Morphoanatomical, Phyto-Histochemical, and *In Vitro* Culture Studies of the Critically Endangered Plant *Dipcadi goaense*

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I hereby declare that the data presented in this Dissertation report entitled, "Morphoanatomical, Phyto-Histochemical, and *In Vitro* Culture Studies of the Critically Endangered Plant *Dipcadi goaense*" is based on the result carried out by me in the Botany discipline at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Aditi Venkatesh Naik. and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report "Morphoanatomical, Phyto-Histochemical, and *In Vitro* Culture Studies of the Critically Endangered Plant *Dipcadi goaense*" is a bonafide work carried out by Ms. Plency Fernandes under my supervision in partial fulfilment of the requirements for the award of degree of M.Sc. in the Discipline of Botany at the School of Biological Sciences and Biotechnology, Goa University.

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CONTENTS

PREFACE

The research described in this dissertation was conducted between 2023 and 2024 at the Botany Department of the School of Biological Sciences and Biotechnology (SBSB), Goa University, under the guidance of Dr. Aditi Venkatesh Naik, Assistant Professor in the Botany Discipline at SBSB. This work serves as the final dissertation report for a Master's Degree in Botany. The primary objective of this study is to undertake Morphoanatomical, Phyto-Histochemical, and *In Vitro* Culture Studies of the Critically Endangered Plant *Dipcadi goaense*. The research is structured around four main objectives, each aimed at contributing to our understanding of this endangered species and its conservation.

Chapter 1 introduces the research, outlining its purpose and emphasizing the importance of conserving endemic species like *Dipcadi goaense*. Chapter 2 comprises a review of literature, summarizing previous research on *Dipcadi* species conducted by various scholars.

Chapter 3 details the materials and methods employed in the study, elucidating the methodologies used to carry out the research objectives. Chapter 4 presents the analysis and conclusions, showcasing the results obtained from the study through figures, tables, and discussions. The conclusions drawn from the research shed light on the best solvent for isolating phytochemicals from *Dipcadi goaense* leaf and bulb extracts, as well as optimal hormone combinations for shoot induction in in vitro cultures. Additionally, the study suggests that *Dipcadi goaense* can potentially thrive in environments with similar soil type, pH, and environmental conditions, as evidenced by successful growth in soil collected from the.Goa university campus In summary, this dissertation contributes valuable insights into the morphology, anatomy, phytochemistry, and cultivation of *Dipcadi goaense*, with implications for its conservation and potential utilization in various applications.

-Ms. Plency Fernandes

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MS. PLENCY FERNANDES

TABLES

LIST OF FIGURES

PICTURE PLATES

ABBREVIATIONS

Entity	Abbreviations
2,4-dichlorophenoxyacetic acid	2,4-D
benzylamino purine	BAP
Centimeter	Cm
degree Celsius	^{o}C
Ethylenediaminetetraacetic acid	EDTA
Grams	G
Grams per liter	g/L
Hours	hrs.
Hydrogen chloride	HCL
Indole -3-butyric acid	IBA
Meter	M
Micrograms	µg
Micromolar	µMol
Milliliters	mL
Millimeters	Mm
Milligrams	Mg
milligrams per liter	mg/L
Normal	N
Number	No
Percent	%
Potential of Hydrogen	pH
pounds per square inch	Psi
Sodium Hydroxide	NaOH
Volume in volume	v/v
Weight in volume	w/v
M.S Medium + 3 mg/mL 2,4-D + 1 mg/mL BAP	C.1
M.S Medium + 3.5 mg/mL 2,4-D + 1 mg/mL BAP	C.2
M.S Medium + 4 mg/mL 2,4-D + 1 mg/mL BAP	C.3
M.S Medium + 3 mg/mL IBA + 1 mg/mL BAP	C.4
M.S Medium + 3.5 mg/mL IBA + 1 mg/mL BAP	C.5
M.S Medium + 4 mg/mL IBA + 1 mg/mL BAP	C.6
M.S Medium + 3 mg/mL NAA + 1 mg/mL BAP	C.7
M.S Medium + 3.5 mg/mL NAA + 1 mg/mL BAP	C.8
M.S Medium + 4 mg/mL NAA + 1 mg/mL BAP	C.9

ABSTRACT

Dipcadi goaense is a bulbous herb belonging to the Hyacinthaceae family, endemic to the type locality Rivona, Kevan foothills of the Western Ghats in South Goa, India. This species holds significance in ethnobotanical practices and pharmaceutical research due to its reported medicinal properties. Morphoanatomical studies of its leaf and bulb revealed unique features, with the bulb exhibiting the presence of calcium oxalate raphide crystals. Histochemical investigations unveiled primary metabolites such as starch, lipids, and proteins, along with secondary metabolites including alkaloids and phenols. Phytochemical analyses using chromatography techniques isolated alkaloids, phenols, steroids, and flavonoids from leaf and bulb extracts. UV-Vis spectrophotometric assays indicated that ethanolic extracts contained higher total phenolic content compared to methanolic and distilled water extracts. Due to its limited population, conservation efforts are essential, necessitating *ex-vitro* and *in vitro* propagation techniques. This study aimed to develop a regeneration protocol for *Dipcadi goaense* using bulb explants on MS Medium supplemented with various concentrations of auxins and cytokines. The combination of 4 mg/mL 2,4-D + 1 mg/mL BAP exhibited the highest shoot response rate of 100%, followed by 3 mg/mL 2,4-D + 1 mg/mL BAP and 3.5 mg/mL 2,4-D + 1 mg/mL BAP, both with an 96.29 ± 57% response. Bulbs grown in laboratory pots also demonstrated successful growth. The findings of this study underscore the importance of conservation efforts for the *Dipcadi goaense* species within the genus *Dipcadi*.

CHAPTER 1: INTRODUCTION

1.1 *DIPCADI GOAENSE*

The genus *Dipcadi*, a part of the family Hyacinthaceae, encompasses diverse plant species primarily distributed across the Western Ghats of India, renowned for their ecological significance, unique floral diversity, and potential therapeutic properties. Among these species, *Dipcadi goaense* emerged as a critical focal point due to its distinctiveness and perilous endangerment, underscoring the need for in-depth scientific investigation and conservation efforts.

1.1.1. Scientific Classification

Kingdom: Plantae

Phylum: Tracheophyta

Class: Liliopsida

Order: Asparagales

Family: Hyacinthaceae

Genus: *Dipcadi*

Species: *goaense*

1.1.2. Origin and Distribution

Dipcadi goaense was apparently found to be located in the lateritic rocky area with gravelly soil and the species is known by only a single population restricted to the locality (Rivona, Kevan -foothills of western Ghats) in South Goa, India. (Prabhugaonkar *et al*., 2009).

1.1.3. Botanical Description

*D. goaense*is a bulbous scapose herb, measuring a hight of 150 – 400mm. The bulbs are spherical in size of 10-19 mm in diameter which are profusely rooting from the base. The leaves are in rosette, 4 -7 per bulb, which are reduced to 1-4 at the end of the season. Leaf is linear, 50 -250 x 250 - 4mm, that are deeply channelled, green sightly broader and white at the base, the margin is entire, while the apex is narrow there is absence of indumentum. The scape is 1.5 - 4 cm x 2 – 3 mm, terete, glabrous with absence of sterile bracts. Inflorescence is raceme type which goes up to 40 - 100mm long, comprising 2 – 8 flowers, floral bracts which are broadly ovate, scarious, 5 - 8 x 4.5 - 7 mm, that acuminate at apex. Flowers pedicellate and shiny, with fully opened pedicels measuring 5-10 x 1 mm in height. The fruit is terete to elliptic in outline, measuring 8-15 x 1.5mm. The perianth tube is 5 - 6 x 5 – 6 mm, lobes of the outer whorl are oblong to elliptic, 9 - 11 x 5 - 6mm, 9 nerved, acute to rounded at apex, the inner lobes are constricted in the middle, 9 - 11 x 4 – 6 mm, which coherent to form a flask shaped structure with apical parts spreading, and with exposing tips of anthers when they are fully opened at night, 7 (-8) nerved acute at apex. Stamens are 6 – 10 mm long whereas filaments 4 – 6 mm long that are strap shaped, originating at the mouth of the perianth tube, adnate to inner lobes throughout their length, anthers 3 - 4 x 1 mm. The pistil is yellow in colour, 13-17 mm, with a stipe of 1 - 1.5 mm long, ovary is 2.5 - 4 x 2.5 – 3.5 mm, with ovules 5 - 7 per locule, style 9-13 x 1 mm, while the stigma is trifid but appearing simple to begin with, papillate. Capsule is distinctly 3 - lobed, 8-11 x 11-13 mm, while the seeds are 2 - 5 per locule, which are ovate to elliptic, rarely semicircular, the middle one is discoid with a rim, while the upper and lower ones are plano-convex to convex which measures to 4.5 – 8 x 3.5 x 1.0 – 1.5 mm, brownish black in colour (Prabhugaonkar *et al*., 2009).

1.1.4. Taxonomic Significance:

Dipcadi goaense, a newly discovered species within the genus *Dipcadi*, stands as a testament to the rich biodiversity of the Western Ghats. It is represented by 30 species distributed in the Mediterranean region, Madagascar, Africa and South West Asia (Mabberley 1997). Ten species of this genus are reported in India with four varieties which include *D. concanense* (Dalz.) Baker; *D. erythraeum* Webb & Berth; *D. goaense*A. Prabhugaonkar, U. S. Yadav & Janarth.; *D. maharshtrensis* Deb et Dasgupta; *D. minor* Hook. f.; *D. montaum* (Dalz.) Baker var. montanum; *D. montaum* (Dalz.) Baker var. madrasicum (Barnes & Fischer) Deb et Dasgupta; D. reidii Deb et Dasgupta; *D. saxorum* Blatt.; *D. serotinum* (L.) Medik.; *D. ursulae* Blatt. var. ursulae and *D. ursulae* Blatt. var. longiracemosaei Deb et Dasgupta).

This species is taxonomically aligned with the Hyacinthaceae family, and exhibits morphological distinctiveness, notably in floral structures, differing significantly from its allied counterparts, such as *D. concanense* and *D. montanum*. The species is characterized by its diminutive flowers, funnel-shaped perianth tubes, and specific floral and vegetative traits, marking its botanical uniqueness in this ecosystem (Prabhugaonkar *et al*., 2009).

All *Dipcadi* species in India flowers from June to July and disperse seeds in august and persist through tunicated bulbs (Mahamble *et al.,* 1960).

1.1.5. Ecological Context:

The Western Ghats, acclaimed as a biodiversity hotspot, host various endemic and rare flora, including *Dipcadi goaense*. These plants often thrive in distinct ecological niches, contributing significantly to the fragile ecosystems of the region, especially in the foothills and sacred groves. The existence of *Dipcadi goaense* in these specific habitats

within the Ghats emphasizes its ecological adaptability and potential role in maintaining the delicate balance of these unique ecosystems (Prabhugaonkar *et al*., 2009).

1.1.6. Medicinal and Phytochemical Significance:

The genus *Dipcadi* has garnered attention in ethnobotanical practices and pharmaceutical research due to its reported medicinal properties. Various studies have explored the phytochemical constituents of *Dipcadi* species, revealing the presence of flavonoids, alkaloids, saponins, and other secondary metabolites saponins (Abdulkareem *et al*., 2014; Adly *et al*., 2015; Ali, 2005). Such compounds have exhibited promising bioactivities, including antioxidant, antimicrobial, and even anticancer properties, indicating the therapeutic potential of these plants' properties (Farida Adly *et al*., 2015; V. Jyothi *et al*., 2018).

