021).

One of the most well-liked bulb plants in the world is the gladiolus, which belongs to Iridaceae family. Starch buildup is a characteristic of its corm growth. Li *et al*. (2021) revealed a relationship between corm development and both carbohydrates (sucrose and starch) and hormones (ABA and GA). Treatment with exogenous hormones and silencing genes involved in endogenous hormone biosynthesis revealed

that ABA controls corm development positively while GA inhibits ABA action. Antagonism between ABA and GA has been linked to the sucrose synthase gene (GhSUS2). GA downregulated GhSUS2 while ABA increased it. The development of corms and cormlets was correlated with an increase in GhSUS2 transcript levels. GhSUS2 silencing inhibited the growth of corms and the accumulation of starch.

Starch and sucrose metabolism play a pivotal role in the formation and growth of corms in *Freesia hybrida*, a member of the *Iridaceae* family. Throughout the development of new corms, there is a notable increase in starch content, making it the predominant carbohydrate form. The levels of starch, sucrose, and soluble sugars generally rise as corm development progresses. This metabolism is closely linked to several key enzymes, including sucrose synthase, sucrose phosphate synthases (SPS), invertase, starch synthase, adenosine diphosphoglucose pyrophosphorylase (APGase), and starch branching enzymes (SBE). By comparing gene expression profiles with enzyme activities and carbohydrate content, it is suggested that AGPase, INV, and SBE play crucial roles in starch accumulation during the formation and growth of corms in *Freesia hybrida*. Additionally, the cleavage of sucrose also contributes to the substrate pool for starch production (Ma *et al*., 2020)

In saffron, the activity patterns of sucrolytic enzymes in the daughter corm suggest that Susy is the primary route for sucrose breakdown, especially when there's a high carbon flux required for growth and storage in the corm. Boosting Susy's activity could serve as an effective strategy for enhancing starch accumulation and yield in potato tubers (Baroja-Fernández *et al*., 2009)

In various ornamental species propagated through corms, such as gladiolus, the Adenosine diphosphate glucose pyrophosphorylase (AGPP) gene has been successfully cloned and characterized at the molecular level. There is a suggestion that augmenting the expression of the GhAGPS1 gene through genetic engineering techniques could potentially enhance both the quantity and quality of gladiolus corms. Additionally, it's

noteworthy that gibberellic acids play a role in regulating AGPPase expression in bulbous plants (Xu *et al*., 2021)

Materials and methods

3. MATERIALS AND METHODS

The present study, "Plant growth regulators for enhancing *in vitro* cormlet production in saffron (*Crocus sativus* L.)" was conducted during the year 2023-2024 at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani. The study aimed to assess the effect of treatment using plant growth regulators (PGRs) in inducing *in vitro* cormlets in *Crocus sativus* and the differential expression of key genes involved in the process.

3.1 COLLECTION OF EXPLANT AND *IN VITRO* TREATMENT USING PLANT GROWTH REGULATORS

3.1.1 Collection of explants

Explants namely corms of *Crocus sativus* were collected from the farmers field in Pampore district of Kashmir during July 2023. Corms were stored in plastic trays and kept on culture racks at a temperature of 24±2°C, 16hrs day/8hrs night (photoperiod) and 60% (humidity) to induce sprouting.

3.1.2 Sterilization of glassware

The tools and glasswares used viz., forceps, scalpel, bottles, petri plates, glass rod, and beakers were thoroughly cleaned and dried in hot air oven at 160°C for 1 hr. Further, the dried glasswares were packed in polypropylene bags and autoclaved at 121°C and 15 Psi for 40 minutes.

3.1.3 Sterilization of explants

Corms were surface sterilized by modifying the protocol of Salwee and Nehvi (2014). Healthy corms were selected for establishing sterile cultures. The protective tunics of the corms (explant) were removed and gently scrubbed under running tap water, followed by rinsing in Tween 20 solution for 15 min. The corms were then thoroughly washed thrice using distilled water and subjected to a rigorous three-step surface sterilization process using 0.02% bavistin for 20min, 0.04% mancozeb for 20min and 1% sodium hypochlorite bleach solutions for 30min with intermittent

shaking. The corms were then dipped in 1.5% mercuric chloride for 2-3 seconds under aseptic conditions inside a laminar airflow chamber which was priorly UV sterilized for 20 minutes.

3.1.4 Culture medium

Modified full-strength MS medium (Murashige and Skoog, 1962) was prepared for the study. Analytical grade chemicals purchased from Hi-Media were used. Stock solutions of macro nutrients, micro nutrients and organic supplements were prepared (as per Appendix IV) by adding the required quantity of chemicals to double distilled water. Stock D which contains Na_2EDTA and FeSO4.7H2O, was kept in amber coloured bottle to prevent iron from reacting photochemically. Stock solutions of plant growth regulators were prepared as per Appendix III. The stock solutions were stored at 4°C in refrigerator.

The stock solutions were pipette out in required volume into a 1L beaker. Other chemicals like sucrose, myoinositol, MS supplement (Himedia) of required quantity were dissolved along with heat stable hormones of required concentration and the volume of the solution was made up to 950 ml using double distilled water. The pH was checked using an electronic pH meter and adjusted to 5.7-5.8 using 1N NaOH and 1N HCl. The medium was then supplemented with 6.5 g L^{-1} agar and the final volume was made up to 1000 ml using double distilled water. Further, the medium was boiled in microwave oven until the agar was melted and a clear solution was obtained. The molten medium was poured into pre-sterilized test tubes, closed with non-absorbent cotton, labelled and autoclaved at 121ºC, 15 psi for 20 minutes. The autoclaved medium was stored in culture room for three days to check media contamination.

3.1.5 *In vitro* induction of multiple shooting

Multiple shoot induction was attempted using MS medium supplemented with 2ppm BA during August 2023. Surface sterilized full corms were used for establishing sterile cultures and were maintained at temperature (24ºC), 16hrs day/8hrs night (photoperiod) and 60% (humidity) for inducing multiple shoots. The cultures were checked regularly and contamination was recorded. Established cultures were subjected

for periodic subculturing at 21-day interval using the same media composition and continued upto December 2023 to induce multiple shoots.

3.1.6 *In vivo* induction of sprouting

Corms at dormant stage were maintained *in vivo* under controlled condition of temperature (24ºC), 16/8 hrs photoperiod with 60% humidity during July – September 2023 for inducing sprouting. Observation on number of corms sprouted, number of sprouts per corms, number of days taken for sprouting were recorded.

3.1.7 *In vitro* elongation of sprouts

180-day-old corms sprouted in-vivo were surface sterilized, transferred to full-strength MS medium under aseptic condition and incubated at temperature (16ºC), 16hrs day/8hrs night (photoperiod) and 90% humidity. Observation on number of multiple shoots per explant were recorded after 15 days.

3.1.8 *In vitro* treatment using plant growth regulators

Stock solutions of all PGRs used in the treatment were prepared using autoclaved water except for PBZ which was dissolved using 80% ethanol (Appendix II). Filter sterilised stock solutions of antigibberillins of required volume was added to sterile medium under aseptic conditions and dispensed into culture tubes. Full corms with multiple shoots developed under *in vitro* condition (3.1.7) were transferred to half-strength MS medium supplemented with 20 µM thidiazuron (TDZ), 10 µM indole acetic acid (IAA), 4% sucrose, and 0.25% of various anti-gibberellins viz., chlorocholine chloride (CCC), paclobutrazol (PBZ), chlormequat chloride (CMC) and mepiquat chloride (MPQ) along with control. Cultures maintained in full strength MS medium was used as absolute control. Completely randomized design was followed with three replications each. Subculturing was done at 21 days interval using same media composition. Due to crystallisation noticed while adding PBZ to sterile culture medium, required volume of PBS was pipetted out and added to the solidified media in culture tubes.

Table 1. *In vitro* treatment of multiple shoots using various PGRs

Treatments	Media composition
T1	½ MS+20µM TDZ+10 µM IAA + 0.25% CCC
T2	½ MS+20µM TDZ+10 µM IAA + 0.25% PBZ
T3	½ MS+20µM TDZ+10 µM IAA + 0.25% CMC
T4	½ MS+20µM TDZ+10 µM IAA + 0.25% MPQ
Control	½ MS+20µM TDZ+10 µM IAA (Parray *et al*., 2012)
Absolute control	Basal MS medium

Observations on number of cormlet initiated and number of days taken for cormlet initiation were recorded after 10, 25 and 50 days of treatment

3.2 GERMINATION AND SIZE ENHANCEMENT OF *IN VITRO* RAISED CORMLET

Cormlets developed were transferred to the full-strength MS medium supplemented with 20 µM TDZ, 10 µM IAA and 9% sucrose for germination and size enhancement of cormlets. Cultures were maintained at temperature (16ºC), 16hrs day/8hrs night (photoperiod) and 60% (humidity). Completely Randomized Design was followed. Three replications were maintained.

3.3 MOLECULAR ANALYSIS

3.3.1 Isolation of RNA using CTAB method

RNA was isolated from the fresh tissues of mother corm of the best two treatments, control and absolute control 21 days after treatment (3 weeks) using modified CTAB method (Chang *et al*., 1993). All experimental materials used for RNA isolation *viz.,* mortar and pestle, microtips, microfuge tubes, forceps, spatulas were treated with DEPC (Diethyl pyro carbonate) overnight and sterilized by double

autoclaving. For the preparation of reagents, DEPC treated water was used. Degradation of RNA by RNases was prevented by the usage of a ribonuclease inhibitor (RNase out) in the work benches and gloves while working.

3.3.1.1 Procedure

Pre chilled mortar and pestle were used for grinding the samples into a fine powder using liquid nitrogen. 200µl beta mercaptoethanol was added to 10 ml CTAB and kept in water bath at 65ºC. 1ml CTAB buffer was added to the powdered sample in the mortar and gently mixed to homogenize the mixture and incubated at room temperature for 15 min after vortexing. Equal volume of chloroform: isoamyl alcohol (24: 1) was added to the homogenate and vortexed for 15 seconds. The homogenate was centrifuged at 12000g for 15 min at 4ºC. Upper aqueous layer was collected to a new microcentrifuge tube, to which 1/3 volume 8M LiCl was added and the mixture was incubated at 4ºC overnight for precipitation of RNA. Incubated RNA samples were collected and centrifuged at 12000g for 20 min at 4ºC. Dissolve the pellet in 300 µl nuclease free water, 30 µl sodium acetate, 750 µl 100% ethanol and incubate at -80ºC for 30 min. After incubation, centrifugation was done at 15000g for 10 min at 4°C. The pellet obtained was washed with 800 µl of 70% ethanol by centrifugation for 1min at 15000g. The RNA pellet was dried in an RNase-free laminar airflow chamber, resuspended in 30 µL of molecular grade water and stored at -80°C.

3.3.2 Quality and quantity determination of RNA

The integrity and quality of isolated RNA were checked using 1% agarose gel electrophoresis carried out at 75 V using 1 X TAE tank buffer and the gel was viewed in a gel documentation system (BioRad Inc., USA) using the software “Image Lab”. The concentration and purity (A260/A280) of isolated RNA were determined using Nanodrop (Thermo Scientific Nanodrop Lite Plus).

$$\text{Quantity of RNA (ng/µL)} = A260 \times 40 \times \text{Dilution factor}$$

$$\text{Purity of RNA} = A260 / A280 \text{ (ratio should be 2 for pure RNA)}$$

3.3.3 cDNA synthesis

The RNA isolated from best two treatment (3.3.1), control and absolute control were subjected to cDNA conversion using reverse transcription protocol (Thermo Scientific Verso cDNA Synthesis Kit #AB-1453/A). First strand cDNA synthesis was performed by the instructions provided in the kit manual. 20 µl reaction mixture was prepared and incubated at 42°C for 30 min. The inactivation of the reverse transcriptase enzyme was performed by incubating the tubes at 95°C for 2min. RNase free tips, tubes, molecular grade water, sterile gloves etc were used for minimizing the intervention of RNases during the process.

Table 2: Reaction mixture composition for cDNA synthesis

Reagents	**Final concentration**	**Volume (µL)**
5X cDNA synthesis buffer	1X	4
dNTP mix	500 µM each	2
Anchored oligo dT	500 ng	1
RT enhancer		1
Verso enzyme mix		1
Template (RNA)	1.5 ng	1-5
Water, nuclease-free		To 20 µL
Total	20 µL	

The reaction conditions for cDNA synthesis are as follows:

Table 3: Reaction conditions for cDNA synthesis

Reaction	**Temperature (°C)**	**Time (min)**	**Cycles**
cDNA synthesis	42	30	1
Inactivation	95	2	1

3.3.4 Primer designing

Nucleotide sequences of the genes involved in synthesis of gibberellin and starch *viz.,* gibberellin3oxiadase (*GA3OX*), starch synthase (*SS*), sucrose synthase (*SUSY*), phosphoglucomutase (*PGM*) from closely related species of *Crocus sativus* were retrieved from NCBI database in FASTA format. Conserved domains of the retrieved genes were identified using Conserverd Domain Search tool (CD search) and subjected to multiple sequence alignment using MEGA 11 (Molecular Evolutionary Genetics Analysis) The tool BioEdit was used for generating consensus sequence which were further used for designing the primers using Primer 3 plus. The quality parameters of primers such as GC content, Tm, secondary structure, self-dimer, cross dimer, hairpin structures were assessed using Thermofisher Scientific – Multiple Primer Analyzer.

3.3.4.1 *Standardization of annealing temperature for primers*

Gradient PCR was carried out using forward and reverse primers of genes viz, *β Actin*, *GA3OX, SS, SUSY* and *PGM* genes at a temperature range between 53.0°C to 61°C using CFX96 Real-Time system (BIO-RAD). Melt curve analysis for each gene was carried out to determine the optimum annealing temperature.

3.3.4.2 *Confirmation of cDNA*

In order to confirm the synthesis of cDNA, PCR amplification was performed using a PCR master mix provided by G Biosciences. The reaction mixture for cDNA confirmation is given below in Table 4.

Table 4. Reaction mixture for cDNA confirmation.

Reagent	Volume (µL)
1X Taq polymerase master mix	5
Forward primer	1
Reverse primer	1
cDNA	1
RNase-Free ddH2O	2
Total volume	10

3.3.4 Differential expression analysis using RT-qPCR

The expression profile of key genes involved in the biosynthesis pathway of gibberellin and starch were studied using reverse transcription - quantitative PCR (RT-qPCR). *β Actin* gene, which is reported as a stable housekeeping gene in *Crocus sativa* was used as the reference gene for the present study (Sohrabi *et al*., 2020). The cDNA synthesised using the samples of best two treatments, control and absolute control (3.3.3) were amplified using SYBR Green qPCR 2X master mix (G-Bioscience) following the reaction cycle (Table 5 and 6) and the threshold cycle (Ct) values were obtained. Reactions were carried out in CFX96 Real-Time PCR system (Bio-Rad).

Table 5. Reaction mix used for RT- qPCR.

Component	Volume (µl)	Final concentration
2X SYBR Green qPCR mix	5	1X
Forward primer	0.5	0.2µM
Reverse primer	0.5	0.2µM
Template (cDNA)	1	1.5 ng/µl
Water, nuclease-free	To 10 µl	
Total	10 µl	

Table 6. Thermal profile for RT- qPCR.

Stage	**Temperature**	**Time**	**Cycles**
Initial denaturation	95	5 min	1
Denaturation	95	45 s	34X
Annealing	Specific to genes	30 s	
Extension	72	45 s	
Final extension	72	1 min	1

After the completion of the RT-qPCR reactions, the threshold cycle (Ct) was recorded and the gene expression level was calculated using the comparative Ct method or delta-delta Ct method. The relative gene expression level of control and treatments were represented as the $2^{-\Delta\Delta Ct}$

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (reference gene)}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct \text{ (control)}$$

Results

4. RESULTS

The study entitled “Plant growth regulators for enhancing *in vitro* cormlet production in saffron (*Crocus sativus* L.)” was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2023-2024. In this study, an attempt was made to enhance the *in vitro* cormlet production in saffron. The first part of the study analyses the effect of various plant growth regulators like TDZ, IAA, CCC, PBZ, CMC, MPQ on early initiation of cormlet formation. The second part of the study analyse the differential expression of key genes involved in the biosynthesis pathway of gibberellin and starch on *in vitro* cormlet formation. The results of the study are presented in this chapter.

4.1 COLLECTION OF EXPLANT AND *IN VITRO* TREATMENT USING PLANT GROWTH REGULATORS

4.1.1 Collection and maintenance of explants

The experimental material for the study *i.e.*, corms of *Crocus sativus* L. were collected from the farmer’s field in Pampore district, Kashmir during July 2023 (Plate 1) and maintained at 24ºC temperature, 16/8hrs photoperiod and 60% humidity.

4.1.2 *In vitro* induction of multiple shooting

Sterile cultures of corms established in MS medium supplemented with 2ppm BA during August 2023 showed sprouting, however did not grow further even after 8 sub culture (Plate 2). No contamination was observed.

4.1.3 *In vivo* induction of sprouting

Another strategy attempted for multiple shoot induction was to induce sprouting *in vivo* and further *in vitro* elongation of sprouts. For this, corms at the dormant stage were kept *in vivo* under controlled conditions of 24ºC temperature, 16/8hrs photoperiod and 60% humidity. The first sprouts emerged on 57th day (Plate 3b) and 100% sprouting was noticed within 65 days with 4 to 8 sprouts per corm.

Further, 180-day old sprouts (Plate 4a) was surface sterilized and transferred to basal MS medium (Plate 4b) for elongation developed healthy multiple shoots in 15 days (Plate 4c). Average number of multiple shoots formed was 5. Number of multiple shoots per explant is shown in Table 8. Healthy and profuse roots were observed in all cultures within 2 days of inoculation (Plate 4d).

Table 7. *In vivo* sprouting of corms

Number of corms kept for sprouting	**Number of corms sprouted**	**Number of sprouts per corm**	**Number of days taken for sprouting**
50	50	6±2	65±10

Table 8. Number of multiple shoots per explant

Explants	**No: of multiple shoots per plant**
P1	11
P2	7
P3	6
P4, P5	5
P6, P7, P8, P9	5
P10, P11, P12, P13, P14, P15	4

Table 9. *In vitro* roots in MS medium

Number of sprouted corms kept for multiple shoots	**Number of corms rooted**	**Number of days taken for rooting**
15	15	3±2

4.1.4 ***In vitro*** **treatment using plant growth regulators**

Full corms with multiple shoots were transferred to half strength MS medium supplemented with 20 µM thidiazuron (TDZ), 10 µM indole acetic acid (IAA), 4% sucrose, and 0.25% of various anti-gibberellins *viz.,* CCC, PBZ, CMC, MPQ. Half strength MS medium supplemented with 20 µM thidiazuron (TDZ), 10 µM indole acetic acid (IAA), 4% sucrose was used as control. Cultures maintained in full strength MS medium was used as absolute control (Plate 5).

Initiation of cormlets was first noticed in treatment using 0.25% MPQ on 10th day of treatment followed by CCC (20 days), control (20 days) and absolute control (30 days) is depicted in Plate 7. Cormlets were initiated in all the 3 replications within 16 days of treatment using MPQ followed by CCC (36 days) and control (38 days) is depicted in Plate 8. Total number of cormlets obtained was highest in treatment using MPQ (7 nos), followed by CCC (5 nos), control (5nos), CMC (4nos) and absolute control (1no). The *in vitro* cormlets generated in treatment using 0.25% MPQ showed prominent size compared to control (Plate 10). In treatment using 0.25% PBZ, no further growth was noticed (Plate 6).

4.2 GERMINATION AND SIZE ENHANCEMENT OF *IN VITRO* RAISED CORMLETS

In vitro cormlets generated in treatment 4 (MPQ), treatment 1 (CCC) and control 55 DAT (Plate 11) were transferred to MS medium supplemented with TDZ (20 µM), IAA (10 µM) and high concentration of sucrose (9%). The cultures were maintained at temperature (16ºC), 16hrs day/8hrs night (photoperiod) and 60% (humidity) and regularly sub cultured at 21-day interval using same media composition.

Table 10. Number /days taken for initiation of cormlets under different treatments

Treatments (T)	No. of replication/ culture tube	No. of cormlets initiated in 15 days			No. of cormlets initiated in 25 days			No. of cormlets initiated in 50 days		
		No. of culture tube	No. of cormlets	No. of days taken	No. of culture tube	No. of cormlets	No. of days taken	No. of culture tube	No. of cormlets	No. of days taken
T1 (CCC)	3	-	-	-	1	2	20	3	5	36
T2 (PBZ)	3	-	-	-	-	-	-	-	-	-
T3 (CMC)	3	-	-	-	-	-	-	2	4	45
T4 (MPQ)	3	2	2	10	3	3	16	3	7	27
Control	3	-	-	-	2	2	20	3	5	38
Absolute control	3	-	-	-	-	-	-	1	1	30

Plate 1. Corms collected from farmers field at Pampore, Kashmir

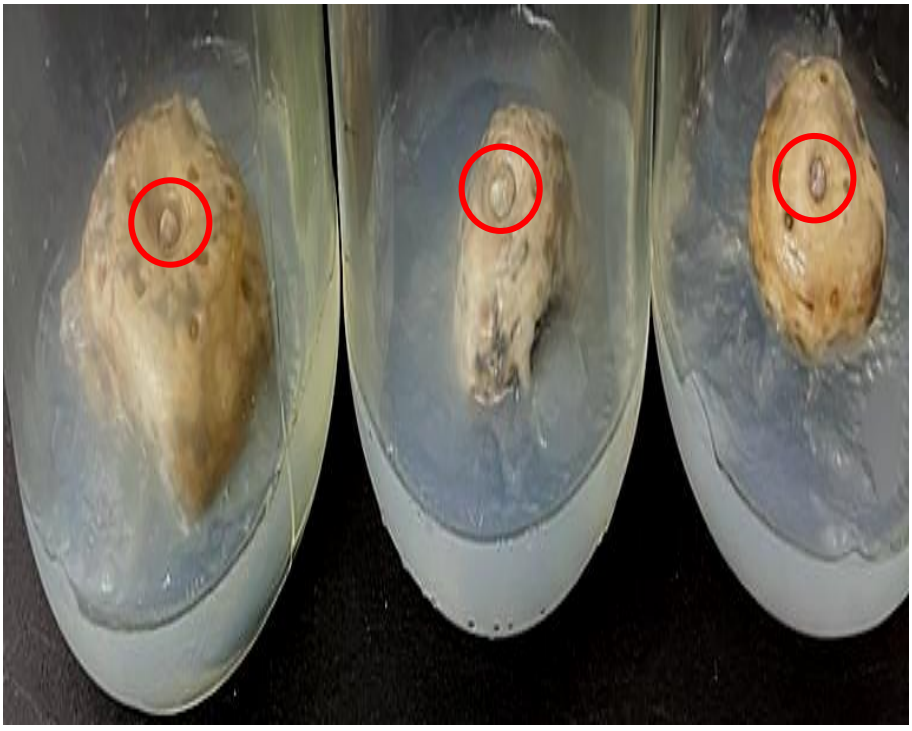


Plate 2. Establishment of sterile cultures of saffron after 8th subculture (S8)

a)

b)

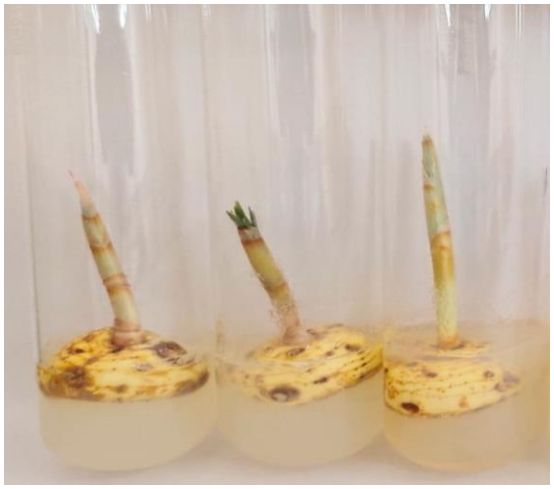
c)