Dipcadi species are medicinal plants with great folk uses. The bulbs and capsules were eaten raw during the famine (Jongbloed *et al*., 2000; Mandaville, 1990). The leaves are laxative and used as an ointment for wounds (Moussaid *et al*., 2013), while the whole plant is used for a cough, biliousness, diabetes, urinary and discharge. Chopped bulbs are fed to animal against stomach pain, scorpion stinging, sweating and discharge (Chouhan, 2023; Mahmood *et al*., 2011; Akhtar *et al*., 2013; Surendra *et al*., 2016).

1.1.7. Conservation Imperative.

Despite their ecological and pharmacological significance, many *Dipcadi* species, including *D. goaense*, confront severe threats of extinction due to habitat degradation, anthropogenic activities, and limited conservation measures. The critical endangerment of *Dipcadi goaense* necessitates urgent scientific attention, comprehensive botanical documentation, and conservation interventions to safeguard its existence and preserve the rich biodiversity of the Western Ghats. *Dipcadi* species along with other plant species has

been conserved as a part of threatened plants and habitats by *in situ* conservation methods (Joshi *et al*., 2012). Ex- situ germplasm conservation of *D. saxorum* by using seeds as propagation material was carried out by Srinivasa *et al*. (2009).

1.1.8. Phytochemistry

The importance of the Indian species of *Dipcadi* resides not only in their endemism and narrow distribution but also in their phytochemical constituents.

Phytochemicals, naturally occurring compounds found in various parts of plants, serve as essential defence mechanisms against herbivores. However, research indicates that these compounds offer significant health benefits to humans as well (Mujjeb *et al*., 2014). Despite appearing non-essential to plants, phytochemicals play a crucial role in their survival by mediating ecological interactions, protecting against diseases, pollution, stress, and UV radiation. Additionally, they contribute to the colour, aroma, and flavour of plants.

The extraction of phytochemicals from plants involves various techniques and solvents. The effectiveness of extraction depends on the methods employed and the types of solvents used. Different solvents have varying polarities and properties, influencing the extraction process and the yield of phytochemicals.

Studies on phytochemical analysis of the genus *Dipcadi* have shown the presence of various phytoconstituents such as tannins, alkaloids, flavonoids, and saponins (Abdulkareem *et al*., 2014; Adly *et al*., 2015; Ali, 2005). In the case of *Dipcadi krishnadevaravae*, the phytochemicals present in the stem and bulb exhibit considerable antioxidant activity when compared with standard antioxidants (Jyothi *et al*., 2018).

Shabrawy *et al*. (2016) isolated two flavonol aglycones (Kaempferol and Quercetin), one flavonol glycoside (quercetin 3-O-rutinoside-7-O-α-rhamnopyranoside), and four C-glycosyl flavones (Vitexin, Isovitexin, Orientin, and Isoorientin) from the fat-

free aqueous methanol extract of *Dipcadi erythraeum* whole plant. Additionally, the bulb extract of *D. erythraeum* revealed the presence of 22 phenolic compounds, among which 14 were identified as C-glycosyl flavonoids. This finding suggests a closer relationship with species of the Asparagaceae family rather than the Liliaceae family (Marzouk *et al*., 2019).

1.1.9. Histochemistry

Histochemistry serves as a methodological approach for the chemical analysis of cells and tissues in correlation with their structural organization. Through histochemistry, biological substances within cells and tissues can be visualized and identified, allowing for the distribution of different chemical compounds to be determined using various stains, indicators, and microscopy techniques (Wick, 2012). Active cell constituents such as starch, proteins, lipids, nucleic acids, and various elements can be detected and localized within cells using histochemical methods (Badria and Aboelmaaty, 2019).

Plants produce secondary metabolites that serve as significant sources of biologically active substances (Antonisamy *et al*., 2015; Balamurugan, 2015; Dhabi *et al*., 2015; Valsalam *et al*., 2019; Rajkumari *et al*., 2019). Syamasundar and Panchaksharappa (1976) conducted histochemical screening to localize the presence of macromolecular substances, including nucleic acids, polysaccharides, and proteins, during the seed development of *Dipcadi montanum*.

1.1.10. *In-Vitro* Culture Studies

Tissue culture an important area of biotechnology can be used to improve the productivity of planting material through enhanced availability of identified planting stock with desired traits. Advanced biotechnological methods such as plant cell, tissue and organ culture are playing a major role in production of valuable secondary metabolites as well as

propagation of important medicinal plant species. Plant tissue culture technology may help in the conservation of many endangered and rare medicinal plants species.

In vitro culture of *Dipcadi montanum* using bulb explant showed highest rate of multiple shoot induction in MS Medium containing BAP (2.0 mg/L) with as response of 98 ±0.24% and 12.4±0.40cm of shoot length. Efficient root induction was achieved by IAA at 1.0mg/L with 89% of response and highest number of roots per shoot (15.2 ± 024) (Radhika *et al*., 2020).

1.2. AIMS AND OBJECTIVES

1. Investigate the anatomical features of *Dipcadi goaense* for comprehensive morphological characterization, enhancing our understanding of its unique structural adaptations.
2. Localize primary and secondary metabolites within *Dipcadi goaense* bulbs to elucidate its biochemical composition and potential medicinal value.
3. Employ chromatographic techniques to identify and quantify phytochemical compounds in *Dipcadi goaense*, contributing to the species-specific chemical profile for conservation and pharmacological insights.
4. Establish preliminary *in vitro* culture protocols for *Dipcadi goaense* to explore its potential for mass propagation, preservation, and sustainable utilization, contributing to conservation efforts of this critically endangered species.

1.3. HYPOTHESIS

The anatomical study of *Dipcadi goaense* may uncover unique structural adaptations, revealing specialized cellular arrangements and tissue types. This exploration could enrich its taxonomic classification and ecological significance. Efforts to localize metabolites will provide insights into the spatial distribution of primary and secondary

metabolites within the plant, offering valuable information about its biochemical composition and potential medicinal properties. Chromatographic techniques will be employed to identify and quantify diverse phytochemical compounds, establishing a species-specific chemical profile crucial for conservation strategies and pharmacological investigations. The establishment of preliminary tissue culture protocols may illuminate pathways for mass propagation, preservation, and sustainable utilization of *Dipcadi goaense*, thereby significantly contributing to its conservation and propagation efforts.

1.4. SCOPE

Considering the taxonomic novelty, ecological importance, and potential medicinal value of *Dipcadi goaense*, this study aims to conduct a multifaceted investigation encompassing anatomical characterization by using safranin stain for comprehensive morphological characterization, enhancing our understanding of its unique structural adaptations. Histochemical localization will help to localize primary and secondary metabolites within *Dipcadi goaense* leaf and bulbs to elucidate its biochemical composition and potential medicinal value.

The phytochemical studies using TLC methods will help to identify different phytochemicals present in the leaf and bulb extract of *Dipcadi goaense* and using UV-Vis spectrophotometer we will be able to quantify phytochemical compounds in *Dipcadi goaense*, contributing to the species-specific chemical profile for conservation and pharmacological insights.

Preliminary *in vitro* culture studies using bulb explants *Dipcadi goaense* will help to explore its potential for mass propagation, preservation, and sustainable utilization, contributing to conservation efforts of this critically endangered species.

CHAPTER 2: REVIEW OF LITERATURE

The genus *Dipcadi* is represented by 30 species distributed in the Mediterranean region, Madagascar, Africa and South West Asia (Mabberley, 1997). *Dipcadi goaense* is a new species in *Dipcadi* genus which is apparently found to be located in the lateritic rocky area with gravelly soil and the species is known by only a single population restricted to Rivone, Kevan foothills of Western Ghats, South Goa, India (Prabhugaonkar *et al*., 2009).

The relevant literature on the present study has been briefly reviewed to understand the different aspects of the study carried out.

This species is closely related *D. concanense* (Dalzell) Baker but differing in its small flowers (13 – 18 mm long vs 35 – 47 mm long) and funnel shaped perianth tube (5 – 6 × 5 – 6 mm vs 18 – 27 × 4 – 5.5mm), which is been proved through phylogenetic tree resulting from ML as well as Bayesian analysis (Deshpande *et al*., 2015).

Kumar *et al*. (2013) reported some critical notes on *D. montanum* var. *madrasicum* and gave detailed description, photographs, distributional and ecological details of the species.

Syamasundar and Panchaksharappa, (1976) localized the presence of macro molecular substances, namely nucleic acids, polysaccharides and proteins during the seed development of *Dipcadi montanum* and also attempted to correlate the observed variations in the cytochemical substances to the morphological changes accompanying the seed development.

Gosavi *et al*. (2011) described distribution, ornamental potential, and karyotype analysis of a *Dipcadi* species, and reported the mitotic count in the species. Samanta *et al*., (2023) compared chromosome banding profiles of different *Dipcadi* species and revealed that the karyotypes of *Dipcadi concanense* and *Dipcadi goaense* exhibited similar

flurochrome banding profile whereas *Dipcardi erytharaeum* differed distinctly in their karyotypes.

Dyka *et al*. (2018) conducted a study on gynoecium micromorphology in different species of Ornithogaloideae. They examined three main parts of the ovary: the parenchymatous ovary base, ovary locules, and ovary roof. The researchers established that this gynoecium consisted of several structural zones, including Synascidiate, Symplicate, Hemisymplicate, and Asymplicate, following W. Leinfellner's concept of the structural zone synascidiate.

They observed that the septal nectary had three separated cavities located on the septal radiuses, which extended from the ovary base to the roof and opened outward at the style base. In the studied species, the nectary exhibited a zone of distinct nectary with congenitally closed nectary cavities in the synascidiate and symplicate zones, a common nectary zone with a postgenitally closed central part in the hemisymplicate zone, and a zone of distinct nectary (nectary split). Nectary deferent channels were located at the level of the asymplicate zone of the gynoecium.

The researchers also noted that the total height of the septal nectary did not exceed the height of the ovary locules in the studied species. Additionally, they found that the walls of the splits were covered with the same secretory epidermis as the walls of the septal nectary cavities. Based on these observations, Dyka *et al*. (2018) concluded that the gynoecium of *Dipcadi brevifolium*, *Ornithogalum fimbriatum*, *Ornithogalum dubium*, and *Ornithogalum orthophyllum* subsp. Kochii could be determined as eusyncarpous in the broad sense with a common septal nectary.

Hemalata and Kameshwari (2015) investigated the detailed morpho-anatomical features of *Urginea indica*, focusing on characteristics of the leaf, flower, and pollen. Their study revealed notable findings in the macerated material of bulbs, where xylem

tracheids/vessels were observed. These elements appeared short and wider in diameter, indicating an advanced state among monocots. The roots exhibited a distinct radial polyarch condition with casparian thickenings in the endodermis. In terms of leaf morphology, stomata were identified as hypostomatic and anomocytic type. The flowers were observed to be trimerous and bisexual. Examination of the anthers in transverse section revealed clear four pollen chambers, indicating dithecous anthers. The pollen shape was characterized as ellipsoid, with a reticulate surface texture.

Pohl*et al*. (2000) reviewed chemical constituents, bioactivity, ethnobotany, and pharmacology of southern African hyacinths. The chemical constituents from plants of this family have been divided into four classes, namely, homoisoflavanones, steroidal compounds, bufadienolides and miscellaneous compounds. A single report on the occurrence of Amaryllidaceae alkaloids in *Urginea altissima* is questioned following a reinvestigation of this species. Plants are employed for traditional medicinal purposes ranging from the treatment of hangovers, rheumatic fever, sprains and syphilis to cancer. Several species have been toxicologically assessed following human deaths and livestock losses after ingestion. The bioactivity and ethnobotany of southern African hyacinthacs is reviewed, as is the pharmacology of isolated constituents and crude extracts.

Adly *et al*. (2015) characterized chemical constituents, from the aqueous extract of *Dipcadi serotinum* (L.) Medik and revealed the presence of flavonoids, alkaloids, sterols, tannins, reducing compounds and saponosides. The amount of total phenolics and total flavonoids were evaluated. The extract contained 65 mg/g of phenolics, and flavonoids 2.34 mg/g. Furthermore, they evaluated the antioxidant activities, anti-radical properties, and antimicrobial activity.

Seven flavonoids were isolated for the first time from the defatted aqueous methanol extract of *Dipcadi erythraeum (*Webb & Berthel). These flavonoids were

identified as kaempferol (1), quercetin (2), quercetin 3-*O*-(6″-α-rhamnopyranosyl)-β-glucopyranoside-7-*O*-α-rhamnopyranoside (3), vitexin (4), isovitexin (5), orientin (6) and isoorientin (7). Their structures were established based on chemical and spectroscopic analysis and by comparison with authentic samples. Summarization of the chemosystematic significance of these compounds was also done (Shabrawy *et al*.2016).

Phytochemical investigations of *Dipcadi Krishnadevarayae* revealed that methanolic extract of both whole stem and bulbous parts possessed flavonoids, cardiac glycosides, alkaloids, saponins and tannins. Significant anthelminthic activity was found with methanolic stem extract against standard drug Albendazole. Antioxidant potential was performed by Folin-Ciocalteu's method, Free Radical Scavenging Activity (FRSA), UV spectrophotometric method and Oyaizu method and the results revealed that both methanolic stem and bulb extracts possessed considerable antioxidant activity when compared with standard antioxidants (Jyothi *et al*. 2018).

Temikotan *et al*. (2013) conducted a comprehensive phytochemical screening and quantitative determination of alkaloids, saponins, flavonoids, phytates, tannins, and cardiac glycosides in selected members of the Hyacinthaceae family. This study aimed to provide valuable tools for assessing the significance of these phytochemicals in plant protection. Similarly, Mulholland *et al*. (2013) investigated compounds such as homoisoflavanones and spirocyclic nortriterpenoids, which are utilized for characterizing the Hyacinthoideae subfamily.

Marzouk *et al*. (2019) conducted a study where the bulbs extract of *D. erythraeum* underwent chemical investigation using Liquid Chromatography-Electrospray Ionization Mass Spectrometry (LC-ESI-MS) technique to identify its polar active constituents. The evaluation against four human carcinoma cell lines (MCF7, HEPG2, A549, and HCT116)

revealed the presence of 22 phenolic compounds, characterized for the first time from the studied species, with 14 of them identified as C-glycosyl flavonoids. Additionally, Hadi and Najjar (2020) reported flavonoids and carbohydrates in *D. erythraeum* through phytochemical screening of the active constituents in the bulbs, stems, leaves, and flowers. While high glycoside levels were found, the phytochemical screening did not indicate significant levels. However, the presence of flavonoids and starchy carbohydrates was notable, prompting further investigation into flavonoids.

Aswal *et al*. (2019) conducted a review on the traditional uses, phytochemistry, pharmacology, and potential clinical applications of *D. indica*. Their findings suggested that the plant, particularly its bulb, contains various bioactive constituents such as alkylresorcinols, bufadienolides, phytosterols, and flavonoids, indicating its potential in treating various diseases, especially microbial infections.

Dormousoglou *et al*. (2023) investigated the phytochemical profile of the methanolic leaf extract of *Drimia numidica* and its potential cyto-genotoxic and cyto/genoprotective effects against mitomycin C (MMC)-mediated effects on healthy human lymphocytes. They concluded that the methanolic leaf extract of *D. numidica*, rich in carotenoids, phenolics, flavonoids, organic acids, and bufadienolides, exhibited protective properties against MMC-mediated cyto/genotoxic effects in healthy human lymphocytes.

Radhika *et al*. (2020) demonstrated efficient shoot and root induction protocols from *D. montanum* bulbs *in vitro*. The study revealed that the highest rate of multiple shoot induction from bulb explants occurred in the MS Medium containing BAP (2.0 mg/L), with a response rate of 98 ± 0.24% and a shoot length of 12.4 ± 0.40 cm. Efficient root induction was achieved using IAA at 1.0 mg/L, resulting in an 89% response rate, with the highest number of roots per shoot (15.2 ± 0.24) obtained.

Özel (2022) developed a regeneration protocol for *Muscari adilii* using different growth regulators like Benzyl aminopurine (BAP) and α-naphthaleneacetic acid (NAA) with twin bulb scales under *in vitro* conditions. The best medium for bulblet regeneration on twin scales was determined as 1 × MS Medium containing 17.76 µM BAP and 2.685 or 10.74 µM NAA, resulting in 15.75 and 14.18 bulblets per explant, respectively. When induced primary bulbs were used as explants, they regenerated 4.25 secondary bulbs on 1 × MS Medium with 17.76 µM BAP and 2.685 µM NAA, along with an increase in the diameter of the induced primary bulbs. The maximum diameter of 0.39 cm for induced primary bulbs was observed on 1 × MS Medium containing 4.44 µM BAP and 5.37 or 10.74 µM NAA.

Baskaran *et al*. (2013) established *in vitro* mass propagation and evaluated antibacterial activity using leaf explants of *D. robusta*. Rapid *in vitro* mass propagation of the species was achieved for commercial cultivation from leaf explants using various concentrations and combinations of plant growth regulators and organic elicitors. The study reported that the highest number of regenerated shoots per explant (14.6 ± 0.54) was obtained on Murashige and Skoog (MS) Medium supplemented with a combination of 2.27 µM Thidiazuron (TDZ), 2.22 µM benzyl adenine (BA), and 20 µM glutamine

Joshi *et al*. (2012) focused on conservation efforts through *in situ* methods to conserve *Dipcadi* species, along with other plant species, as a part of threatened plants and habitats. Srinivasa *et al*. (2009) concentrated on *ex-situ* germplasm conservation of *D. saxorum* using seeds. Panchaksharappa and Syamasundar (1975) investigated ovule development in *D. montanum*, highlighting protein and polysaccharide concentrations. They reported low concentrations of proteins in the 2-nucleate embryo sac, while the nucellus exhibited higher concentrations of proteins. Additionally, they observed low

concentrations of polysaccharides in the 4-nucleate embryo sac, whereas the hypostase cells showed slightly higher concentrations of polysaccharides.

Bothaa and Penrith (2008) investigated the use of bulbs for consumption in Africa, noting that some species of *Dipcadi* contain toxins that affect the central nervous system. Specifically, they found that *Dipcadi glaucum* causes incoordination and diarrhea in ruminants.

CHAPTER 3. METHODS AND METHODOLOGY

3.1. COLLECTION OF PLANT MATERIALS

Bulbs and other plant parts of *Dipcadi goaense* were collected from the month of July - September from the type locality Rivona, Kevan – foothills of Western Ghats, Goa India, 15º08'34.14" N, 74º08'03.66" E. During the study period of 2023-2024, healthy plant parts were collected in separate Zip-lock bags and brought to Goa University laboratory which were used for study purpose.

3.2. SAMPLE PREPARATION

The bulbs of *Dipcadi goaense*, collected from their original location, were cultivated in laboratory pots using soil obtained from the university campus. The leaves that sprouted from these bulbs were utilized for anatomical, histochemical, and phytochemical studies.

3.3. ANATOMICAL STUDIES OF *DIPCADI GOAENSE* PLANT PARTS

Anatomical studies of the plant species *Dipcadi goaense* were carried out to understand different anatomical features such as cell types, arrangement presence of specialised structures, and adaptations for ecological niche survival in leaf and bulb. Free-hand thin transverse section of leaf and bulb of *Dipcadi goaense* were stained with safranin. Then the sections were washed thoroughly with distilled water and mounted on the clean glass slide using 10% glycerine. The sections were examined and photographed using bright field microscopy.

The following standard procedure was performed for preparation of stains.

Safranin: 0.1% w/v Safranin was prepared by dissolving 0.1g of safranin in 100 mL of distilled water. Thin free-hand sections were placed in water and then kept for 2-3 minutes

in safranin stain. Excess of stain was removed by washing the sections in distilled water. The sections were mounted on clean stain- free glass slide using 10% glycerine and observed under bright –field Nikon Eclipse E200 microscope under 4x,10, and 40x magnification.

3.4. HISTOCHEMICAL LOCALIZATION IN *DIPCADI GOAENSE* PLANT PARTS

Histochemical tests were performed on thin sections of leaf and bulb sections of *Dipacdi goaense*. Thin free-hand sections were applied with Specific histochemical stains for the histochemical localization of plant primary (starch, proteins and lipids) and secondary metabolites.

3.4.1. Localization of plant primary metabolites

Plant organs such as leaf and bulb were used. The sections of the same were taken and employed with following test for detection of starch, proteins and lipids.

3.4.1.a. Localization of starch (I_2KI)

Preparation of stain: 0.2 g of iodine was dissolved in 2% iodine potassium iodide solution (I_2KI).

Sample preparation: Sample preparation involved placing thin free-hand sections in distilled water, followed by staining them with potassium iodide solution for 1-2 minutes. Excess stain was removed by washing with distilled water. Subsequently, the sections were mounted with 10% glycerine and observed under bright-field microscopy. Starch grains appeared blue to black in color, while newly formed starch granules appeared red to purple (Krishnan *et al*., 2001).

3.4.1.b. Localization of lipids (Sudan-IV)

Preparation of stain: 0.7 g of Sudan IV was dissolved in 100mL of 95% ethanol to prepare Sudan IV dye. Filter the dye before use.

Sample preparation: Thin free-hand sections were pre-treated with 70% ethanol for 1-2 minutes and were stained with filtered solution of Sudan IV for 5-20 minutes. The sections were then thoroughly washed with 50% ethanol for few seconds, dried mounted in 10% glycerine, and observed under bright-field microscope. Pink or red colour reactions indicated the presence of lipids (Krishnamurthy, 1988; Krishnan *et al*., 2001).

3.4.1.c. Localization of proteins (Coomassie brilliant blue) (CBB)

Preparation of stain: 0.02% Coomassie brilliant blue was prepared in Clark's solution at pH 2. Clark's solution was prepared by adding 1 part of glacial acetic acid to 3 parts of absolute ethanol.

Sample preparation: Thin free-hand sections were initially placed in 50% ethanol for 5 minutes, followed by transfer into 0.02% Coomassie brilliant blue stain for 10-15 minutes. Subsequently, the sections were thoroughly washed in Clark's solution to remove excess stain. Finally, they were mounted with 10% glycerine and observed under bright-field microscopy, where proteins appeared blue due to staining.

3.4.2. Plant secondary metabolites localization

For histochemical plant secondary metabolites localization plant part sections of leaf and bulb were subjected with the following stains for detection of alkaloids and phenolic compounds.

3.4.3.a. Detection of alkaloids (Dragendroff's reagent)

Preparation of stain: 5.2 g of bismuth carbonate and 4 g of sodium iodide were boiled for a few minutes with 50 mL of glacial acetic acid. This mixture was then allowed to stand for 12 hours. The precipitated sodium acetate crystals were filtered using a glass funnel. Subsequently, 40 mL of the clear red-brown filtrate was mixed with 160 mL of

ethyl acetate and 1 mL of water, and the resulting solution was stored in an amber-colored bottle.

To prepare the working solution, 10 mL of the stock solution was mixed with 20 mL of acetic acid and made up to 100 mL with water, as described by Svendsen and Verpoorte (1983).

Sample preparation: Free-hand transverse sections of leaf and bulb were stained with Dragendroff's reagent, followed by washing the sections with 5% sodium nitrate, yellow to reddish- orange colour indicated the presence of alkaloids.

3.4.2.b. Detection of Phenolic compounds (2% Ferrous sulphate in 10% formalin)

Preparation of stain: 2g of ferrous sulphate was dissolved in 100 mL distilled water to make 2% of ferrous sulphate and 10 mL of formalin was diluted in 90 mL distilled water to make 10% formalin.