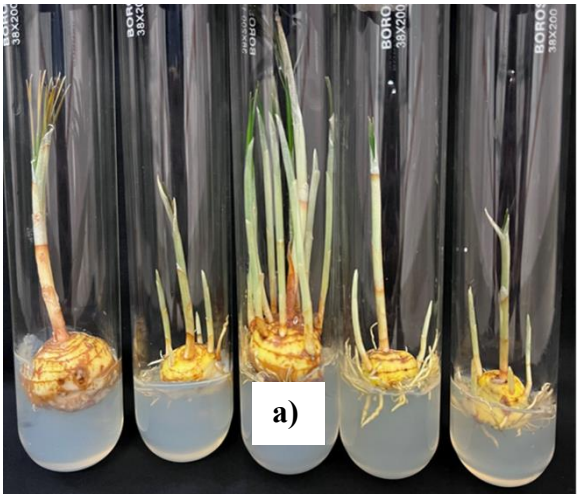
Plate 3. Sprouting of corms *in vivo*

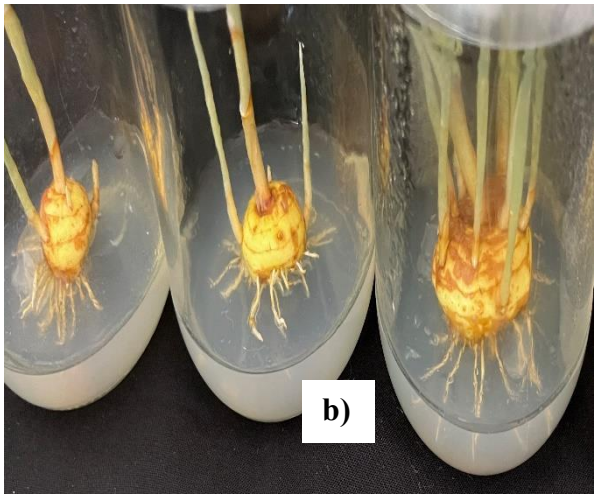
a) Dormant corms

b) *In vivo* sprouts emerged on 57th day

c) 150-day old sprouts induced *in vivo*







c) **d)**

Plate 4. *In vitro* elongation of sprouts

a) 180-day old sprouts induced *in vivo*

b) *In vitro* establishment of 180-day old sprouts for elongation

c) *In vitro* elongation of sprouts 15 days after inoculation

d) Sprouts with *in vitro* roots

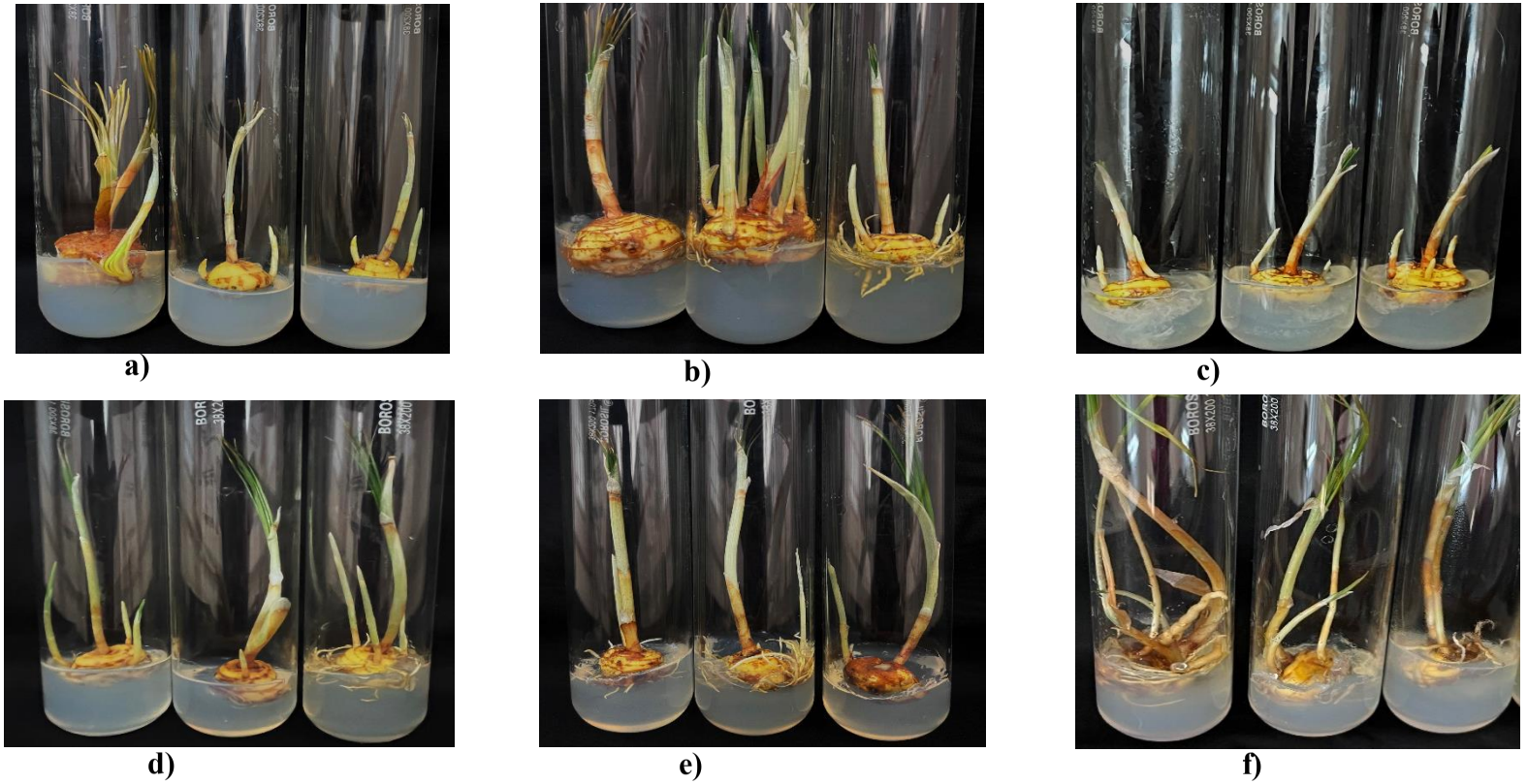
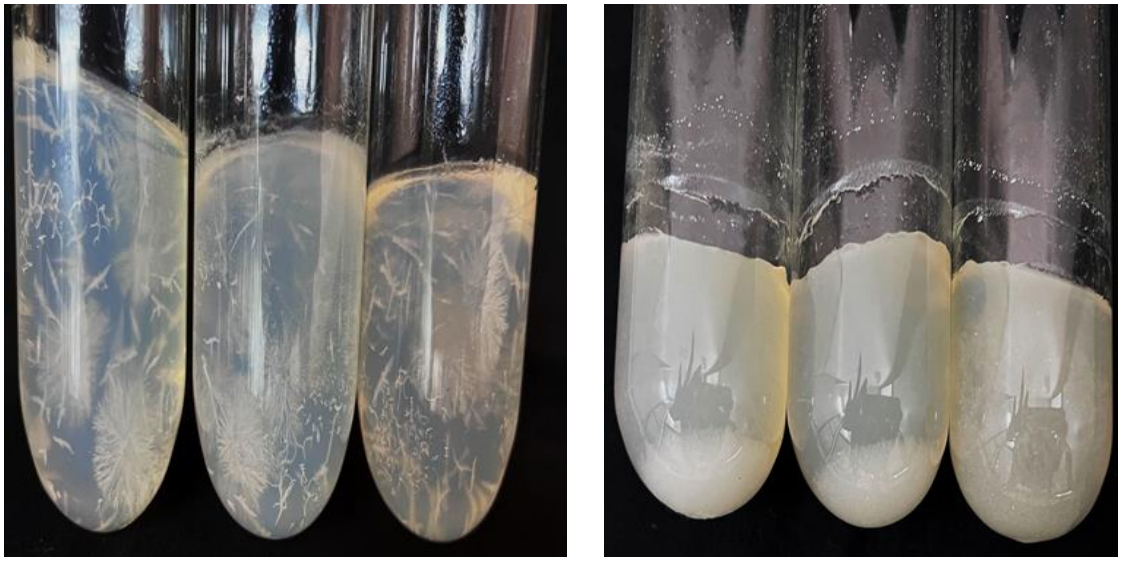
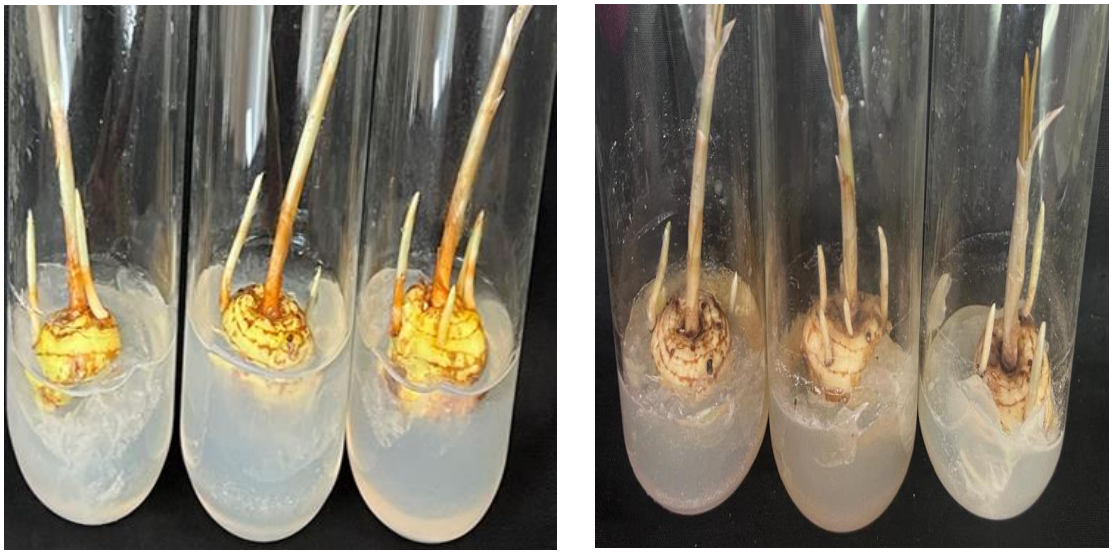


Plate 5. *In vitro* treatment using PGRs

a) Control: ½ MS medium + TDZ (20 µM) + IAA (10 µM) + sucrose (40g/L)
b) T1: ½ MS medium + TDZ (20 µM) + IAA (10 µM) + sucrose (40g/L) + CCC (0.25%)
c) T2: ½ MS medium + TDZ (20 µM) + IAA (10 µM) + sucrose (40g/L) + PBZ (0.25%)
d) T3: ½ MS medium + TDZ (20 µM) + IAA (10 µM) + sucrose (40g/L) + CMC (0.25%)
e) T4: ½ MS medium + TDZ (20 µM) + IAA (10 µM) + sucrose (40g/L) + MPQ (0.25%)
f) Absolute control (basal MS medium)



a1) **a2)**



b1) **b2)**

Plate 6. *In vitro* treatment using 0.25% PBZ

a1) Crystallisation noticed in T2- Half strength MS medium supplemented with 0.25 % PBZ (DMSO as solvent)

a2) Crystallisation noticed in T2- Half strength MS medium supplemented with 0.25 % PBZ (80% ethanol as solvent)

b1) Treatment 2 on day1

b2) Treatment 2-50 DAT

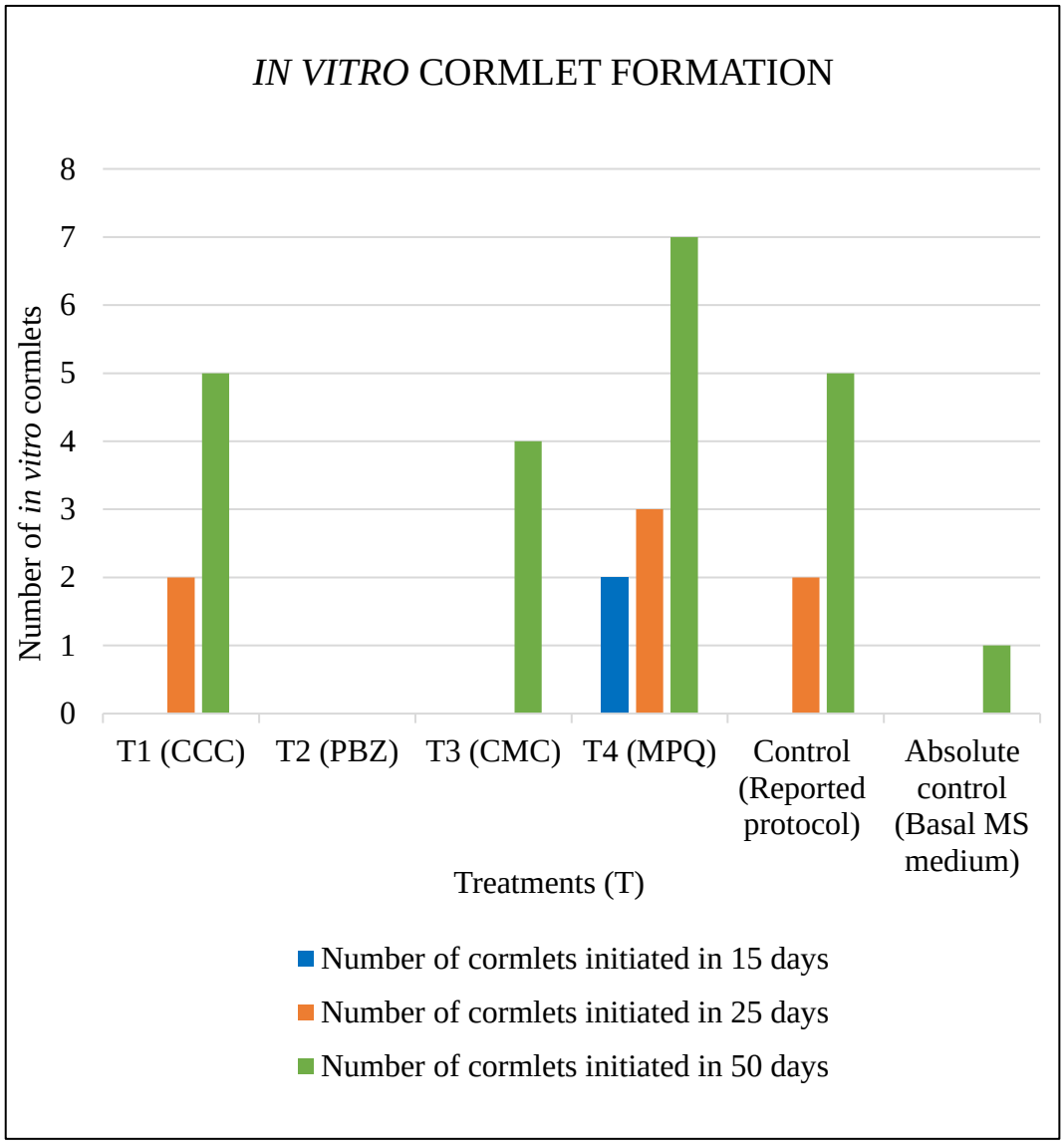


Fig1: *In vitro* cormlet initiation in saffron using different PGRs

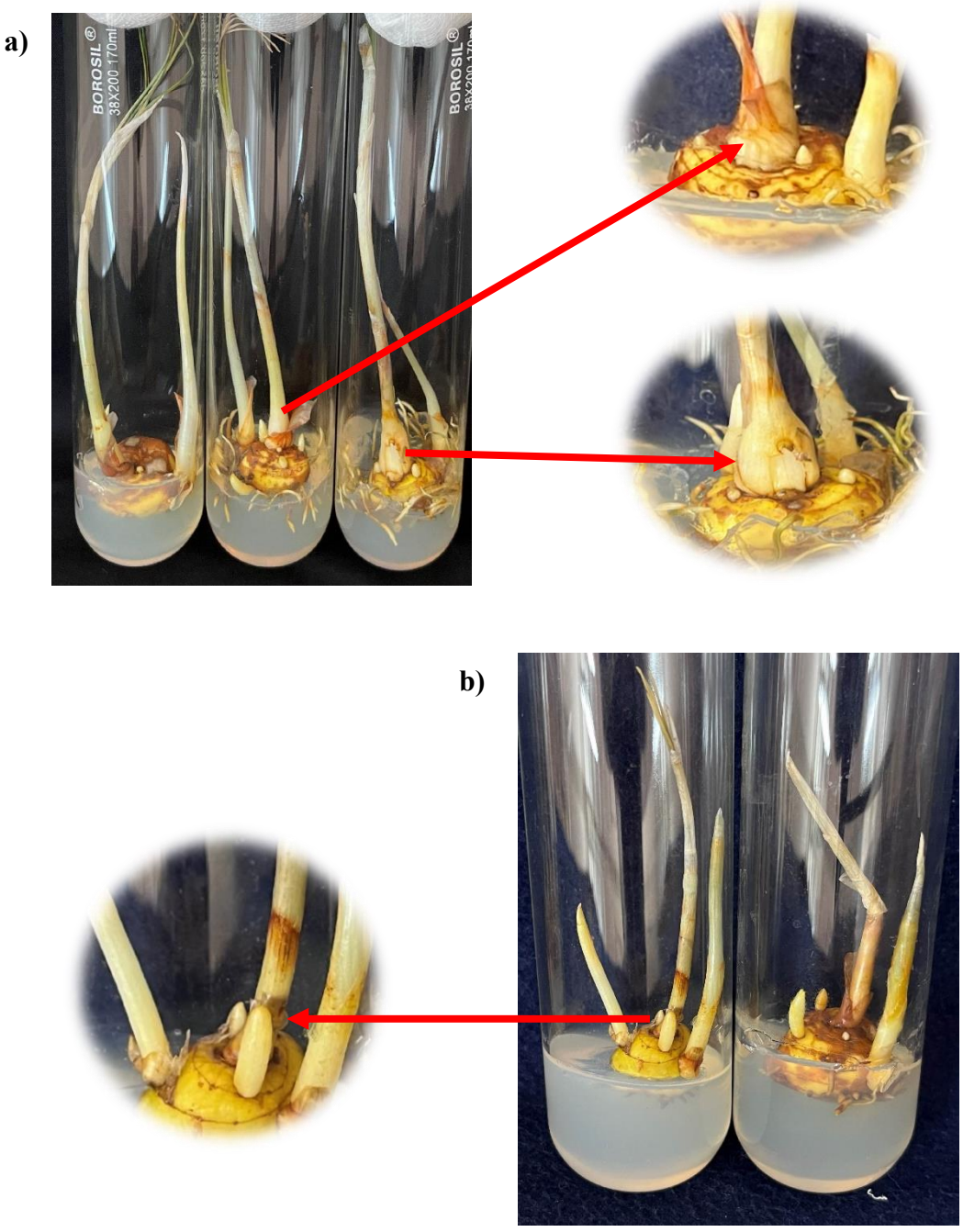


Plate 7. *In vitro* initiation of cormlets

a) *In vitro* cormlet initiation in treatment 4 (MPQ) on 10th day of treatment

b) Control on 10th day of treatment

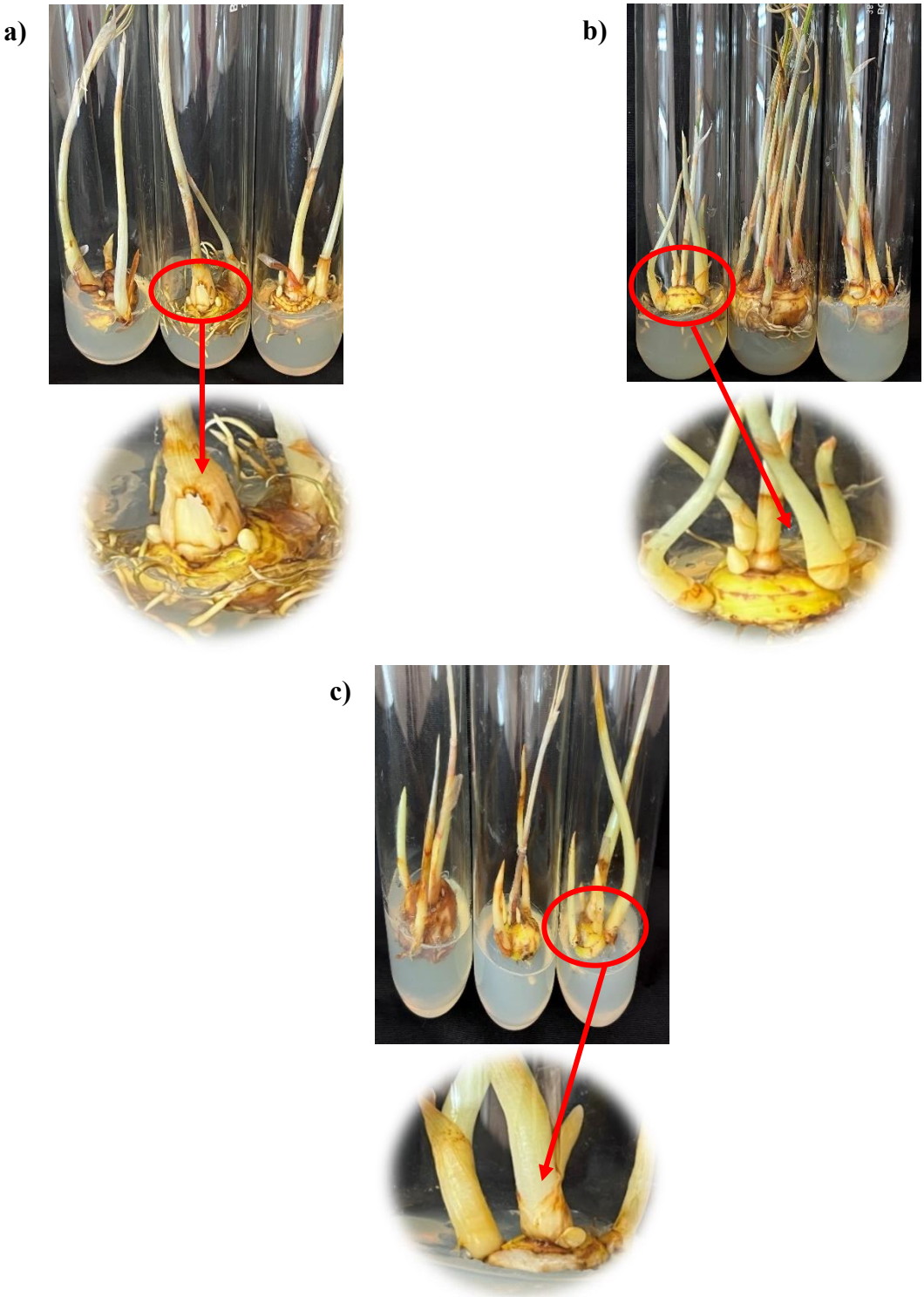


Plate 8. *In vitro* initiation of cormlets in 25 days of treatment (DAT)

a) *In vitro* cormlets initiation in T4 – 16 DAT

b) *In vitro* cormlets initiation in T1 – 20 DAT

c) *In vitro* cormlets initiation in control – 20 DAT

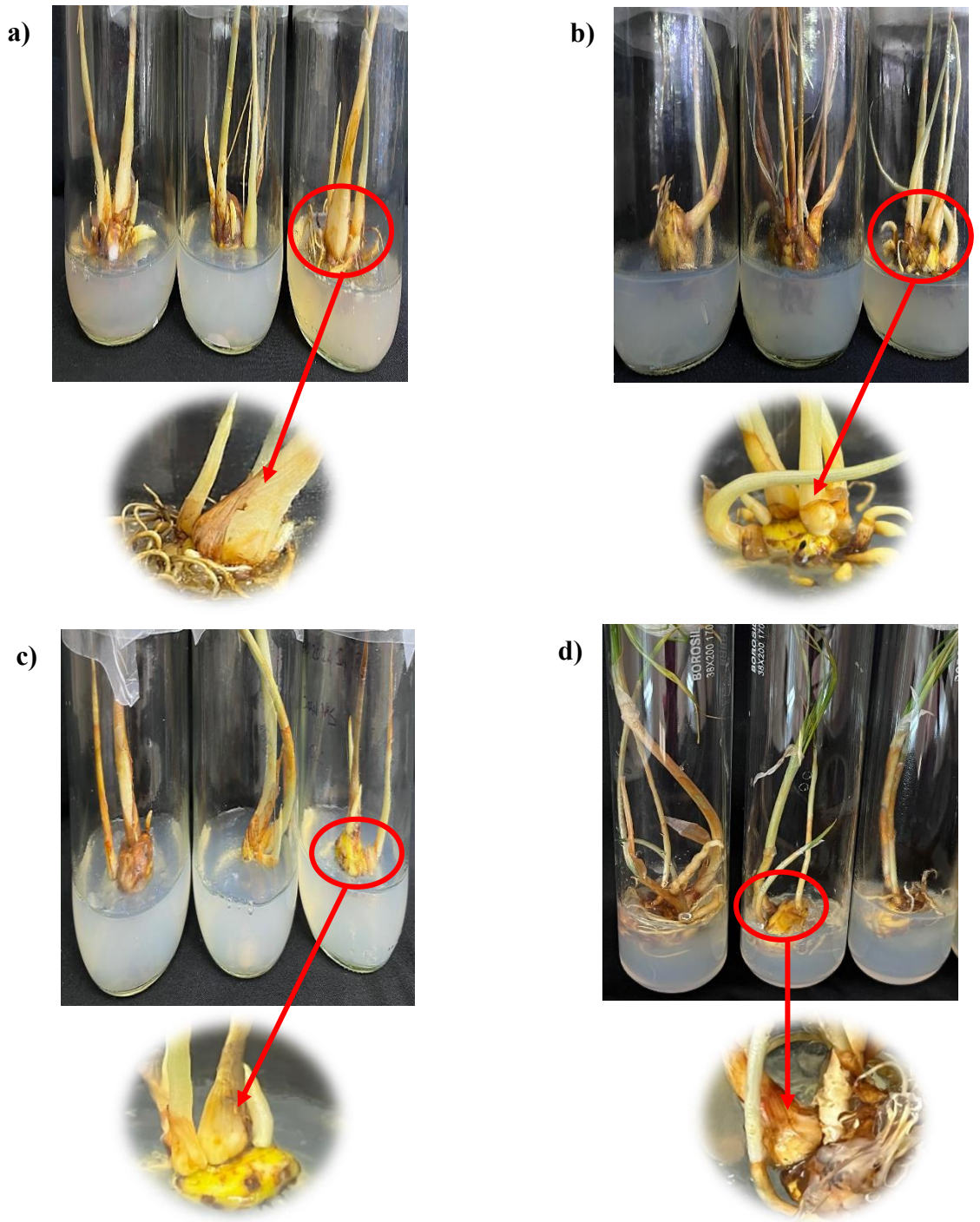
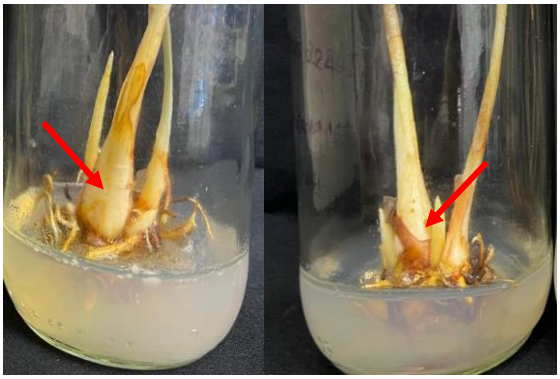
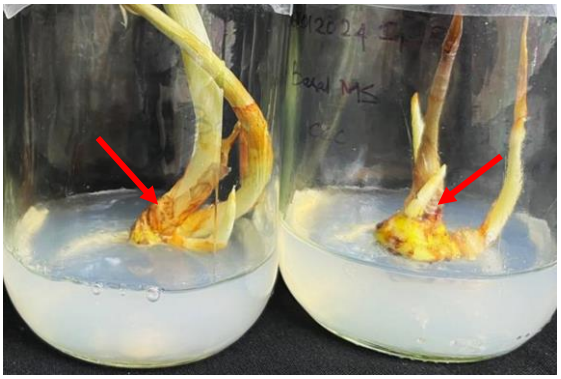


Plate 9. *In vitro* initiation of cormlets within 50 days of treatment

a) *In vitro* cormlets initiation in T4 – 27 DAT
b) *In vitro* cormlets initiation in T1 – 36 DAT
c) *In vitro* cormlets initiation in control – 38 DAT
d) *In vitro* cormlets initiation in absolute control – 30 DAT



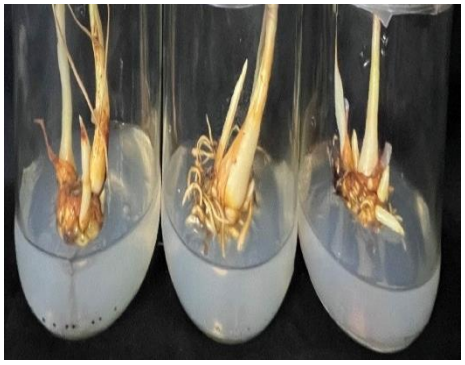
a)



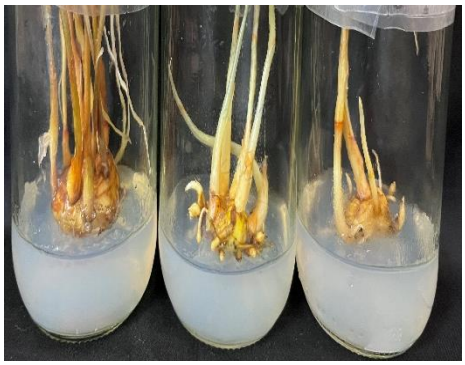
b)

Plate 10. Size of *in vitro* cormlet – 55 DAT

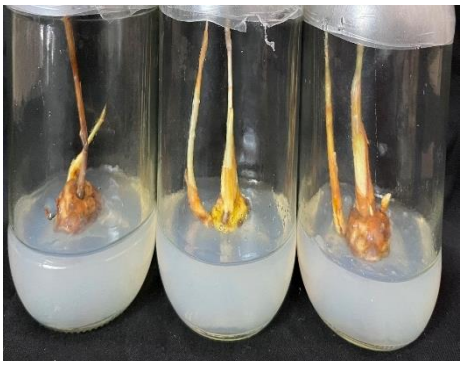
a) Treatment 4 (0.25% MPQ)
b) Control



a)



b)



c)

Plate 11. Germination and size enhancement of *in vitro* raised cormlets

a) Cormlets developed in T4
b) Cormlets developed in T1
c) Cormlets developed in control

4.3 MOLECULAR ANALYSIS

4.3.1 Isolation of RNA

RNA isolated from the corm tissues of best two treatments *ie*, T4 (0.25% MPQ), T1 (0.25% CCC), control and absolute control using CTAB method showed good quality.

4.3.2 Quality and quantity determination of RNA

The concentration and purity of the isolated RNA was checked using NanoDrop (Table 8). The integrity and quality of isolated RNA was checked using agarose gel electrophoresis. The RNA was observed as distinct intact bands at 28 S and 18 S on 1% agarose gel (Plate 12.).

Table 11. Concentration and purity of isolated RNA from best two treatment, control and absolute control

Samples	Concentration (ng/µl)	A260/A280
T1 (0.25% CCC)	815.7	2.06
T2 (0.25% PBZ)	1359.3	2.09
Control	1234.1	2.06
Absolute control	1424.2	2.12

4.3.3 Primer designing

Primers were designed using the gene sequence of closely related crop species of *Crocus sativus* retrieved from NCBI is mentioned as Table 12. Multiple sequence alignment of retrieved sequences using MEGA 11 showed conserved region as depicted in Fig 2-5. The consensus sequence generated from conserved region using BioEdit is shown in Table 13. The sequences of primers for genes viz., *gibberellin3oxidase (GA3OX), phosphoglucomutase (PGM), starch synthase (SS), sucrose synthase (SUSY)* was designed using Primer3plus and the quality parameters analyzed using Thermofisher Scientific – Multiple Primer Analyzer are provided in the Table 14.