Sample preparation: Free-hand transverse sections of the leaf and bulb were placed in distilled water. These sections were then stained with 2% ferrous sulphate in 10% formalin (1:1 v/v). After staining, the sections were rinsed with distilled water and mounted with 10% glycerine. Subsequently, the sections were observed under a bright-field microscope. Positive reaction for phenolic compounds was marked by grey to black coloration, as described by Gahan (1984).

3.5. PHYTOCHEMICAL ANALYSIS

3.5.1. Extraction of plant extract

The bulbs and leaves of *Dipcadi goaense* were first washed thoroughly with distilled water to remove any impurities. Subsequently, the cleaned leaves and bulbs were cut into small pieces and dried at 60°C in an oven. Once completely dried, they were

pulverized into a powder using a grinder and stored in a container for further experimentation.

For the extraction process, 10 g of the powdered material was successively extracted in 250 mL of methanol and ethanol solvents for 24 hours using the Soxhlet extraction method. After extraction, the solvents were concentrated to dryness under reduced pressure using a rotary evaporator. The resulting dried extract was then stored at 4°C for further analysis.

3.5.2. Thin layer chromatography (TLC) analysis

To distinguish the different bio-active chemical present in crude extract of plant, aluminium foil backed silica gel plates, dimensions 5 cm X 10 cm (Merck, Darmastat, Germany) were used to analyse leaf and bulb extract of *Dipcadi goaense* for their phytochemical constituent compositions. The plant sample dissolved in methanol were spotted on a single TLC plate and dried. **Table 3.1** lists the mobile phases, conditions and derivatizing agents used for detection of various secondary metabolites. The spots were visualized and examined before and after dipping with derivatizing agent (Wagner and Bladt, 1996; Naik and Sellappan, 2020).

The Rf values were calculated by using following formula:

$$\mathrm{Rf} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

Table 3.1. Mobile phases and derivatizing agents used for TLC for respective secondary metabolites.

TLC analysis			
Class of compound	**Mobile Phase**	**Derivatisation**	**Observation**
Alkaloids	Toluene: Ethyl acetate: Methanol: 25% Ammonia (30:30:15:1) v/v/v/v	Dragendroff's reagent	Orange bands
Phenolic compounds	Tetra Hydrofuran: Toluene: Formic Acid: Water (16:8:2:1)	Alcoholic Ferric Chloride	Dark Blue zones
Steroids	n-Butanol: Methanol: Water (3:1:1) v/v/v	Anisaldehyde sulphuric Acid	Purple, Maroon bands
Flavonoids	Tetrahydrofuran: Toluene: Formic acid: Water (16:8:2:1) v/v/v	Natural Product or 10% Methanolic Sulphuric Acid.	Florescent compounds, red fluorescence plant Pigment –chlorophyll.

3.5.3. Phytochemical Estimation of secondary metabolites

3.5.3.a. Determination of Phenolic Contents

The total phenolic contents were determined as Gallic acid equivalents (mg GAE/g) of dry extract as per Folin and Ciocalteu reagent method (Singleton and Rossi, 1965). Briefly, 1 mL of extract or Gallic acid (10-100 µg/mL) was mixed with 5 mL 10 % Folin-Ciocalteu phenol reagent. 5 mL of 1M sodium carbonate was added after 15 minutes. After allowing the mixture to incubate for 30 minutes the absorbance was measured at 760 and 765nm. The total phenol content was measured from calibration curve where $y = 0.0075x+0.9052$, $R^2 = 0.9838$ was utilized for determination of content.

3.6. TISSUE CULTURE STUDIES

3.6.1 Plant material

Bulbs of *Dipcadi goaense* were collected in the month of September from the type locality Rivona, Kevan – foothills of Western Ghats, Goa-India. During the study period of 2023-2024.

3.6.2. Sterilization Procedure

3.6.2.a. Sterilization of Glassware

The glassware intended for tissue culture studies underwent a thorough cleaning process. Initially, they were washed with tap water and then immersed in a teepol solution for 15 minutes. Following this, they were rinsed with water to remove any remaining residue. Subsequently, the glassware was subjected to heat sterilization by exposure to dry hot air at temperatures ranging from 130 to 170°C for a duration of 2 to 4 hours in a hot air oven.

Similarly, all equipment required for media preparation and inoculation, including forceps, scalpels, and cotton, were wrapped in newspaper and sterilized using the wet sterilization method. This involved autoclaving the equipment at a temperature of 121°C under 15 psi pressure for a duration of 15 minutes.

3.6.3. MS culture Medium

3.6.3.a. Preparation of stock solution

During the study, Murashige and Skoog (MS) Medium (1962) was utilized. The chemical composition of the medium is provided in **Table 3.2**. Stock solutions of all the media constituents were prepared by accurately weighing the required amount of chemicals and dissolving them in sterile distilled water at a concentration of g/L. The

details of these stock solutions are provided in **Table 3.3**. For the preparation of stock solution E, iron sulfate and sodium EDTA were dissolved separately in sterile distilled water to achieve the desired concentrations. Subsequently, the iron sulfate solution was added to the sodium EDTA solution, and the final volume was adjusted using sterile distilled water. This prepared stock solution was then stored in an amber bottle. All the stock solutions were stored at 4°C in a refrigerator for further use in the study.

Table 3.2. Composition of MS Medium (Murashige and Skoog,1962).

Nutrients	***Constituents***	***(mg/L)***
Major salts	Ammonium nitrate ($NH_4NO_{3)}$	1650
	Potassium nitrate (KNO_3)	1900
	Calcium chloride ($CaCl_2.2H_2O$)	440
	Magnesium sulphate ($MgSO_4.7H_2O$)	370
	Potassium dihydrogen orthophosphate (KH_2PO_4)	170
Minor salts	Boric acid	6.2
	Manganese sulphate ($MnSO_4.4H_2O$)	22.3
	Zinc sulphate ($ZnSO_4.7H_2O$)	8.6
	Potassium iodide (KI)	0.83
	Sodium molybdate ($NaMoO_4.2H_2O$)	0.25
	Cobalt chloride ($CoCl_2.6H_2O$)	0.025
	Copper sulphate ($CuSO_4.5H_2O$)	0.025
Iron source	Ferrous sulphate ($FeSO_4.7H_2O$)	27.84
	Sodium ethylenediaminetetraacetic acid ($Na_2EDTA.2H_2O$)	37.24
Organic supplements	Thiamine HCl	0.10
	Nicotinic acid	0.50
	Pyridoxine HCl	0.50
	Glycine	2.00
	myo-Inositol	100

Table 3.3. Stock solution of MS Media.

Stocks	*Constituents*	*Amount (g/L)*	*Strength of stocks*	*Stocks to be taken for 1 litre Medium*
	NH_4NO_3	16.5		
A	KNO_3	19.0	10x	100 mL
	$MgSO_4.7H_2O$	37.0		
B	$MnSO_4.4H_2O$	2.23	100x	10 mL
	$ZnSO_4.7H_2O$	0.86		
	$CuSO_4.5H_2O$	0.0025		
	$CaCl_2.2H_2O$	44.0		
C	KI	0.083	100x	10 mL
	$COCl_2.6H_2O$	0.0025		
	KH_2PO_4	17.0		
D	H_3BO_3	0.62	100x	10 mL
	$NaMoO_4.2H_2O$	0.0025		
	$FeSO_4.7H2O$	2.784		
E	Na_2 EDTA.$2H_2O$	3.724	100x	10 mL
	Thiamine HCl	0.010		
F	Nicotinic acid	0.050	100x	10 mL
	Pyridoxine HCl	0.050		
	Glycine	0.200		
	myo-Inositol	10.0		

3.6.3.b. Preparation of M.S media

The preparation of culture medium is crucial for the success of plant tissue culture, with the choice of nutrient components and growth factors playing a significant role. In the present study, Murashige and Skoog solid basal medium was employed. To prepare 1 Litre of MS medium, 400 mL of warm double-distilled water was added to a 1 L conical flask. Subsequently, 100 mL of stock solution A was added, followed by 10 mL each of stock solutions B, C, D, E, and F (refer to **Table 3.3**). Additionally, 30 g of sucrose (as the carbon

source) was added to the medium and dissolved using a magnetic stirrer. The final volume was adjusted by adding distilled water, and the medium was thoroughly mixed with the help of a magnetic stirrer.

After adding the required plant growth hormones (auxins and cytokinins), the pH of the medium was adjusted to 5.7 using either 0.1N NaOH or 0.1N HCl. Subsequently, 8 grams of agar (0.8%) was added to the medium, and the culture vessel containing the medium was autoclaved at 15 psi at 121°C for 15 minutes. Once autoclaved, 15 mL of molten agar medium was dispensed into sterile test tubes (150 x 25mm) to prepare slants. The culture tubes and vessels were sourced from Borosil Glass Works Pvt. Ltd, Mumbai, India. After solidification of the medium, the test tubes were covered with autoclavable test tube caps (Tarsons Products Ltd.) and were ready to be used for culturing purposes.

3.6.3.c. Preparation of stock solutions of plant growth regulators

Stock solution of all plant growth regulators, cytokines such as 6-benzylaminopurine (BAP) and auxins such as indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) were prepared as mg/mL stock solution (**Table 3.4**). All plant growth regulators were obtained from HiMedia Laboratories Private Limited. 100 mg of plant growth regulator was dissolved in few drops of appropriate solvent (auxins in 95% ethanol and cytokines in NaOH) and final volume was made up 100 mL with sterile distilled water. All the stock solutions were sterilised and stored in amber bottles at 4ºC.

3.6.4. Explant

Mature bulbs of *Dipcadi goaense* were used as explant.

3.6.4.a. Surface Sterilization of Explant

Dipcadi goaense bulbs were collected from the healthy plant and washed thoroughly for 20-30 min in running tap water until all the mud or dust particles were cleaned. Washed bulbs were surface sterilized in laminar air flow. The bulb explants were treated with 70 % ethanol for 30s after that rinsed thrice with sterile distilled water followed by 0.1 % $NaOCl_2$ for 3 minutes. The explants were washed 5 times with sterile distilled water to remove all the traces of sterilant.

Table 3.4. Plant growth regulators.

Combination No	***Plant Growth Regulators (PGR's) Combination***	***Concentration (mg/mL)***	***Molecular Weight***
Auxin + Cytokines			
1	2,4-D + BAP	3 + 1	221.04 + 225.25
		3.5 + 1	
		4 + 1	
2	NAA + BAP	3 + 1	186.21+ 225.25
		3.5 + 1	
		4 + 1	
3	IBA + BAP	3 + 1	203.24 + 225.25
		3.5 + 1	
		4 + 1	

3.6.5. Inoculation

The inoculation of the explant was carried out in laminar air-flow cabinet. The inner surface of the laminar air flow was swabbed with 70% alcohol using absorbent cotton. Prior to inoculation all the necessary equipment's such as pre-autoclaved forceps, surgical blades, surgical blade holders, cotton, petri dishes, culture vials and culture vials with Media were kept inside the laminar air cabinet in the presence of UV light for about 15-20 minutes. Later, culture Medium, explant etc. were transferred inside the laminar air flow cabinet. Prior to inoculation the forceps and the surgical blade were dipped in 95%

ethyl alcohol, and was flame sterilised. With the help of this sterile forceps, the surface sterilised explant was carefully inoculated on slants of culture medium containing different concentration of auxins and cytokines.

3.6.6. Incubation and culture conditions

The cultures were incubated at 25+°C 25 at 16/8 hours photoperiod. The relative humidity was maintained at 50-60%. The results were recorded based on visuals observations and photographs were taken. In case of contamination the requisite re-inoculation was done.