Table 12. Accession number of gene sequence in related crops retrieved from NCBI

Crops	Accession number			
	GA3OX	**SS**	**SUSY**	**PGM**
Phoenix dactylifera	-	-	XM_039134347.1	XM_039118690.1
Elaeisguineensis	-	-	XM_010938975.3	XM_029262070.1
Asparagus officinalis	-	-	XM_020414598.1	XM_020406105.1
Dendrobium catenatum	-	-	-	XM_028692266.1
Corylus avellana	XM_059605341.1	XM_059603773.1	-	-
Lyciumferocissimum	XM_059447346.1	-	-	-
Phalaenopsis equestris	-	-	XM_020731693.1	XM_020727170.1
Zea mays	-	NM_001111900.1	NM_001111724.2	XM_008677133.4
Solanum tuberosum	-	Y08786.1	NM_001287982.1	AJ240053.1
Solanum verrucosum	XM_049511295.1	-	-	-
Arabidopsis thaliana	-	NM_129196.4	NM_124296.3	NM_124561.3
Cucumis sativus	NM_001280696.1	-	-	-
Raphanus sativus	XM_018623943.2	XM_018585389.2	-	XM_018602381.2
Oryza sativa	-	MF678475.1	-	AF455812.1
Beta vulgaris	XM_010672441.3	-	AY457173.1	XM_010695137.4
Solanum stenotomum	XM_049539324.1	-	-	-
Solanum dulcamara	XM_055972727.1	-	-	-
Oryza glaberrima	XM_052300720.1	-	-	-
Ipomoea batatas	-	AB194726.1	-	-
Triticum aestivum	-	AF286318.1	-	-
P.sativum	-	X80009.1	-	-

Table 13. Consensus sequence of genes generated using BioEdit tool

Target	Consensus sequence
GA3OX	CGGTGGGTGATGGTGGAGCCGGTAACCGGTGCACT GGTGGTTCAAGTCGGAGACTTGCTACATATTCTTAC AAACGGGTTGTACCCGAGTTCAGCCCATCAGGCCGT CGTGAACCAAAACCGAAAACGTATATCGATAGCGTA CTTTTTTGGACCATCGGAAAGTGCCGAAATTTCACC
SS	ATGGCTGTGATGGAGCATTCCTATTACGGATCATTCG GGTATCATGTTACGAACTTCTTTGCTGTAAGCAGTAG ATCCGGGACCCCAGAGGACTTGAAATATTTAGTTGA TAAGGCCCATAGCTTAGGTCTGCGGGTTTTGATGGAT ATTGTTCACAGCCATGCAAGCAACAATGTGACTGAT GG
SUSY	GAGGAGAAGTACCACTTCTCTTGCCAGTTCACTGCT GATCTAATTGCCATGAACCACACTGACTTTATTATTA CCAGTACATTCCAAGAGATTGCTGGAAACAAGGAC ACCGTTGGACAATATGAGAGTCACATGGCTTTCACG CTCCCCG
PGM	GAGGTTCCCACTGGTTGGAAATTCTTTGGTAATCTTA TGGATGCTGGAAAATTGTCTATCTGTGGAGAAGAAA GCTTTGGAACAGGTTCTGATCACATCCGTGAGAAGG ATGGCATATGGGCTGTGTTGGCTTGGCTTTCGATCAT TGC

Table 14. Details of primers designed using Primer 3 plus

Target gene	Primers		No. of bases	Tm (ºC)	Product size (bp)
GA3oxidase (GA3OX)	F	CACTGGTGGTTCA AGTCGGA	20	59.9	124
	R	GGCACTTTCCGAT GGTCCAA	20	60.6	
Starch synthase (SS)	F	TCCTATTACGGATC ATTCGGGT	22	58.5	128
	R	TCCATCAAAACCC GCAGACC	20	60.6	
Sucrose synthase (SUSY)	F	ACCACTTCTCTTG CCAGTTCA	21	59.5	106
	R	CCAACGGTGTCCT TGTTTCC	20	59.3	
Phosphoglucomutase (PGM)	F	CACTGGTTGGAAA TTCTTTGGT	22	57.3	118
	R	CACAGCCCATATG CCATCCT	20	59.9	
Beta actin (β Actin)	F	CCGGTGTCATGGT TGGTAT	19	57.13	267
	R	GCAGGCACATTGA AGGTCT	19	58.05	

4.3.3.1 *Optimization of annealing temperatures of primers*

Optimisation of annealing temperatures of primers corresponding to the genes under study was carried out using Gradient PCR -CFX96 Real-Time system (BIO-RAD). Melt curve pattern for each primer at different temperatures is shown in Fig 6. The optimal annealing temperature for genes under study is mentioned in Table 15.

Table 15. Standardized annealing temperature of genes

Genes	**Annealing temperature (°C)**
GA3OX	53.6
SS	53.6
SUSY	53.6
PGM	53.6
β Actin	53.6

4.3.3.2 *Confirmation of cDNA synthesis*

The isolated RNA (4.4.1) converted to complementary DNA (cDNA) using reverse transcriptase was amplified by PCR using the primers of genes viz., *GA3OX, SS, SUSY, PGM*, *β Actin* (reference gene) and depicted as Plate 13.

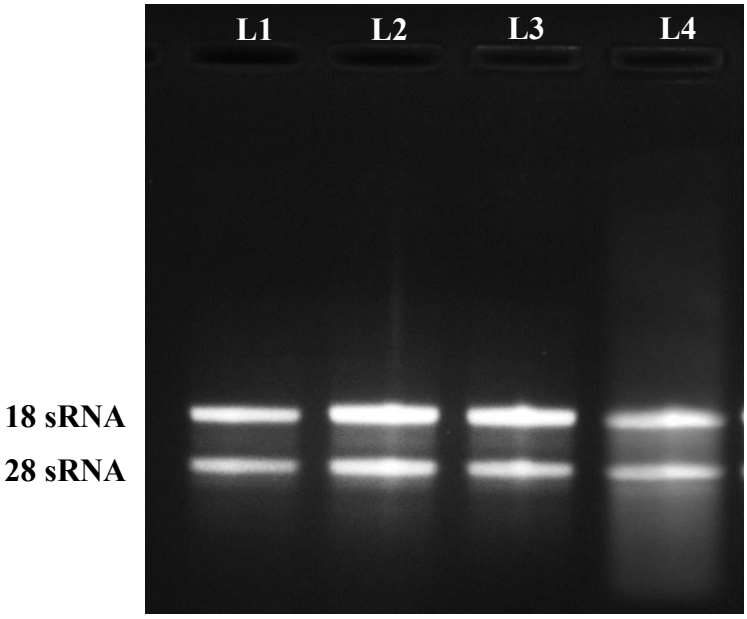


Plate 12. Gel profile of isolated RNA

L1 – Control
L2 – Treatment 2 (0.25% CCC)
L3 – Treatment 4 (0.25% MPQ)
L4 – Absolute control

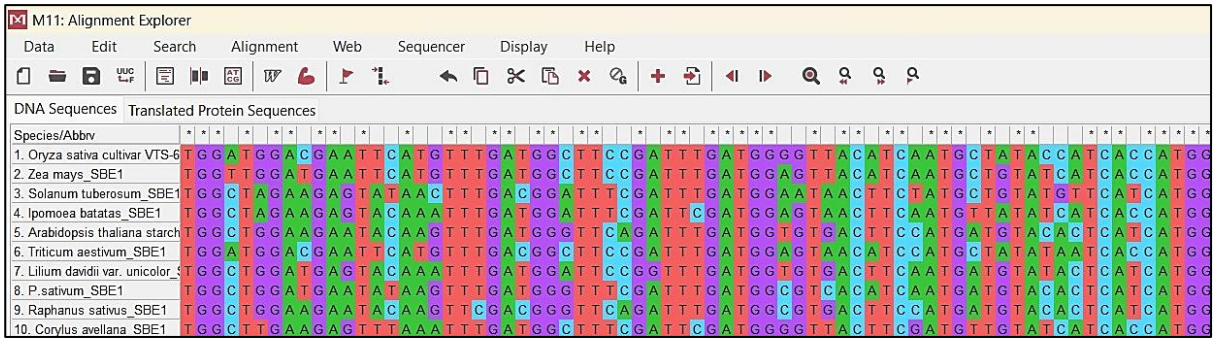


Fig 2. Conserved regions in *starch synthase* (*SS*)

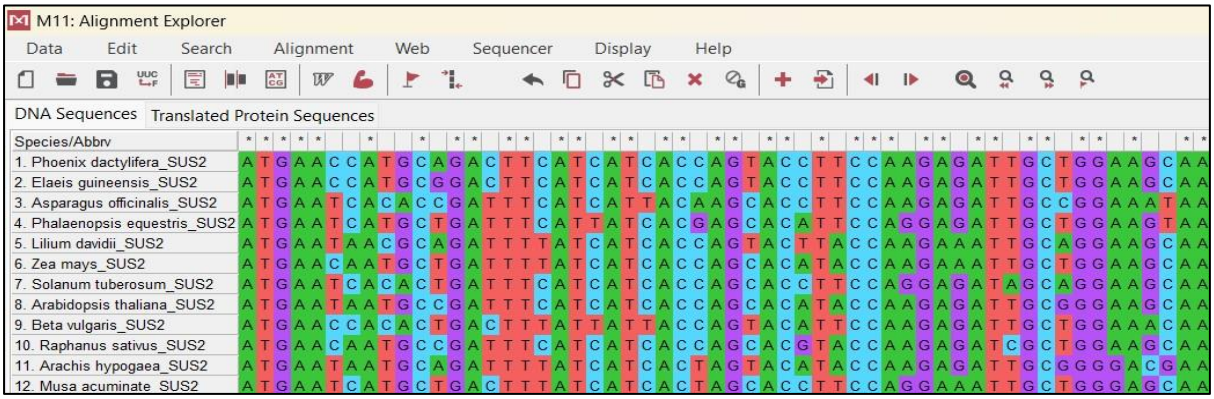


Fig 3. Conserved regions in *sucrose synthase* (*SUSY*)

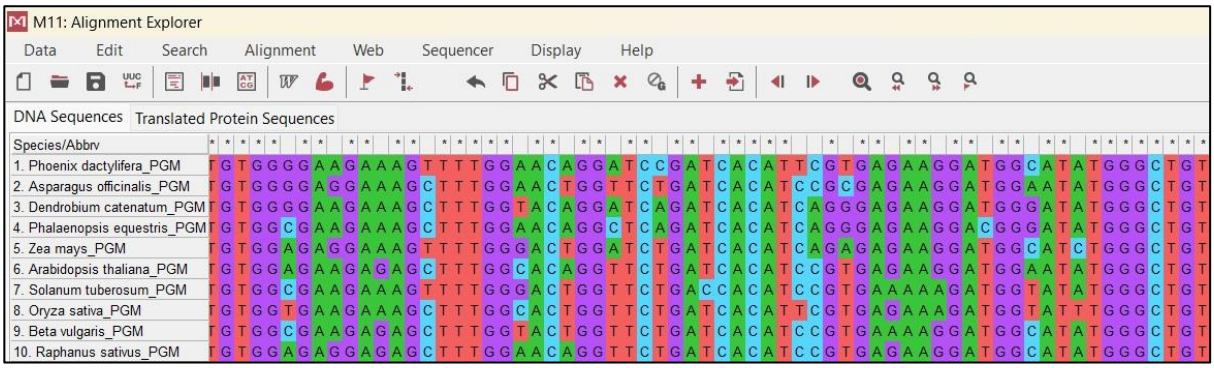


Fig 4. Conserved regions in *phosphoglucomutase* (*PGM*)

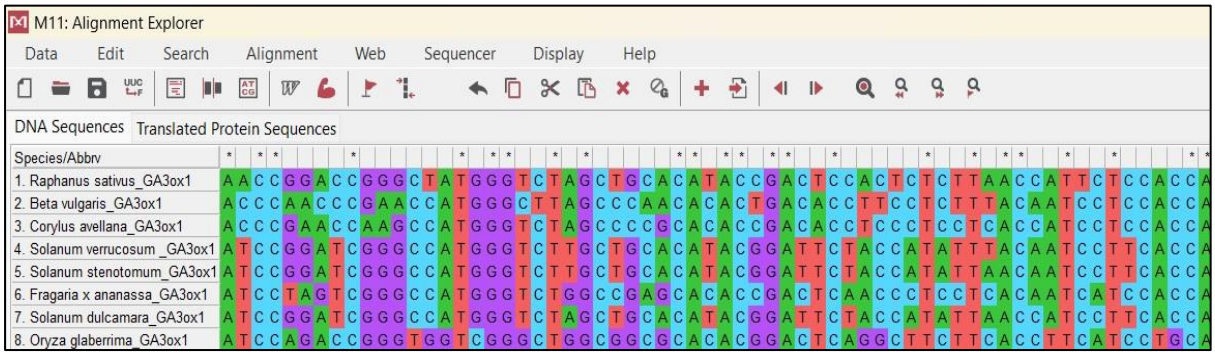


Fig 5. Conserved regions in *gibberellin3oxidase* (*GA3OX*)

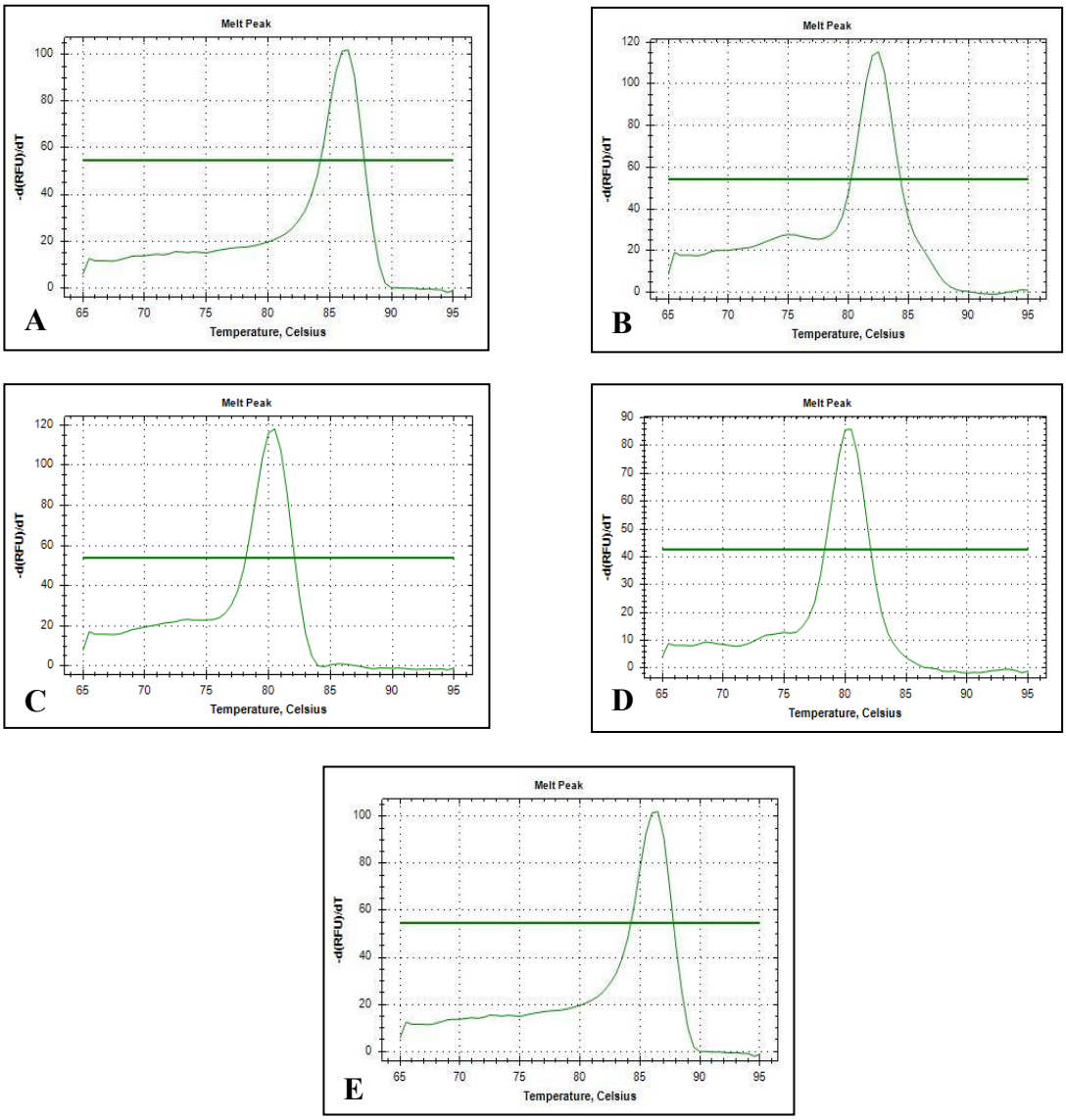


Fig 6: Melt curve of primers in the optimum annealing temperature

A – *β Actin* (53.6°C); B – G*A3OX* (53.6°C)
C – *SS* (53.6°C); D – *SUSY* (53.6°C)
E – *PGM* (53.6°C)