3.6.7. Observation and Data collection

The cultures were observed at regular intervals of every 4 days and percentage of bulb induction was determined after 6 weeks of inoculation.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. GROWTH OF *DIPCADI GOAENSE* BULB

The bulbs of *Dipcadi goaense*, cultivated in pots using soil from the Goa University campus, exhibited growth (see **Plate 4.3**). Notably, full plant growth was observed after 7 weeks (see **Plate 4.4**) from the initial planting of the bulbs. This response in a different environment compared to its natural habitat may be attributed to the similarity in soil type between the original location and the soil at Goa University, both being of the lateritic soil type. The cultivated plants of *Dipcadi goaense* were subsequently utilized for anatomical, histochemical, and phytochemical studies.

4.2. ANATOMICAL STUDIES

The study on the transverse section of the leaf and bulb of *Dipcadi goaense* revealed the following features.

4.2.1. T. S of Leaf

The transverse section (T.S) of the leaf revealed the presence of a thick layer of cuticle on the epidermis. Both the upper and lower epidermis appeared compact and radially arranged, hosting stomata. The leaf exhibited an amphistomatic nature, with an abundance of anomocytic stomata, resembling findings reported by Hemalata and Kameshwari (2015). These stomata were slightly sunken to flush with the surface and consisted of two kidney-shaped guard cells containing chloroplasts, positioned at the same level as the epidermal cells. Notably, leaf hairs were absent. Within the leaf, a ground tissue known as the mesophyll tissue was observed between the upper and lower epidermis. Interestingly, this tissue did not show differentiation into palisade and spongy parenchyma. The mesophyll cells appeared isodiametric. Vascular bundles within the leaf were collateral and typically oval or round in shape **(Plate 4.5)**.

Plate 4.1: Habitat of *Dipcadi goaense*, (a) Overview, (b) Closer view

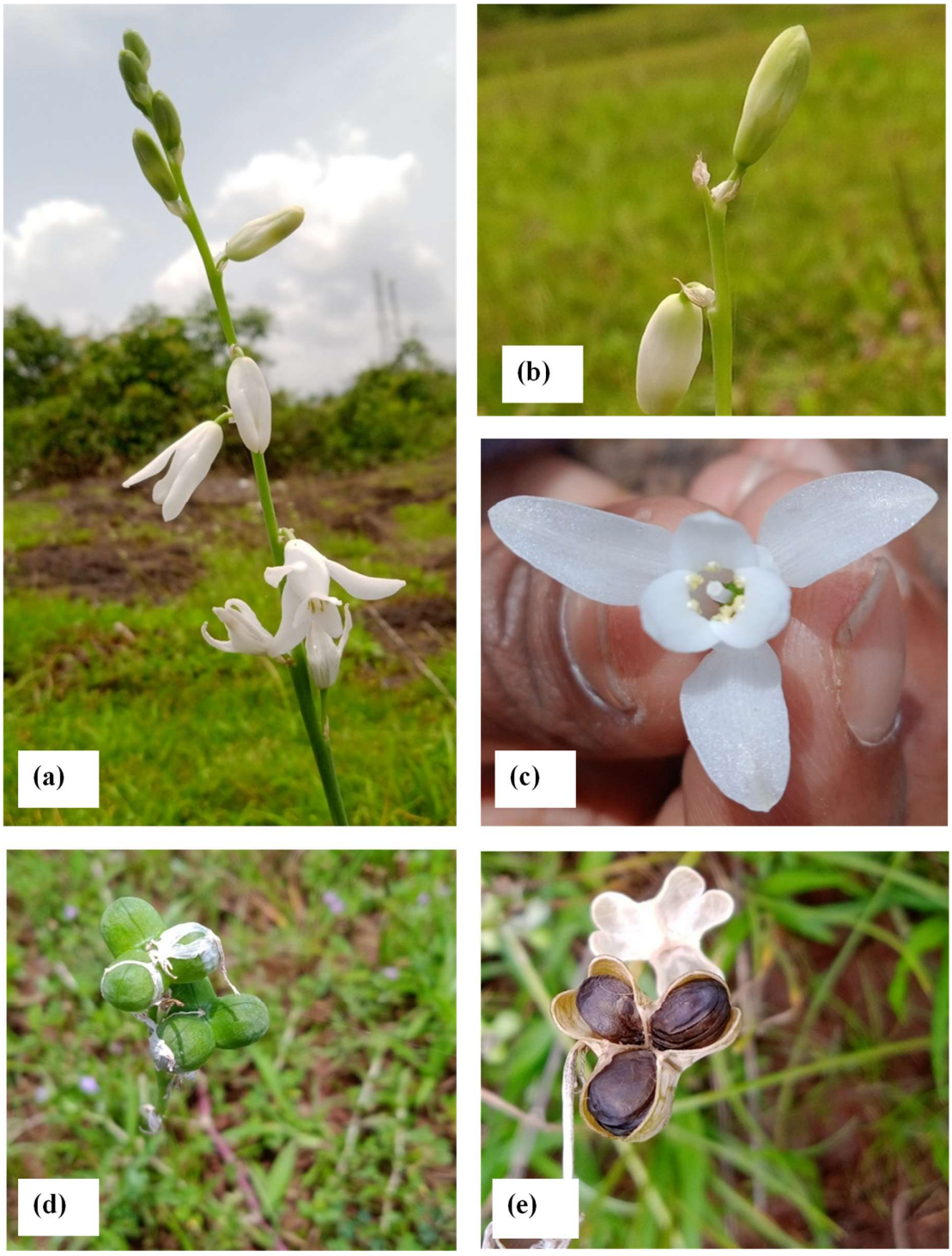

Plate 4.2: Showing *Dipcadi goaense,* (a) Habit, (b) Flower bud, (c) Full bloom flower, (d) Capsule, (e) Dehiscent seed.

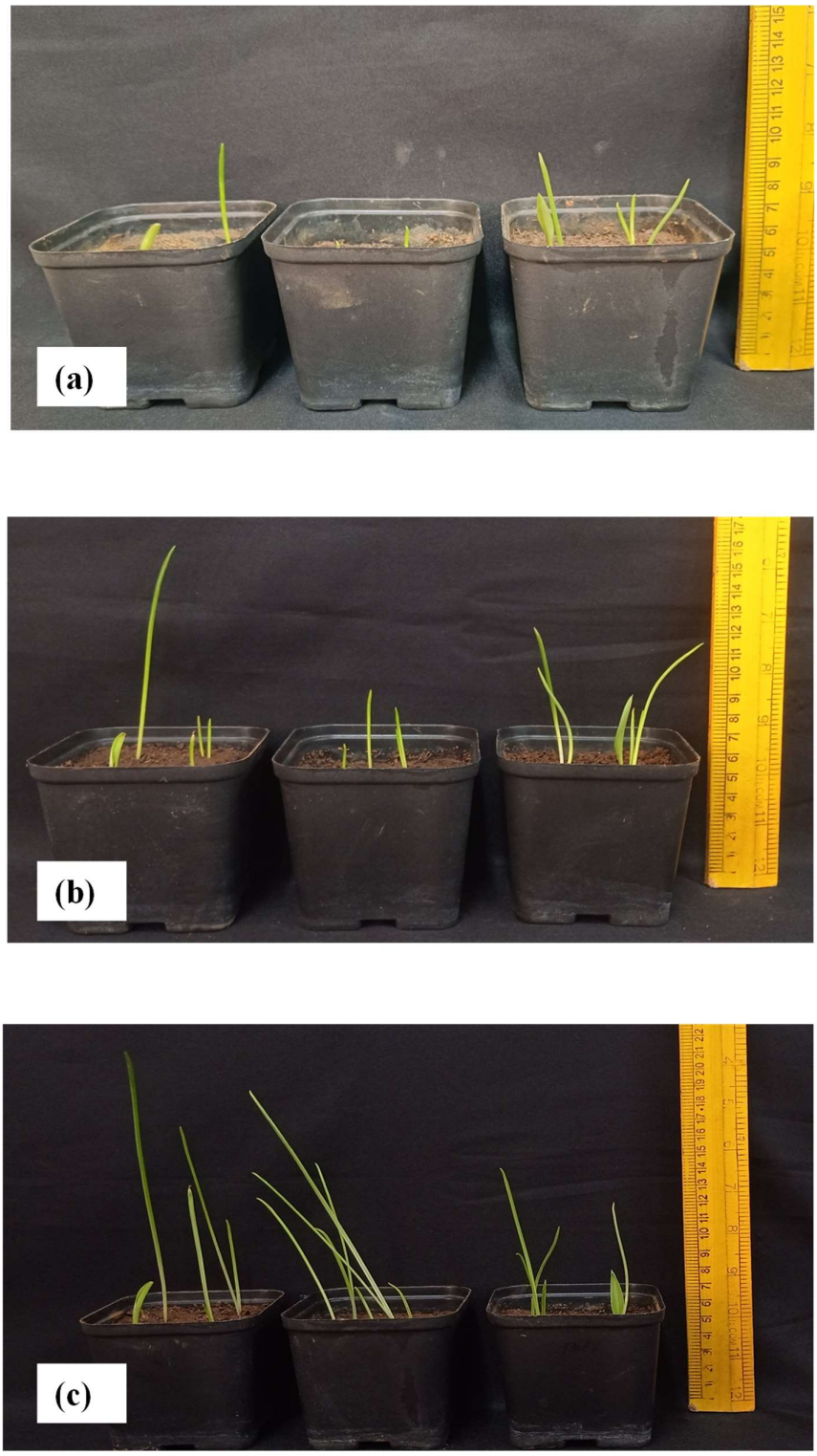

Plate 4.3: Bulb of *Dipcadi Goaense* grown in lab showing shoot growth (a) after 7 days, (b) after 14 days, (c) after 21 days.

Plate 4.4: Bulb of *Dipcadi Goaense* grown in lab showing full growth after 7 weeks.