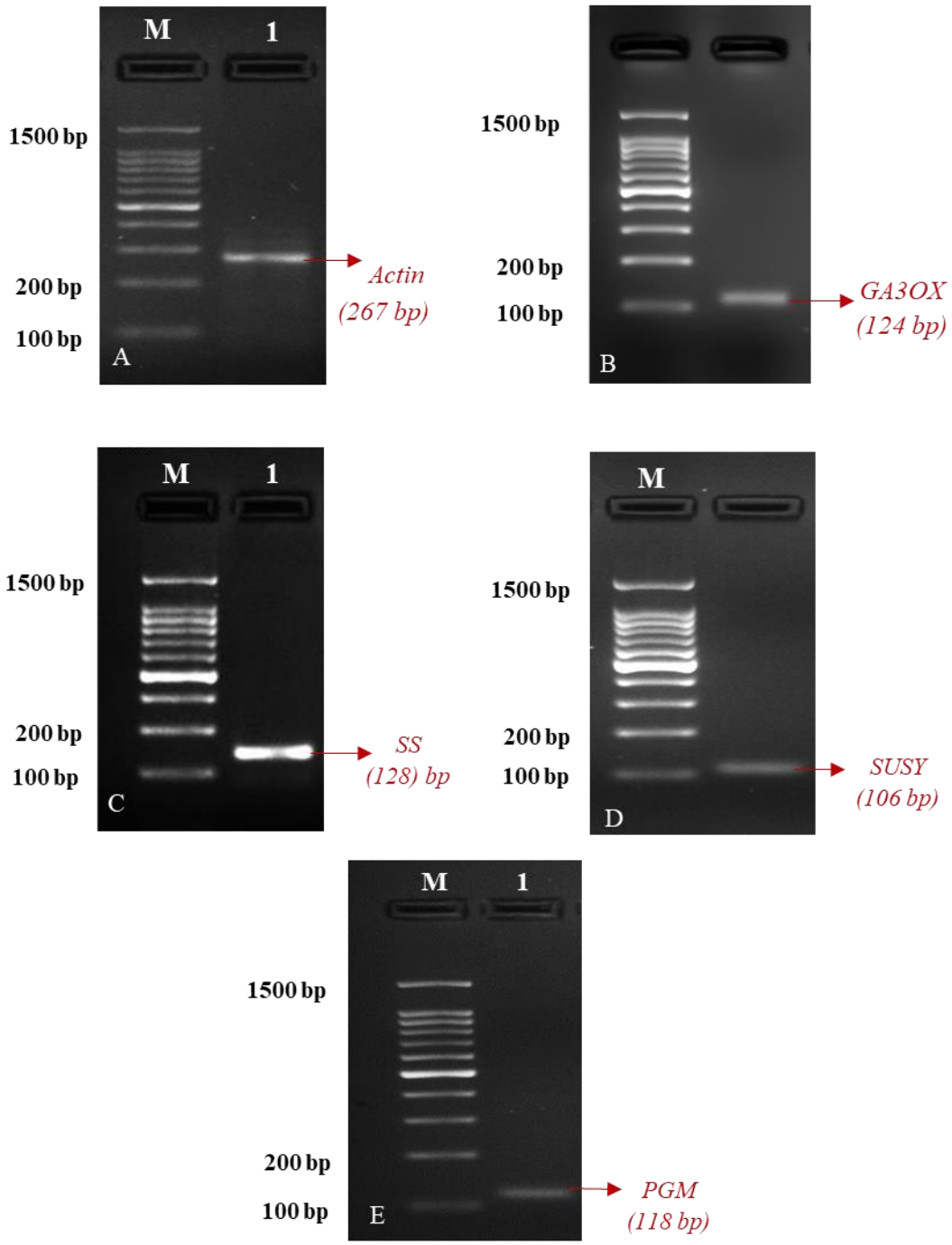


Plate 13. Gel profile of cDNA amplified using forward and reverse primers of different genes.

A – *β Actin* with an expected amplicon size of 267 bp
B – *GA3OX* with an expected amplicon size of 124 bp
C – *SS* with an expected amplicon size of 128 bp
D – *SUSY* with an expected amplicon size of 106 bp
M – 100 bp ladder

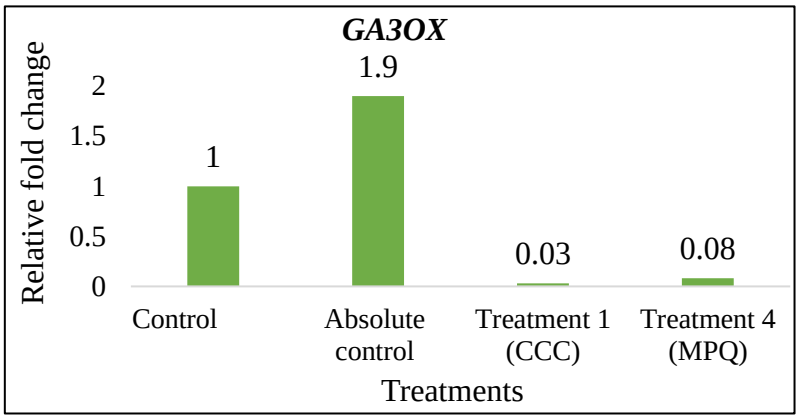


Fig 7. Relative fold change of *gibberellin 3 oxidase* on different treatments

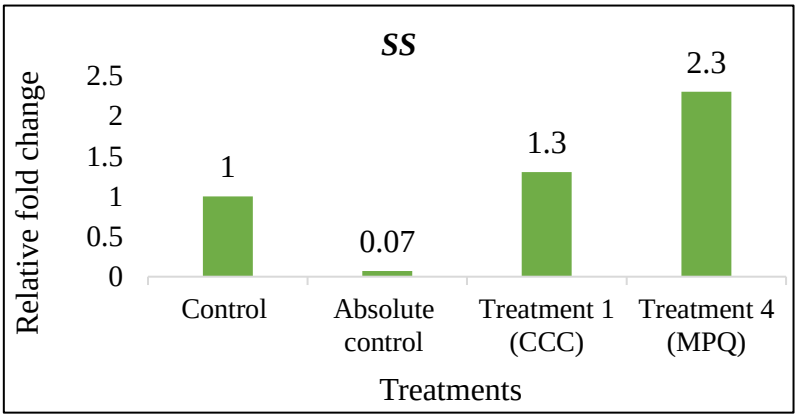


Fig 8. Relative fold change of *starch synthase* on different treatments

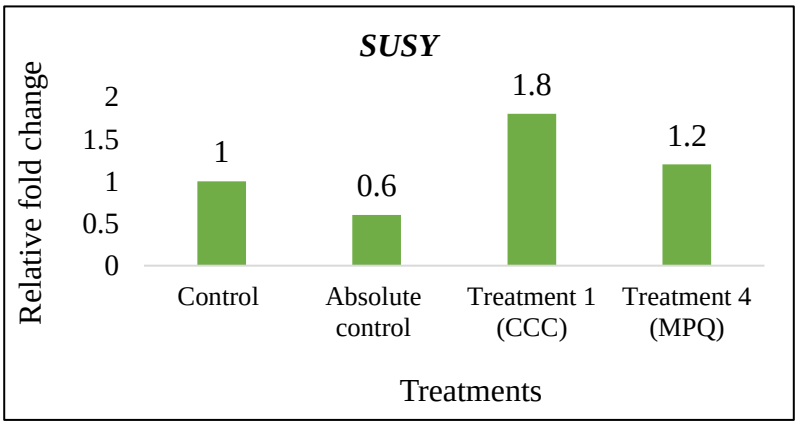


Fig 9. Relative fold change of *sucrose synthase* on different treatments

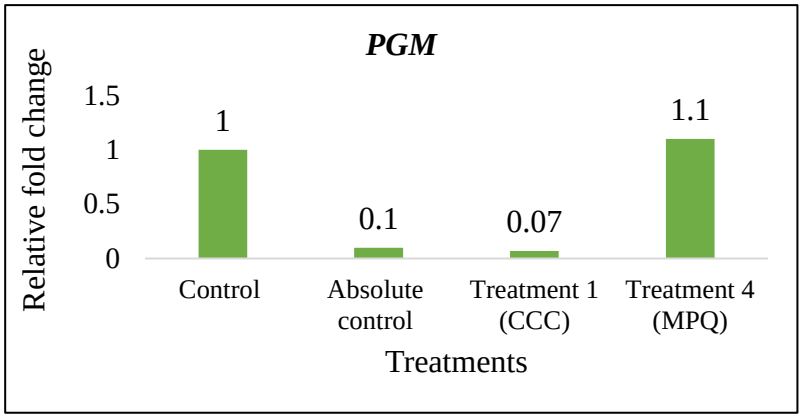


Figure 10. Relative fold change of phosphoglucomutase on different treatments

Discussion

5. DISCUSSION

Saffron (*Crocus sativus* L.) also known as 'Red Gold', stands as one of the most valuable and expensive crops in the world. The dried stigma of commerce serves multifaceted roles as a spice, a natural dye and as a medicinal resource with historical roots dating back to ancient civilizations (Tarraf *et al*., 2024).

Due to triploid nature, saffron is inherently sterile and is propagated vegetatively via corms. Under natural field conditions, this slow-growing species develops 3–4 daughter corms from each mother corm during a crop season. Selection of high-quality corms is paramount for successful saffron cultivation as larger sized corms is reported to influence flowering and cormlet formation owing to higher food reserve (Kaushal and Upadhyay, 2002).

Conventional method of propagation in saffron faces numerous challenges and is influenced by various factors such as low rate of corm multiplication, biotic and abiotic stresses, and inadequate crop management practices (Parray *et al*., 2012; Rubio Moraga *et al*., 2014; Menia *et al*., 2018). Consequently, the natural propagation of saffron became intricate and insufficient to meet the demand for planting material. Biotechnological advancements, particularly *in vitro* culture techniques, hold significant promise for addressing these limitations and enhancing saffron propagation (Tahiri *et al*., 2023). Several protocols for producing *in vitro* cormlets have been developed by various research institutes in India, particularly in Kashmir (Parray *et al*., 2012; Salwee and Nehvi, 2014).

Results of preliminary attempts on indoor farming of saffron at CoA, Vellayani indicated the possibility of its commercial cultivation in Kerala. Standardization of technology for cormlet production suitable for Kerala condition is necessary to make indoor farming more economically feasible. At present there are no reports on *in vitro* cormlet production of saffron from Kerala. Considering the huge economic value of saffron, the present study was taken with an objective to evaluate the effect of various plant growth regulators for enhancing cormlet production in saffron and the differential expression analysis of key genes involved in the process. The results of the study are discussed in this chapter.

5.1 INFLUENCE OF CORM SIZE AND STORAGE CONDITIONS ON GERMINATION AND GROWTH OF SAFFRON

The corms for the present study were collected from farmers field in Pampore, Kashmir during July 2023 and the average diameter of the collected corms was 2.8 cm having average weight of 6.4g. Corms having an average weight of 8-10g and diameter of 2.5–3 cm are reported to be ideal for higher saffron production and productivity (Menia *et al*., 2018) whereas the *in vitro* daughter corm formation are reported from corms having 3.2 cm diameter (Dewir *et al*., 2022). In the present study, 25% of the collected corms were infected with fungal mycelium and were discarded. Only healthy corms were taken for further research works as association of fungal species of Fusarium, *Rhizoctonia*, *Penicillium*, *Aspergillus* is reported to affect sprouting and further growth in saffron (Gupta *et al*., 2021; Mirghasempour *et al*., 2022).

The corms incubated *in vivo* at16°C in the present study sprouted on 57th day during September and showed profuse growth, produced healthy multiple shoots, profuse roots, dark green leaves and daughter corms under *in vitro* condition. Similar data on sprouting during September was reported by Yasmin and Nehvi (2018). How ever corms incubated and maintained at 24°C in the present study though showed sprouting did not show further growth under *in vitro* condition. According to Renau-Morata *et al*., (2013) storage of corms at cold temperature could affect sprouting, *in vitro* propagation efficiency and daughter corm production in saffron.

5.2 INDUCING *IN VITRO* CORMLETS USING PGR's

In vitro cormlet formation in saffron is regulated by PGRs, anti-gibberellins, elevated sucrose levels, and polyamines (Steinitz *et al*., 1991; Kumar *et al*., 2011). A combination of PGRs *viz*, TDZ and IAA has been reported by Parray *et al* (2012) in inducing multiple cormlets *in vitro*. The present study attempted to analyse the combinatorial effect of various anti-gibberellins *viz.*, chlorocholine chloride (CCC), paclobutrazol (PBZ), chlormequat chloride (CMC) and mepiquat chloride (MPQ) with 20µM TDZ and 10µM IAA on *in vitro* cormlet formation. Anti-gibberellins serve as effective plant growth retardants by impeding stem elongation through the inhibition of

gibberellic acid biosynthesis, thereby promoting development of storage organs (Bisht *et al.*, 2018)

The combination of 0.25% MPQ with 20µM TDZ and 10µM IAA was found to induce early cormlet initiation in 10 days of treatment compared to control (20 days) and absolute control (30 days) with the highest number of total cormlets of 7 as against control (5nos) and absolute control (1no) in 50 days. Similar results were reported in onion whereby mepiquat chloride was found to enhance yield and weight of bulbs (Pal *et al*., 2017). Treatment using 0.25% CCC in the present study could initiate cormlets in 20 days of treatment and generated total 5 cormlets in 50 days. Similar attempts in saffron were made by Salwee and Nehvi during 2014 where they had treated slices of multiple shoots using 0.2% CCC. According to Sharma *et al*. 2023, CCC could be used effectively for enhancing *in vitro* micro tuberization in potato. The treatment using CMC generated total 4 *in vitro* cormlets in 50 days. Similar results were reported by Buldakov, 2021 whereby, chlormequat chloride could efficiently boost tuberization of potato *in vitro*. The treatment using PBZ in the present study doesn't show *in vitro* cormlet initiation and might be due to the improper distribution of the compound in the solidified medium leading to poor absorption. However, PBZ has been reported as an effective inducer of *in vitro* cormlet formation in saffron by Devi *et al.* (2010).

Among the anti-gibberellins used for the study, only treatment using MPQ showed a positive combinatorial effect on early initiation of cormlets, number of cormlets and size of cormlets whereas the second-best treatment *ie*, using CCC was on par with control (20µM TDZ and 10µM IAA) indicating the potential of TDZ as a growth retardant. Application of TDZ as a potential growth retardant has been reported in potted rose plants (Mundhara *et al*., 2005), strawberry and blueberry cultivars (Cappelletti *et al*., 2016). Cormlets were initiated in all the 3 replications within 16 days in treatment using MPQ followed by CCC (36 days) and control (38 days) whereas in absolute control cormlets were generated in only one replication out of the three in 45 days, confirming the role of growth regulators and anti-gibberellins in *in vitro* cormlet formation.

According to parray *et al.* (2012) treatment using TDZ and IAA could generate multiple cormlets via somatic embryogenesis in saffron. The potential of MPQ in

inducing early initiation and more number of cormlets of prominent size compared to control, revealed in the present study may be exploited in future towards *in vitro* cormlet production via somatic embryogenesis.

5.3 MOLECULAR ANALYSIS OF KEY GENES IN *IN VITRO* CORMLET FORMATION

The second objective of the study was to elucidate the molecular basis of cormlet formation. An attempt was made to understand the molecular crosstalk in the biosynthesis pathway of gibberellin and starch during *in vitro* cormlet formation by studying the expression profile of key genes *viz*., *gibberellin3oxidase (GA3OX), starch synthase (SS), sucrose synthase (SUSY) and phosphoglucomutase (PGM)*.

5.3.1 Genes involved in gibberellin biosynthesis (*GA3OX*)

Gibberellin 3-oxidase (*GA3OX*) is a crucial enzyme responsible for the conversion of precursor GAs into their active forms, thus directly influencing the concentrations of active GAs within plants (Mitchum *et al*., 2006). The formation of tubers in plants relies on the intricate interplay of hormone signalling. GA is reported to inhibit the growth and development of potato tubers (Chen *et al.,* 2022) and taproot formation in turnip (Liu *et al.,* 2021). Xu *et al.* 1998 reported that external application of bioactive GA can delay tuber development in potato. Similarly, Li *et al*. (2021) found that high concentration of gibberellin can inhibit the cormlet formation in gladiolus. Vecchio *et al.* 1994 reported reduced gibberellin content and enhanced tuber formation in *in vitro* cultures of potato treated using antigibberillin CCC.

The present study incorporated anti-gibberellins *viz*., CCC, PBZ, CMC, MPQ in culture medium to block the synthesis of bioactive gibberellins and to study their effect on *GA3OX* by RT-qPCR. Significant reduction in the expression level of *GA3OX* (0.08, 0.03-fold) was revealed in treatment using 0.25% MPQ and 0.25% CCC respectively whereas significant upregulation of *GA3OX* (1.9 fold) was noticed in absolute control indicating the inhibitory effect of MPQ on *GA3OX* oxidase and suggest the potential of these compounds in blocking the conversion of inactive GA to bioactive GA. The inhibitory action of MPQ and CCC on *Copalyl diphosphate synthase* in GA biosynthesis pathway has been reported (Rademacher, 2000) however the inhibitory

action of MPQ and CCC on *GA3OX* revealed in the present study is the first report of its kind. Similar findings on significant downregulation of gibberellin biosynthesis genes upon application of plant growth retardants are reported in tomatoes (Ji *et al.,* 2019).

5.3.2 Genes involved in starch biosynthesis (*SS, SUSY, PGM*)

Starch, being the primary carbohydrate in storage organs, have critical functions in their development, including corms, tubers, and seeds (Zhang *et al*., 2020). Starch is formed from sucrose and glucose 6 phosphate via intermediaries *viz*., uridine diphosphate glucose (UDP-G), Adenosine diphosphate glucose (ADP-G), Glucose 1 phosphate. S*tarch synthase* (*SS*) is an enzyme which converts ADP-G to starch by adding the glucosyl moiety to existing glucan chains whereas sucrose synthase (SUSY) converts sucrose to ADP-G ie, unloading of sucrose sourced from photosynthetic organs like leaves to non-photosynthetic organs (known as sink organs), where it undergoes conversion into starch for long-term storage in amyloplasts (Yu *et al*., 2022). Phosphoglucomutase catalyzes the interconversion between glucose-1-phosphate (Glc-1-P) and glucose-6-phosphate (Glc-6-P), which is essential for the transitory starch synthesis (Malinova *et al*., 2014). The formation and development of tubers is achieved by accelerating starch accumulation and starch granule enlargement in potato (Gukasyan *et al*., 2005; Roumeliotis *et al*., 2012), taro (Zhang *et al*., 2023).

In treatment using MPQ in the present study, there was a significant upregulation of all the key genes involved in starch biosynthesis *viz*., *SS* (2.3-fold), *SUSY* (1.3-fold), *PGM* (1.1-fold) whereas in treatment using CCC upregulation of *SS* (1.3-fold), *SUSY* (1.8-fold) was noticed. The result suggests that cormlets observed on 21 days in the present study is highly correlated with the upregulation of starch biosynthesis genes and is supported by the findings of Li *et al.* 2021 whereby the upregulation of sucrose synthase gene (*GhSUS2*) correlates with the emergence of cormlets in *Gladiolus hybridus*, a member of the Iridaceae family.

Molecular analysis could reveal a negative co-relation in the expression pattern of genes involved in biosynthesis pathway of gibberellin and starch metabolism during the early phase of cormlet initiation and suggest a low gibberellin mediated starch

accumulation during *in vitro* cormlet formation in saffron. Desta and Amare (2021) reported that the application of anti-gibberellins could enhance starch accumulation by increasing the activities of enzymes involved in starch biosynthesis. Similar results on gladiolus were reported Li *et al* (2021) whereby high concentration of gibberellin was found to reduce starch synthesis. Reports on enhanced starch content in the endosperm of maize upon application of CCC supports the result of present study (Cao and Shannon, 1997). Similar results in lily were reported by Zheng *et al*. (2012) whereby treatment using CCC had led to a reduction in gibberellin levels in lily bulbs and they suggested that a decrease in gibberellin content could potentially stimulated starch accumulation within the bulbs.

To conclude, the various plant growth regulators including anti-gibberellins used in the present study *viz*., TDZ, IAA, MPQ, CCC and CMC could induce *in vitro* cormlets in saffron. MPQ (0.25%) is found as the best treatment for early induction of cormlets, number of cormlets and size of cormlets initiated. The present study is the first report on differential expression analysis of key genes involved in *in vitro* cormlet initiation in saffron. Future studies include assessment of the potential of *in vitro* raised cormlets for flowering *in vivo.*

Summary

6. SUMMARY

The study entitled "Plant growth regulators for enhancing *in vitro* cormlet production in saffron (Crocus sativus L.)" was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani during 2023-2024. The objective of the study was to evaluate the effect of various plant growth regulators in enhancing *in vitro* cormlet formation and differential expression of key genes involved in cormlet production.

Saffron (*Crocus sativus* L.) is the most expensive spice in the world. It has been used in food, dairy and dye industries and as medicine due to its colouring, flavouring and therapeutical properties. Cultivation of saffron is restricted to certain geographical areas due to its requirement of peculiar agroclimatic conditions. The global demand for saffron is high due to its wide variety of uses. Saffron is propagated by corms as the flowers are sterile and fail to produce viable seeds. Conventional propagation methods are very slow, and propagation by tissue culture is having great potential. Large-scale propagation of saffron is achieved through *in vitro* cormlet production.

Corms of saffron were collected from farmer's fields in Pampore, district of Kashmir. The surface sterilized corms were established in MS medium supplemented with 2 ppm BA for *in vitro* multiple shooting during August 2023 and subcultured at 21-day interval in the same medium upto December 2023. However, only sprouting was noticed in the culture but didn't grow further. And further growth was not observed. Corms were maintained under *in vivo* conditions of 16ºC temperature, 16/8hrs photoperiod and 60% humidity for sprouting. The first sprout was initiated on 57th day and 100 per cent sprouting observed within 65 days. 180-day old *in vivo* sprouted corms were surface sterilized and transferred to basal MS medium for elongation and shooting. Healthy multiple shoots were observed in 15 days with an average of 5 multiple shoots per culture and profuse roots were noticed in 2 days.

Further, the *in vitro* multiple shoots were transferred to half-strength MS medium supplemented with 20 µM thidiazuron (TDZ), 10 µM indole acetic acid (IAA), 40% sucrose, and 0.25% of various anti-gibberellins viz., chlorocholine chloride (CCC), paclobutrazol (PBZ), chlormequat chloride (CMC) and mepiquat chloride

(MPQ) along with control. Cultures maintained in full strength MS medium was used as absolute control.

Cormlet initiation was first noticed in treatment using MPQ on 10th day of treatment followed by CCC (20 days), control (20 days) and absolute control (30 days). Treatment using 0.25% MPQ showed maximum number of cormlets in 50 days (7) followed by treatment using 0.25% CCC (5), 0.25% CMC (4), control (5) and absolute control (1). *In vitro* cormlets generated in treatment using 0.25% MPQ showed early induction of cormlets, more number of cormlets and prominent size compared to control. Further, *in vitro* raised cormlets were transferred to MS medium supplemented with TDZ (20 µM), IAA (10 µM) and 9% sucrose for germination and size enhancement.

Differential expression analysis of key genes involved in *in vitro* cormlet formation was carried out using Real Time quantitative PCR (RT-qPCR). RNA was isolated from corms of best two treatment (MPQ and CCC) 21 days after treatment (3 weeks) along with control and absolute control using CTAB method. The purity and concentration of the isolated RNA was checked using NanoDrop. The integrity and quality of RNA were confirmed using agarose gel electrophoresis. Further, RNA was converted into complementary DNA (cDNA). Primers for the amplification of the genes was designed after subjecting the retrieved nucleotide sequence of closely related crops of saffron to multiple sequence alignment using MEGA 11 and generated consensus sequence using BioEdit. Further, primers were designed from the consensus sequence using Primer 3 Plus.

Differential expression analysis using (RT-qPCR) was carried out to understand the molecular crosstalk in the biosynthesis pathway of gibberellin and starch during *in vitro* cormlet formation by studying the expression profile of key genes *viz*., *gibberellin3oxidase (GA3OX), starch synthase (SS), sucrose synthase (SUSY) and phosphoglucomutase (PGM)*. The gene expression showed that cormlet formation is correlated with the upregulation of starch biosynthesis genes and downregulation of gibberellin biosynthesis genes. In the treatment using 0.25% mepiquat chloride significant reduction was noticed in the expression level of GA3OX (0.08-fold) and

upregulation of all the key genes involved in starch biosynthesis viz., SS (2.3-fold), SUSY (1.3-fold), PGM (1.1-fold). Similar results were noticed in treatment using 0.25% CCC, whereby significant reduction in the expression level of GA3OX (0.03-fold) and upregulation of SS (1.3-fold) and SUSY (1.8-fold) was found.

Gene expression analysis revealed a negative co-relation in the expression pattern of genes involved in biosynthesis pathway of gibberellin and starch, which correlated with the *in vitro* cormlets observed after 21 days of treatment. A 4-fold reduction in the expression pattern of *GA3OX* noticed in the best treatment (0.25% MPQ) indicates the potential anti-gibberllin activity of MPQ in blocking the conversion of inactive gibberellins to bioactive gibberellins.

To conclude, plant growth regulators viz., mepiquat chloride and chlorocholine chloride can be used effectively for early cormlet initiation and to enhance *in vitro* cormlet production in saffron. The present study is the first report on differential expression analysis of key genes involved in *in vitro* cormlet formation in saffron and could reveal a low gibberellin mediated starch accumulation during early phase of cormlet production.

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Appendices

8. APPENDICES

APPENDIX I

1. Composition of basal Murashige and Skoog (MS) medium

Murashige and Skoog medium						
Stock	Chemical	Quantity for 1L of MS (mg)	Conc. of Stock	Volume of Stock (ml)	Quantity for preparing Stock (mg)	Volume for 1L MS Media (ml)
Macro nutrients						
A	$MgSO_4.7H_2O$	370	40X	100ml	1480	25 ml
	KH_2PO_4	170			680	
B	$CaCl_2.2H_2O$	440	50X	100 ml	2200	20 ml
Micro nutrients						
C	KI	0.83	1000X	100 ml	83	1 ml
	H_3BO_3	6.2			620	
	$MnSO_4.H_2O$	16.9			1690	
	$ZnSO_4.7H_2O$	8.6			860	
	$Na_2MoO_4.2H_2O$	0.25			25	
D	$FeSO_4.7H_2O$	27.8	50X	100 ml	139	20 ml
	Na_2 EDTA.$2H_2O$	37.3			186.5	
E	$CuSO_4.5H_2O$	0.025	10000X	100 ml	25	0.1 ml
	$CoCl_2.6H_2O$	0.025			25	
Organic Supplements						
F	Nicotinic acid	0.5	2500X	100 ml	125	0.4 ml
	Pyridoxine -HCl	0.5			125	
	Thiamine - HCl	0.1			25	
	Glycine	2			500	
Components directly added						
	MS Supplement	3.3 g				
	Myoinositol	100 mg				
	Sucrose	30 g				
	Agar	6.5 g				

APPENDIX II

2. Stock solution of plant growth regulators

Stock	Concentration of stock	Volume of stock (mL)	Quantity for preparing stock (mg)	Solvent
Hormones				
BA	1000 ppm	50	50	Autoclaved H_2O
IAA				
TDZ				
Anti-gibberellins				
CCC	1%	50	0.5 g	Autoclaved H_2O
CMC				
MPQ				
PBZ	1%	50	0.5 g	80% ethanol

APPENDIX III

3. Reagents required for isolation of RNA

CTAB RNA extraction buffer - 1L (pH - 8.5-9.0)	
CTAB	20 g
0.5 M EDTA (pH - 8)	50 mL
Tris base	12.1 g
PVP	20 g
NaCl	116.9 g
β-mercaptoethanol	2% (Should be freshly added)
Chloroform: isoamyl alcohol	
Chloroform	24 mL
Isoamyl alcohol	1 mL
8 M Lithium chloride	
Lithium chloride	339.12 g
Distilled H_2O	Made up to 1000mL
3M Sodium acetate	
Sodium acetate	246.09 g
Distilled H_2O	Made up to 1000mL
70% ethanol	
Ethanol	70 mL
Distilled H_2O	30 mL

APPENDIX - VI

4. Reagents required for agarose gel electrophoresis

50X TAE buffer – 1L (pH - 8)	
Tris base	242 g
Glacial Acetic acid	57.1 mL
0.5 M EDTA	100 mL
6X Gel loading dye	
Bromophenol Blue	0.2 g
50 % glycerol	6mL
Millipore H_2O	4mL

PLANT GROWTH REGULATORS FOR ENHANCING *IN VITRO* CORMLET PRODUCTION IN SAFFRON

(*Crocus sativus* L.)