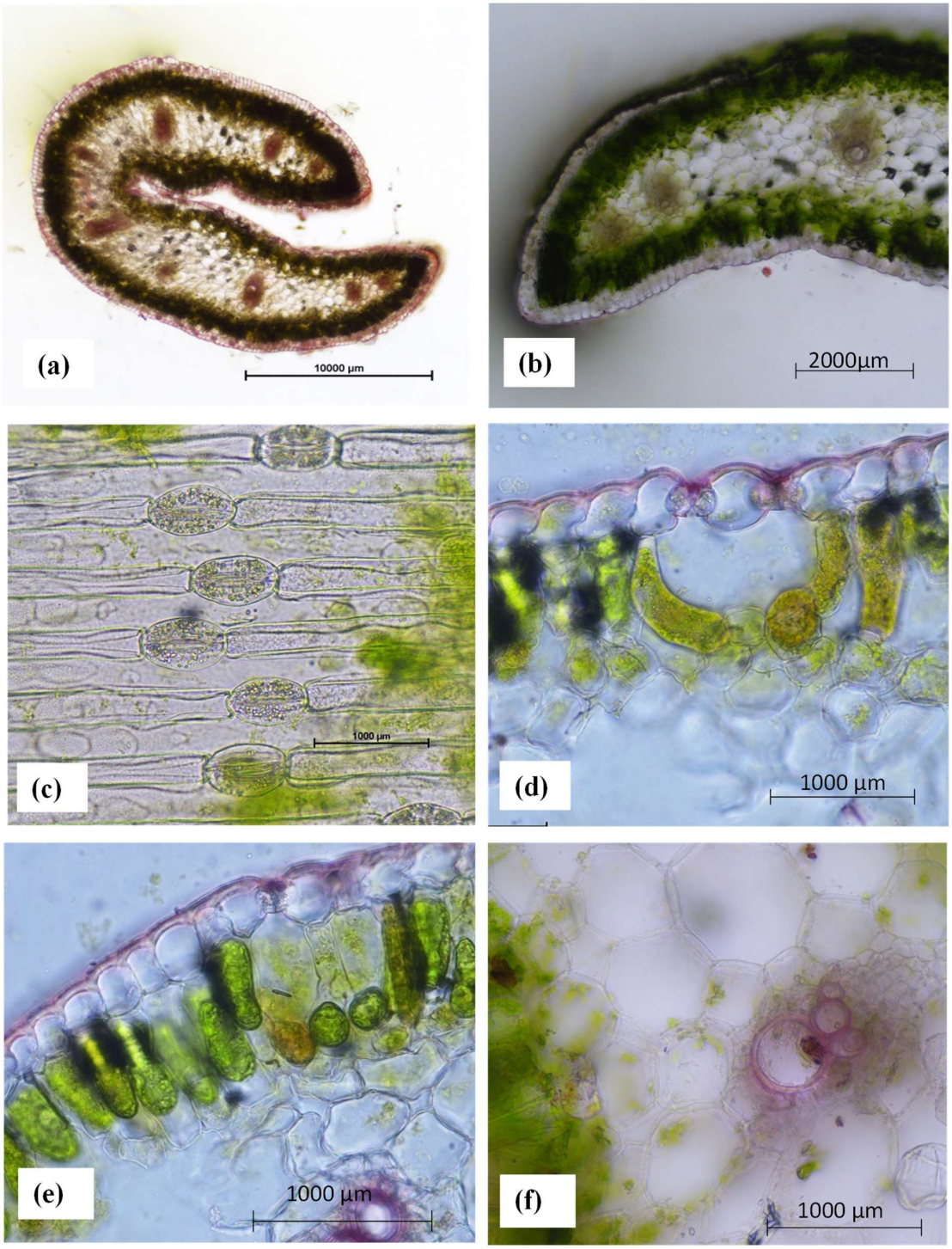

Plate 4.5: Anatomy of *D.goaense* leaf.(a) Overview of the leaf(4X), (b) T.S of leaf showing cuticle ,upper and lower epidermis, stomata(10X), (c) Epidermis peel showing anomocytic stomata (40X) , (d) T.s of leaf with sunken stomata in the epidermis layer,(e) portion of leaf showing mesophyll tissue below the epidermis,(f) T.S of leaf showing Vascular bundles.

4.2.2. T.S of Bulb

In the transverse section (T.S) of the bulb, a single layer of epidermal cells was evident, appearing slightly flat and oval-shaped, with a thin cuticle covering their surface. The space between the upper and lower epidermis was filled with oval-shaped parenchyma cells, characterized by thin membranes. Notably, the formation of numerous needle-shaped calcium oxalate raphide crystals was observed, consistent with findings reported by Lawrie *et al*. (2023). Additionally, starch grains were detected among the parenchyma cells. Within the parenchymatous tissue, vascular bundles were present. These bundles appeared to be of the collateral type **(Plate4. 6)**.

4.3 HISTOCHEMICAL STUDIES

4.3.1. Primary metabolites localization

4.3.1.a. Localization of starch

a.i. Localization of starch in the leaf

In the leaf of *D. goaense*, dark blue grains of amyloplasts were prominently observed, distributed within various layers of the leaf anatomy. These amyloplasts were particularly abundant in the epidermal layer, where they appeared as dense clusters, indicating a high concentration of starch. Within the mesophyll tissue, amyloplasts were also present, albeit in slightly lower quantities compared to the epidermis. Additionally, minute quantities of starch were detected in the bundle sheath cells surrounding the vascular bundles.

The presence of these dark blue grains, indicative of starch accumulation, suggests an active process of starch synthesis and storage within the leaf tissues of *D. goaense*. Starch serves as a crucial reserve carbohydrate in plants, playing a vital role in energy storage and metabolism.

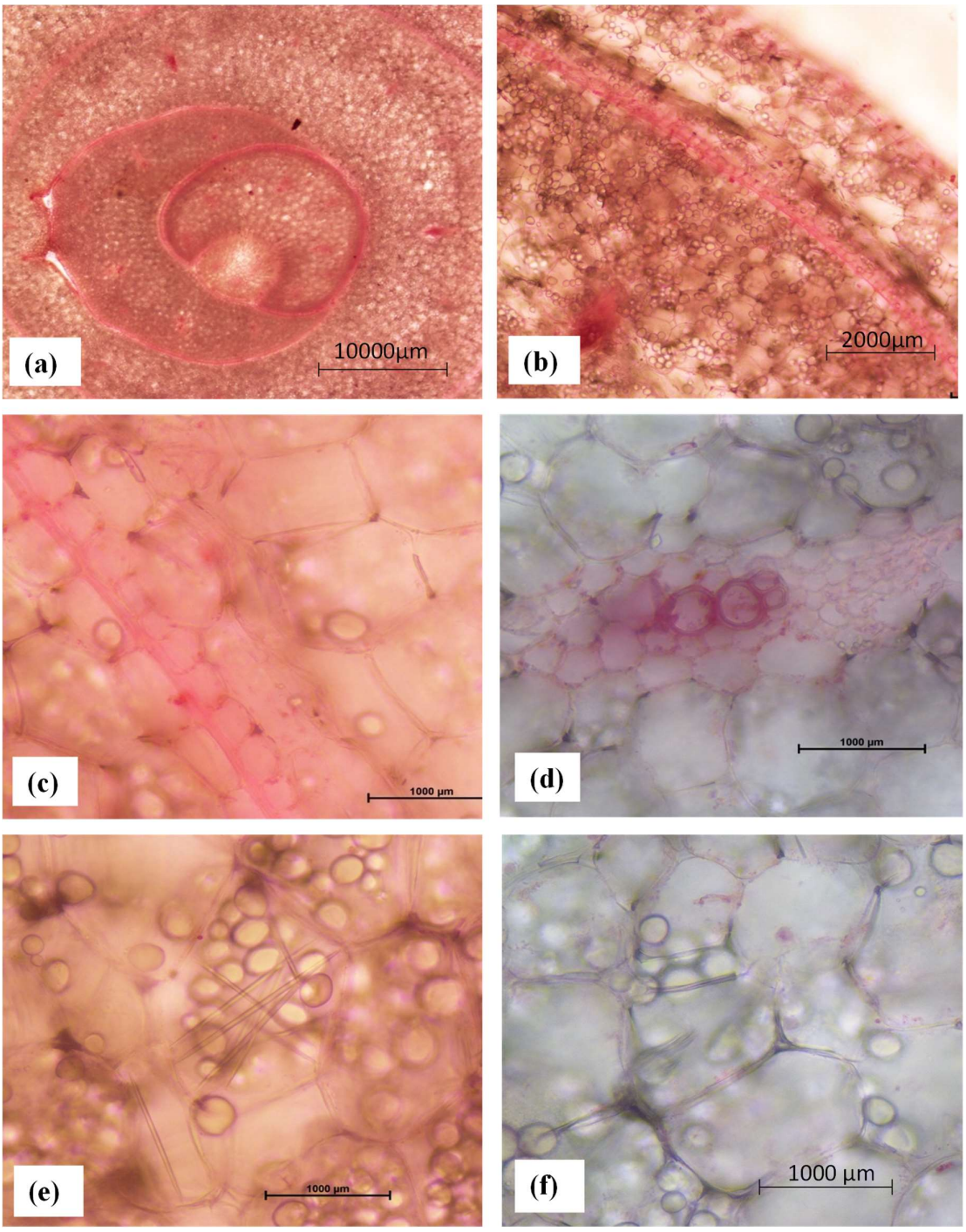

Plate 4.6: Anatomy of *D.goaense* bulb (a) Overview of the T.S of bulb(4X), (b) T.S of leaf showing cuticle and epidermis layer and vascular bundle (10X), (c) Anlarged view showing single layer of Epidermis (40X), (d) T.S of bulb showing vascular bundles with xylem and phloem (40X), (e) Parenchymatic cells showing the presence of needle shape calcium oxalate raphides crystals(40X), (f) T.S of bulb showing the presence of stach grains in the parenchymatic cells (40X).

The abundance of starch in the epidermis and mesophyll tissues suggests that *D. goaense*may employ starch as a readily accessible energy source for metabolic activities and growth. Further investigation into the regulation and utilization of starch reserves in *D. goaense*could provide valuable insights into its physiological adaptations and metabolic processes **(Plate 4.7)**.

a.ii. Localization of starch in bulb

In the bulb of *D. goaense*, extensive accumulation of dark blue stained starch grains was observed within the parenchymatic cells, indicative of significant starch storage within this tissue. The starch grains appeared as dense clusters, filling the cytoplasm of the parenchyma, and suggesting a crucial role of this tissue in starch synthesis and storage. This abundance of starch in the parenchymatic cells reflects the plant's strategy for energy storage and utilization, particularly during periods of dormancy or adverse environmental conditions.

Additionally, minute quantities of starch were detected in the epidermal layer of the bulb, albeit in lesser amounts compared to the parenchymatic cells. While the primary function of the epidermis is often associated with protection and regulation of water loss, the presence of starch in this layer suggests a potential role in metabolic processes or as a reserve for energy utilization. The localization of starch in both the parenchymatic cells and epidermal layer underscores the importance of starch as a vital carbohydrate reserve in the bulb of *D. goaense*. This starch reserve likely serves as an essential source of energy for metabolic activities, growth, and survival during unfavourable conditions. Further investigation into the regulation and dynamics of starch accumulation in the bulb tissues could provide valuable insights into the plant's physiological adaptations and survival strategies **(Plate 4.7)**.

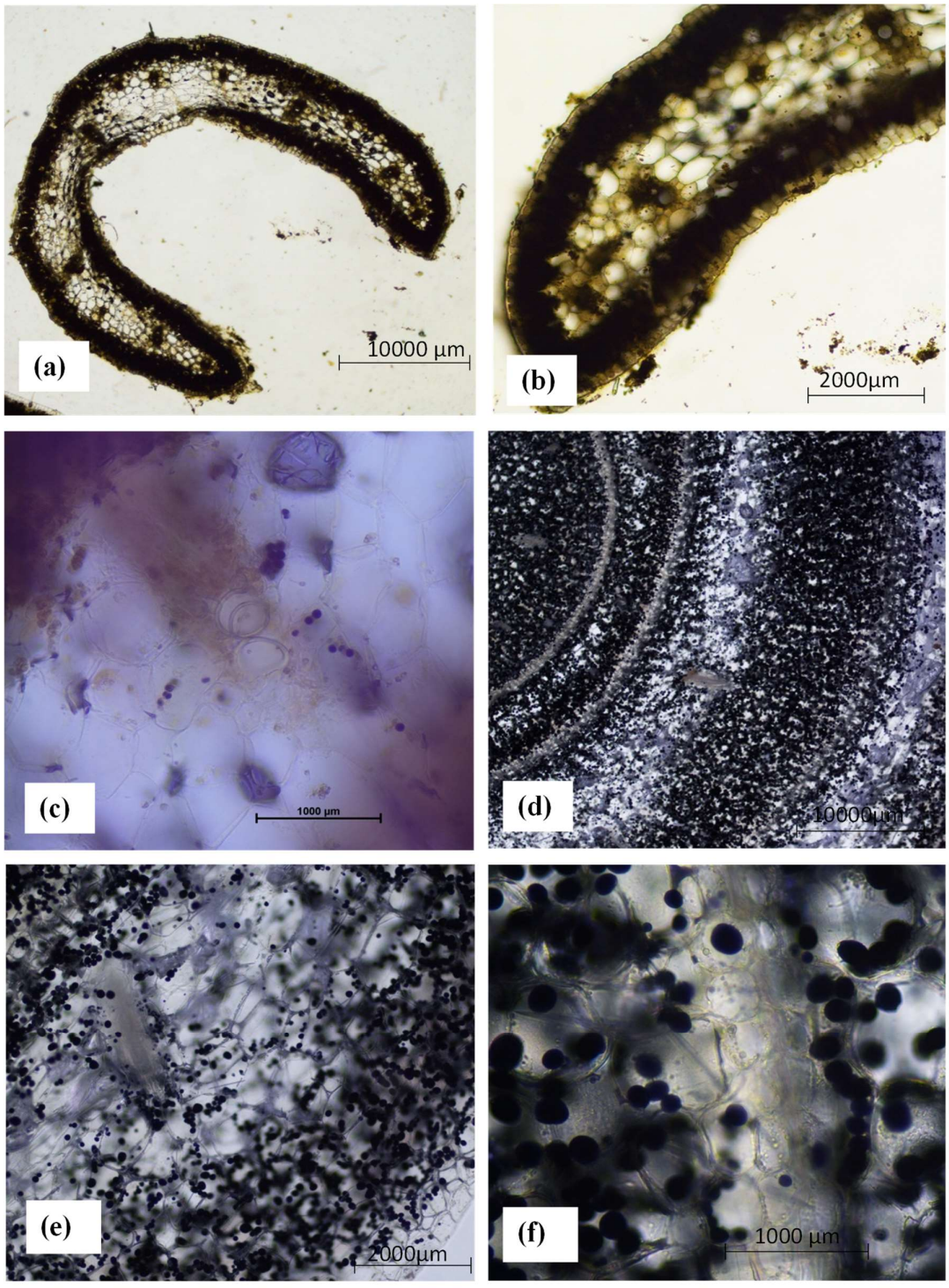

Plate 4.7: T.S of leaf and bulb of *Dipcadi goaense* showing localization of Starch in, (a) T.S of leaf (4X), (b) T.S of leaf (10X), (c) T.S of leaf (40X), (d) T.S of bulb (4X), (e) T.S of bulb (10X), (f) T.S of bulb (40X).