BY

NEETHU R. S.

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ABSTRACT OF THESIS

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9. ABSTRACT

The study entitled "Plant growth regulators for enhancing *in vitro* cormlet production in saffron (*Crocus sativus* L.)" was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani during 2023-2024. The objective of the study was to evaluate the effect of plant growth regulators in enhancing *in vitro* cormlet formation and differential expression of key genes involved in cormlet production. Experimental design followed was CRD with five treatments and three replications each.

The explant, corms were collected from farmer's fields in Pampore, Kashmir, and subjected to a rigorous three-step surface sterilization process using 0.02% bavistin (20min), 0.04% mancozeb (20min), 1% sodium hypochlorite bleach solutions (30min) and a final dip in 1.5% mercuric chloride solution. *In vitro* multiple shooting attempted using Murashige and Skoog (MS) medium supplemented with 2ppm Benzyl adenine (BA) showed sprouting, but did not grow further. Corms maintained under *in vivo* conditions of 16ºC temperature, 16/8hrs photoperiod and 60% humidity showed 100 per cent sprouting in 65 days. The sprouted corms were transferred to basal MS medium for elongation and shooting. Healthy multiple shoots were observed in 15 days with an average of 5 multiple shoots per culture and profuse roots were noticed in 2 days in all replications.

Further, the *in vitro* multiple shoots were transferred to half-strength MS medium supplemented with 20 µM thidiazuron (TDZ), 10 µM indole acetic acid (IAA), 40% sucrose, and 0.25% of various anti-gibberellins *viz*., chlorocholine chloride (CCC), paclobutrazol (PBZ), chlormequat chloride (CMC) and mepiquat chloride (MPQ) along with control. Cultures maintained in full strength MS medium was used as absolute control.

Cormlet initiation was first noticed in treatment using MPQ on 10^{th} day followed by CCC (20 days), control (20 days) and absolute control (30 days). Cormlets were initiated in all 3 replications within 16 days in treatment using MPQ followed by CCC (36 days) and control (38 days). Total number of cormlets obtained was highest in treatment using MPQ (7 nos), followed by CCC (5 nos), control

(5nos), CMC (4nos) and absolute control (1no). The present study showed 0.25% MPQ as the best treatment with early initiation and maximum number of cormlet production. *In vitro* cormlets generated in treatment using 0.25% MPQ showed prominent size compared to control.

In the treatment using 0.25% mepiquat chloride significant reduction was noticed in the expression level of *GA3OX* (0.08-fold) and upregulation of all the key genes involved in starch biosynthesis viz., *SS* (2.3-fold), *SUSY* (1.3-fold), *PGM* (1.1-fold). Similar results were noticed in treatment using 0.25% CCC, whereby significant reduction in the expression level of *GA3OX* (0.03-fold) and upregulation of *SS* (1.3-fold) and *SUSY* (1.8-fold) was found. Molecular studies could reveal a negative co-relation in the expression pattern of genes involved in biosynthesis pathway of gibberellin and starch metabolism during the early phase of cormlet initiation.

To conclude, plant growth regulators *viz.*, mepiquat chloride and chlorocholine chloride can be used effectively for early cormlet initiation and to enhance *in vitro* cormlet production in saffron. The inhibitory activity of MPQ & CCC against *GA3oxidase* noticed in the present study suggest the potential of these compounds in blocking the conversion of inactive GA to bioactive GA. The present study is the first report on differential expression analysis of key genes involved in *in vitro* cormlet formation in saffron and could reveal a low gibberellin mediated starch accumulation during early phase of cormlet production.

സംഗ്രഹം

2022-23 കാലയളവിൽ തിരുവനന്തപുരം വെള്ളായണിയിലെ കോളേജ് ഓഫ് അഗ്രികൾച്ചറിലുള്ള ഡിപ്പാർട്ട്മെന്റ് ഓഫ് മോളിക്യുലർ ബയോളജി ആൻഡ് ബയോടെക്നോളജിയിൽ വച്ച് "സസ്യവളർച്ചാ റെഗുലേറ്ററുകൾ ഉപയോഗിച്ച് കുങ്കുമപ്പൂവിന്റെ ഇൻവിട്രോ കോംലെറ്റ് ഉൽപ്പാദനം വർദ്ധിപ്പിക്കുക (*ക്രോക്കസ് സാറ്റൈവസ്* എൽ.)" എന്ന തലക്കെട്ടിൽ പഠനം നടത്തി. കുങ്കുമപ്പൂവിലെ ഇൻവിട്രോ കോംലെറ്റ് ഉൽപ്പാദനം വർദ്ധിപ്പിക്കുന്നതിന് വിവിധ സസ്യ വളർച്ചാ നിയന്ത്രണങ്ങളുടെ ഫലവും കോർമ്മെറ്റ് രൂപീകരണത്തിൽ ഉൾപ്പെടുന്ന പ്രധാന ജീനുകളുടെ ഡിഫറൻഷ്യൽ എക്സ്പ്രഷനും വിലയിരുത്തുക എന്നതാണ് പഠനത്തിന്റെ ലക്ഷ്യം. അഞ്ച് ചികിത്സകളും മൂന്ന് റെപ്ലിക്കേഷനുകളും ഉള്ള സിആർഡിയായിരുന്നു തുടർന്നുള്ള പരീക്ഷണാത്മക രൂപകൽപ്പന.

കശ്മീരിലെ പാംപോറിലെ കർഷകരിൽ നിന്ന് ശേഖരിച്ച കോർമുകൾ പഠനത്തിനായി ഉപയോഗിച്ചു. 0.02% ബാവിസ്റ്റിൻ (20 മിനിറ്റിനും), 0.04% മാൻകോസെബ് (20 മിനിറ്റിനും), 1% സോഡിയം ഹൈപ്പോക്ലോറൈറ്റ് ബ്ലീച്ച് സൊല്യൂഷൻ (30 മിനിറ്റിനും), 1.5% മെർക്കുറിക് ക്ലോറൈഡ് ലായനി എന്നിവ ഉപയോഗിച്ച് കോർമുകൾ അണുവിമുക്തമാക്കി. 2 പിപിഎം ബെൻസെയിൽ അഡെനിൻ (BA) അടങ്ങിയ മുരാഷിഗ്, സ്കൂഗ് (MS) മീഡിയം ഇൻ വിട്രോ മൾട്ടിപ്പിൾ ഷൂട്ടിംഗിനായി ഉപയോഗിച്ചിരുന്നു, അതിൽ കോർമുകൾ മുളയ്ക്കുന്നതു നിരീക്ഷിച്ചുവെങ്കിലും കൂടുതൽ വളർന്നില്ല. 16 ഡിഗ്രി സെൽഷ്യസ് താപനില, 16/8 മണിക്കൂർ ഫോട്ടോപീരിയഡ്, 60% ഈർപ്പം എന്നിവയിൽ നിലനിർത്തിയ

കോർമുകൾ 65 ദിവസത്തിനുള്ളിൽ 100 ശതമാനം മുളച്ചു. മുളപ്പിച്ച കോർമുകൾ വിപുലീകരണത്തിനും ഷൂട്ടിംഗിനുമായി ബേസൽ MS മീഡിയത്തിലേക്ക് മാറ്റി.

15 ദിവസത്തിനുള്ളിൽ ഇൻ വിട്രോ കൾച്ചറുകളിൽ ശരാശരി 5 മൾട്ടിപ്പിൾ ഷൂട്ടുകൾ നിരീക്ഷിക്കപ്പെട്ടു, കൂടാതെ എല്ലാ റെപ്ലിക്കേഷനുകളിലും 2 ദിവസത്തിനുള്ളിൽ സമൃദ്ധമായ വേരുകൾ ശ്രദ്ധിക്കപ്പെട്ടു. തുടർന്നു ഇൻ വിട്രോ മൾട്ടിപ്പിൾ ഷൂട്ടുകൾ 20 µM തിഡിയാസുറോൺ (TDZ) 10 µM ഇൻഡോൾ അസറ്റിക് ആസിഡ് (IAA) 40% സുക്രോസ്, 0.25% ആന്റിജിബർലിനുകൾ ആയ ക്ലോറോകോളിൻ ക്ലോറൈഡ് (CCC) പാക്ലോബ്യൂട്രസോൾ (PBZ), ക്ലോർമെകാറ്റ് ക്ലോറൈഡ് (CMC), മെപികാറ്റ് ക്ലോറൈഡ് (MPQ) എന്നിവ അടങ്ങിയ ഹാഫ്-സ്ട്രെങ്ത് എംഎസ് മീഡിയത്തിലേക്ക് കൺട്രോളിനോപ്പം മാറ്റി കുത്തിവച്ചു. ബേസൽ MS മീഡിയത്തിൽ പരിപാലിക്കുന്ന കൾച്ചറുകൾ അബ്സല്യൂട്ട് കൺട്രോളായി ഉപയോഗിച്ചു.

പത്താം ദിവസം MPQ അടങ്ങിയ മീഡിയത്തിലാണ് കോംലെറ്റ് ആദ്യം ശ്രദ്ധിക്കപ്പെട്ടത്, തുടർന്ന് CCC (20 ദിവസം) കൺട്രോൾ (20 ദിവസം). അബ്സല്യൂട്ട് കൺട്രോൾ (30 ദിവസം). MPQ അടങ്ങിയ മീഡിയത്തിൽ 16 ദിവസത്തിനുള്ളിൽ 3 റെപ്ലിക്കേഷനുകളിലും കോംലെറ്റുകൾ ആരംഭിക്കുകയും തുടർന്ന് 36 ദിവസത്തിനുള്ളിൽ CCC-യിൽ, 38 ദിവസത്തിനുള്ളിൽ കൺട്രോളിലും കോംലെറ്റുകൾ വരാൻ ആരംഭിച്ചു. MPQ അടങ്ങിയ മീഡിയത്തിൽ ഏറ്റവും കൂടുതൽ കോർംലെറ്റുകൾ ലഭിച്ചത്, അതായത് 7 എണ്ണം, തുടർന്ന് CCC-യിൽ 5 എണ്ണം, കൺട്രോളിൽ 5 എണ്ണം, CMC-യിൽ 4 എണ്ണം,

അബ്സല്യൂട്ട് കൺട്രോളിൽ 1 എണ്ണം എന്നിങ്ങനെയാണ് കോംലെറ്റുകൾ കാണപെട്ടത്.

0.25% MPQ ഉപയോഗിച്ച് ഉൽപാദിപ്പിക്കുന്ന ഇൻ വിട്രോ കോംലെറ്റുകൾ കൺട്രോളുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ വലുപ്പം കാണിക്കുന്നു. കോംലെറ്റ് രൂപീകരണത്തിൽ ഉൾപ്പെട്ടിരിക്കുന്ന പ്രധാന ജീനുകളുടെ തന്മാത്രാ വിശകലനം സൂചിപ്പിക്കുന്നത്, കോർംലെറ്റ് രൂപീകരണം *സ്റ്റാർച്* ബയോസിന്തസിസ് ജീനുകളുടെ വർദ്ധനവുമായും ഗിബ്ബെറെലിൻ ബയോസിന്തസിസ് ജീനുകളുടെ നിയന്ത്രണം കുറയ്ക്കുന്നതുമായും ബന്ധപ്പെട്ടിരിക്കുന്നു. 0.25% MPQ ഉപയോഗിച്ചുള്ള ചികിത്സയിൽ ഗിബ്ബെറെലിൻ ബയോസിന്തസിസ് ജീനിൽ, GA3OX (0.08 മടങ്ങ്) ഗണ്യമായ കുറവും, സ്റ്റാർച്ച് ബയോസിന്തസിസിൽ ഉൾപ്പെടുന്ന എല്ലാ പ്രധാന ജീനുകളായ SS (2.3 മടങ്ങ്), SUSY (1.3 മടങ്ങ്), PGM (1.1 മടങ്ങ്) *എന്നിവയിൽ* വർദ്ധനവും കാണാൻ *സാധിക്കുന്നതാണ്*.

ഉപസംഹാരമായി, സസ്യവളർച്ചാ നിയന്ത്രണങ്ങളായ മെപികഛാറ്റ് ക്ലോറൈഡ്, ക്ലോറോകോളിൻ ക്ലോറൈഡ് എന്നിവ ആദ്യകാല കോർമ്മെറ്റ് ആരംഭിക്കുന്നതിനും കുങ്കുമപ്പൂവിൽ ഇൻ വിട്രോ കോർമ്മെറ്റ് ഉൽപാദനം വർദ്ധിപ്പിക്കുന്നതിനും ഫലപ്രദമായി ഉപയോഗിക്കാം. നിലവിലെ പഠനത്തിൽ GA3OX-നെതിരായ MPQ & CCC യുടെ പ്രതിരോധ പ്രവർത്തനം നിഷ്ക്രിയമായ ഗിബ്ബെരെല്ലിനെ ബയോ ആക്റ്റീവ് ഗിബ്ബെരെല്ലിനിലേക്ക് പരിവർത്തനം ചെയ്യുന്നത് തടയുന്നതിൽ ഈ സംയുക്തങ്ങളുടെ കഴിവ് സൂചിപ്പിക്കുന്നു. കുങ്കുമപ്പൂവിലെ ഇൻ വിട്രോ കോർമ്മെറ്റ് രൂപീകരണത്തിൽ ഉൾപ്പെട്ടിരിക്കുന്ന പ്രധാന ജീനുകളുടെ ഡിഫറൻഷ്യൽ എക്സ്പ്രഷൻ

വിശകലനത്തെക്കുറിച്ചുള്ള ആദ്യ റിപ്പോർട്ടാണ് ഇപ്പോഴത്തെ പഠനം, കൂടാതെ കോർമ്മെറ്റ് ഉൽപ്പന്നത്തിന്റെ ആദ്യഘട്ടത്തിൽ കുറഞ്ഞ ഗിബ്ബെരെല്ലിൻ മധ്യസ്ഥ *സ്റ്റാർച്* അടിഞ്ഞുകൂടുന്നത് വെളിപ്പെടുത്താൻ *സാധിക്കും*.