4.3.1.b. Localization of lipids

b.i. Localization of lipids in leaf

In the leaf of *D. goaense*, the presence of lipids was observed in various regions, as indicated by the red oil droplets visualized upon staining with Sudan IV. Specifically, lipids were detected in the epidermal layer, mesophyll cells, and vasculature region of the leaf tissue. The distribution of lipids across these different cell types suggests their diverse roles and metabolic functions within the leaf structure.

In the epidermal layer, the presence of lipids is significant as it forms the outer protective barrier of the leaf, contributing to its impermeability and resistance against environmental stresses. The accumulation of lipids in this layer may serve to enhance the water-repellent properties of the leaf surface, thereby reducing transpiration and preventing excessive water loss. Within the mesophyll cells, lipids likely play a crucial role in membrane structure and function, as well as in various metabolic processes. Lipid-rich membranes are integral components of chloroplasts, where they participate in photosynthesis and the synthesis of energy-rich compounds. Additionally, lipids may serve as energy reserves or signaling molecules within the mesophyll cells, contributing to cellular homeostasis and adaptation to changing environmental conditions.

The presence of lipids in the vasculature region suggests their involvement in nutrient transport and storage, as well as in the maintenance of vascular integrity and function. Lipids may be associated with specialized structures within the vasculature, such as the phloem sieve tubes and xylem vessels, where they facilitate the long-distance transport of organic and inorganic solutes throughout the plant. Overall, the localization of lipids in the various regions of the leaf highlights their diverse roles in membrane structure, metabolic processes, and physiological adaptation. Further investigation into the

composition and distribution of lipids in *D. goaense*leaf tissue could provide valuable insights into the plant's lipid metabolism and its adaptation to environmental challenges **(Plate 4.8)**

b.ii. Localization of lipids in bulb

In the bulb of *D. goaense*, the presence of lipids was observed in several key regions, as indicated by the visualization of red dots upon staining. Specifically, lipids were detected in the cuticle, epidermal layer, parenchymatic cells, and vasculature layer of the bulb tissue. The cuticle, which forms the outermost layer of the bulb, showed the presence of lipids. This finding suggests that lipids play a role in providing a protective barrier against environmental stresses such as water loss, pathogens, and mechanical damage. The accumulation of lipids in the cuticle may contribute to the resilience and integrity of the bulb structure, ensuring its survival and adaptation in varying environmental conditions.

Within the epidermal layer, lipids were also observed, indicating their involvement in maintaining the structural integrity and function of the outermost cell layer. Lipids in the epidermis may contribute to the impermeability of the bulb surface, regulating water and nutrient uptake, as well as protecting against external threats. The presence of lipids in the parenchymatic cells suggests their involvement in various metabolic processes and cellular functions within the bulb tissue. Lipids may serve as energy reserves, structural components of membranes, and signaling molecules, contributing to the overall physiological functioning and homeostasis of the bulb.

In addition, lipids were detected in the vasculature layer of the bulb, indicating their role in nutrient transport and vascular function. Lipids may be associated with specialized structures within the vasculature, such as the xylem and phloem, where they facilitate the transport of water, minerals, and organic solutes throughout the bulb. Overall, the localization of lipids in the cuticle, epidermal layer, parenchymatic cells, and vasculature layer of the bulb highlights their diverse roles in structural integrity, metabolic processes, and physiological adaptation **(Plate 4.8)**.

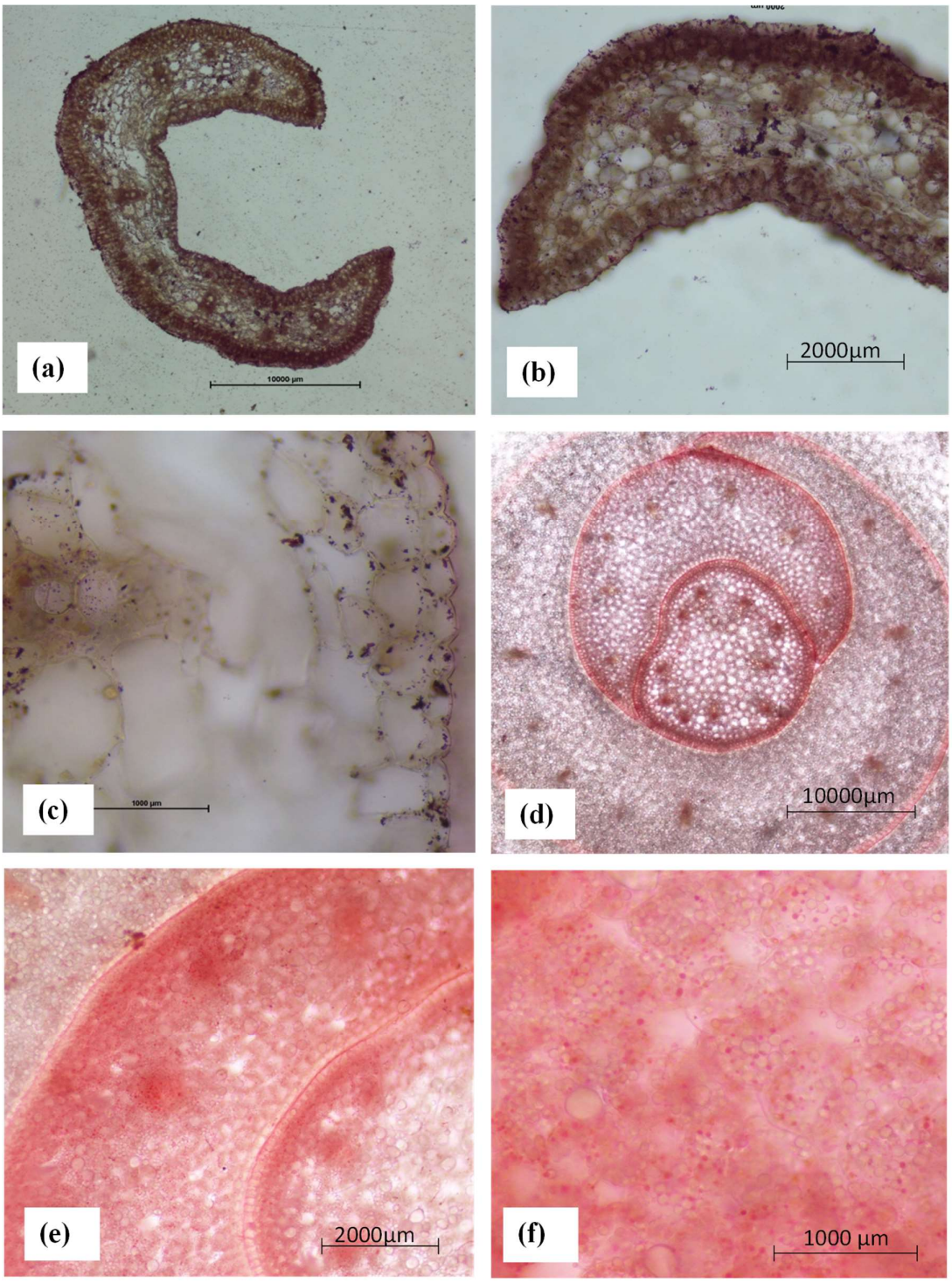

Plate 4.8: T.S of leaf and bulb of *Dipcadi goaense* showing localization of Lipids in, (a) T.S of leaf (4X), (b) T.S of leaf (10X), (c) T.S of leaf (40X), (d) T.S of bulb (4X), (e) T.S of bulb (10X), (f) T.S of bulb (40X).

4.3.1.c. Localization of proteins

c.i. Localization of proteins in leaf

In the leaf of *D. goaense*, the presence of proteins was observed in specific tissue regions, as indicated by light blue coloration upon staining. Specifically, proteins were localized in the mesophyll tissue and the vascular region of the leaf.

Within the mesophyll tissue, proteins were detected, suggesting their involvement in various metabolic processes and cellular functions. Mesophyll cells are responsible for photosynthesis, gas exchange, and nutrient storage, and proteins play essential roles in these physiological processes. Proteins may serve as enzymes, structural components, and signalling molecules within the mesophyll cells, contributing to the overall functioning and health of the leaf tissue. In addition to the mesophyll tissue, proteins were also found in the vascular region of the leaf. The vascular system, consisting of xylem and phloem tissues, is responsible for the transport of water, minerals, and organic solutes throughout the plant. Proteins in the vascular region may be associated with specialized structures such as sieve tubes, companion cells, and vessel elements, where they facilitate the long-distance transport of nutrients and signalling molecules.

The localization of proteins in both the mesophyll tissue and the vascular region highlights their importance in various physiological processes essential for leaf function and plant growth **(Plate 4.9)**.

c.ii. Localization of proteins in bulb

In the bulbs of *D. goaense*, the presence of proteins was confirmed through observation of light blue coloration in specific tissue regions. This staining pattern indicated the distribution of proteins within various anatomical structures of the bulb.

The cuticle, which forms the outermost protective layer of the bulb, exhibited light blue coloration upon staining. This suggests that proteins may be present in the cuticular layer, where they could contribute to the structural integrity and protective function of the cuticle, helping to prevent water loss and protect against environmental stresses.

Similarly, proteins were detected in the epidermal layer of the bulb. The epidermis serves as a barrier between the internal tissues of the bulb and the external environment, and proteins in this layer may play roles in defence against pathogens, regulation of water and nutrient uptake, and communication with neighbouring cells.

Within the parenchymatic cells of the bulb, light blue coloration indicative of protein presence was observed. Parenchyma cells are the most abundant type of plant cells and are involved in various metabolic activities such as storage of reserves, photosynthesis, and secretion. Proteins within parenchyma cells may serve diverse functions, including enzymatic activities, structural support, and signalling.

Furthermore, proteins were found in the vasculature region of the bulb. The vascular tissues, including xylem and phloem, are essential for the transport of water, nutrients, and signaling molecules throughout the plant. Proteins in the vascular system may participate in processes related to nutrient loading, long-distance signalling, and defence against pathogens.

Overall, the presence of proteins in the cuticle, epidermal layer, parenchymatic cells, and vasculature region of the bulbs of *D. goaense*suggests their involvement in various physiological and metabolic processes essential for bulb development, growth, and adaptation to environmental conditions. Further characterization of these proteins could provide valuable insights into the molecular mechanisms underlying bulb physiology and function **(Plate 4.9)**.

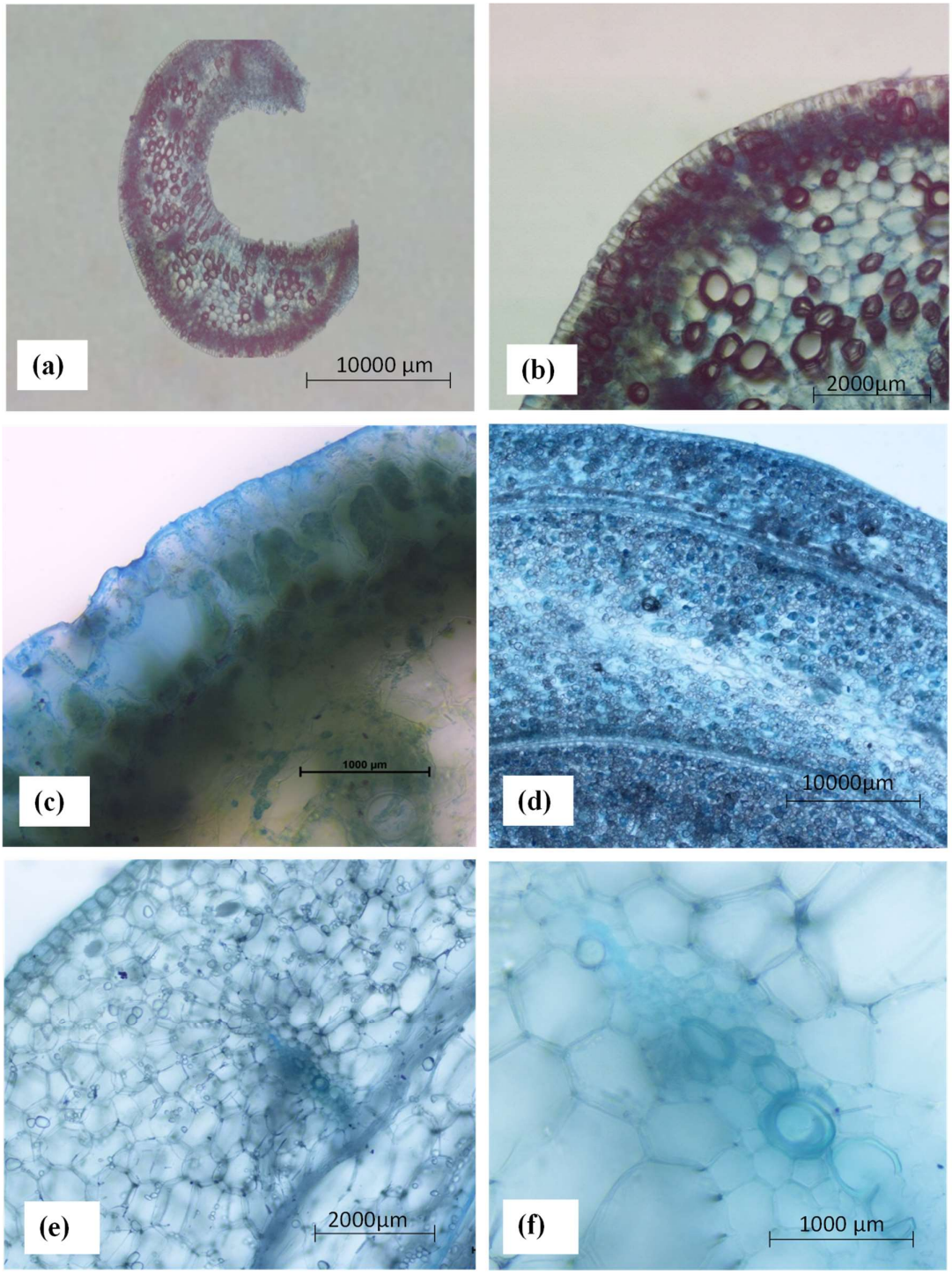

Plate 4.9: T.S of leaf and bulb of *Dipcadi goaense* showing localization of Proteins in, (a) T.S of leaf (4X), (b) T.S of leaf (10X), (c) T.S of leaf (40X), (d) T.S of bulb (4X), (e) T.S of bulb (10X), (f) T.S of bulb (40X).

4.3.2 Secondary metabolize localization

4.3.2.a. Localization of alkaloids

a.i. Localization of alkaloids in leaf

In the transverse section (T.S) of the leaf, alkaloids were localized primarily in two main tissue regions: the epidermal layer and the mesophyll tissue. The epidermal layer, which forms the outermost protective covering of the leaf, exhibited the presence of alkaloids. This suggests that alkaloids may play a role in defending the leaf against herbivores, pathogens, or other environmental stresses. Alkaloids are known for their diverse biological activities, including antimicrobial and insecticidal properties, and their presence in the epidermal layer could contribute to the leaf's defence mechanisms.

Additionally, alkaloids were detected in the mesophyll tissue of the leaf. The mesophyll tissue is where most of the photosynthetic activity occurs, and it consists of two main types of cells: the palisade mesophyll and the spongy mesophyll. Alkaloids in the mesophyll tissue may have implications for the plant's metabolic processes or could serve as secondary metabolites involved in defence or signalling pathways. The localization of alkaloids in both the epidermal layer and mesophyll tissue of the leaf suggests that these compounds may have multifaceted roles in the physiology and ecology of the plant **(Plate 4.10)**.

b.ii. Localization of alkaloids in bulb

In the parenchymatic cells, alkaloids were detected using a golden staining, which revealed the presence of these compounds. Alkaloids are known to exhibit a wide range of pharmacological activities and are often found in plant tissues as secondary metabolites.

Interestingly, the amyloplasts present in the parenchyma cells exhibited a dark blue staining reaction when treated with Dragendorff’s reagent. This staining reaction is typically associated with the detection of alkaloids. However, it's important to note that

amyloplasts are specialized organelles responsible for starch storage in plant cells. The dark blue staining of amyloplasts in response to Dragendorff's reagent could potentially obscure or interfere with the positive reaction for alkaloids. This observation highlights the importance of carefully interpreting staining reactions and considering the specific characteristics of cellular components when analyzing tissue samples. Further investigation may be necessary to confirm the presence of alkaloids in the parenchymatic cells and to elucidate their potential roles and significance in the plant's physiology and metabolism **(Plate 4.10)**.

4.3.2.b. Localization of phenols

b.i. Localization of phenols in leaf

In the transverse section (T.S) of the leaf of *D. goaense*, both the upper and lower epidermis, as well as the mesophyll tissue, exhibited black staining when subjected to staining procedures. This black staining is indicative of the presence of phenolic compounds, which are known for their antioxidant properties and various biological activities. The localization of phenolics in these regions suggests their potential roles in plant defense mechanisms and cellular processes within the leaf tissues. **(Plate 4.11)**.

b.ii. Localization of phenols in bulb

Similarly, in the bulb of *D. goaense*, phenolic compounds were detected in the parenchymatic cells located below the epidermis. The reaction with 2% ferrous sulphate in 10% formalin resulted in grey to black coloration in these cells, confirming the presence of phenolics. Phenolic compounds are often associated with defence mechanisms in plants, as they can act as antioxidants and play roles in protecting against environmental stressors. The localization of phenolics in the parenchymatic cells of the bulb suggests their involvement in cellular processes and responses to external stimuli in this plant **(Plate 4.11).**

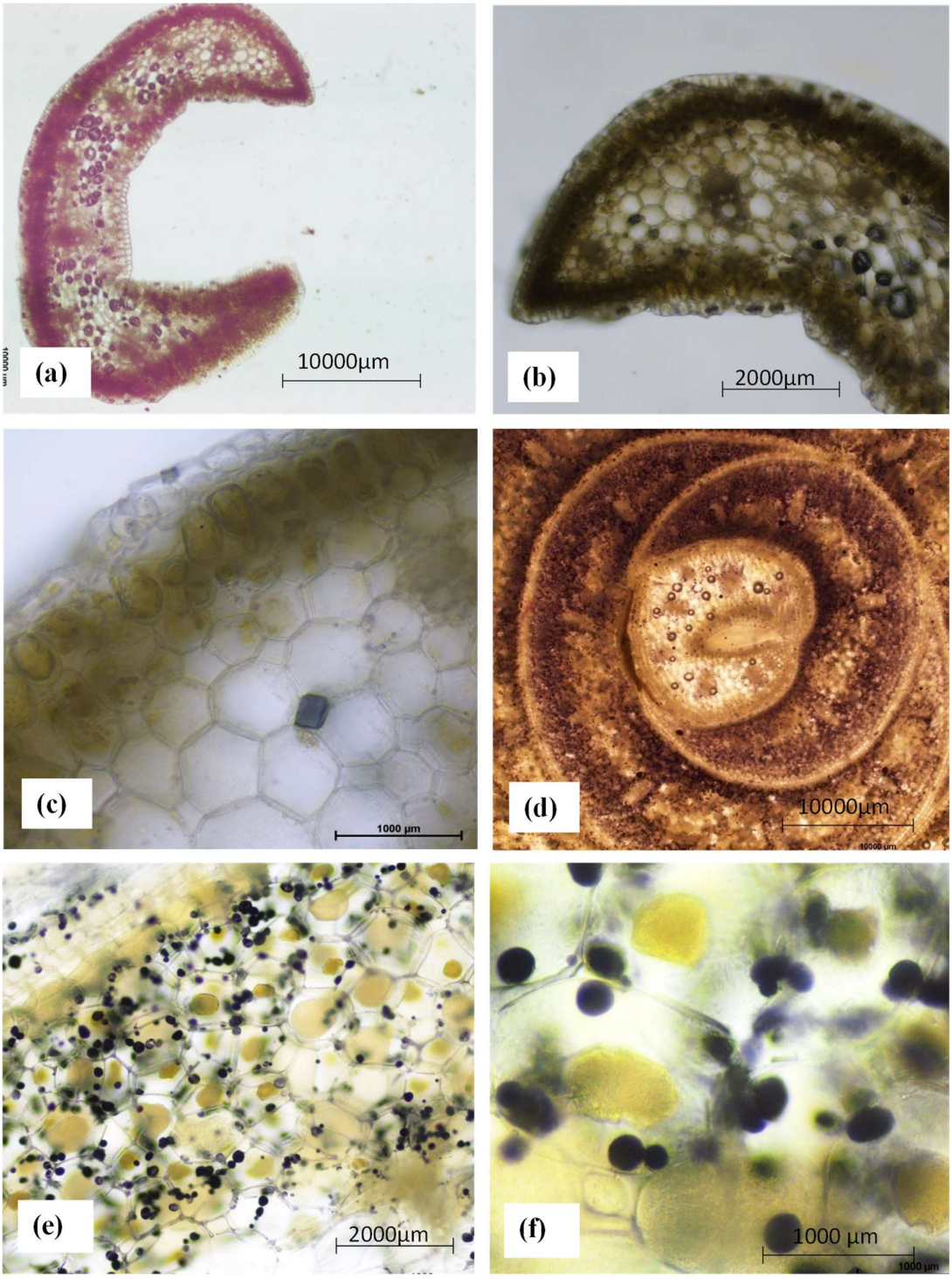

Plate 4.10: T.S of leaf and bulb of *Dipcadi goaense* showing localization of Alkaloids in, (a) T.S of leaf (4X), (b) T.S of leaf (10X), (c) T.S of leaf (40X), (d) T.S of bulb (4X), (e) T.S of bulb (10X), (f) T.S of bulb (40X).

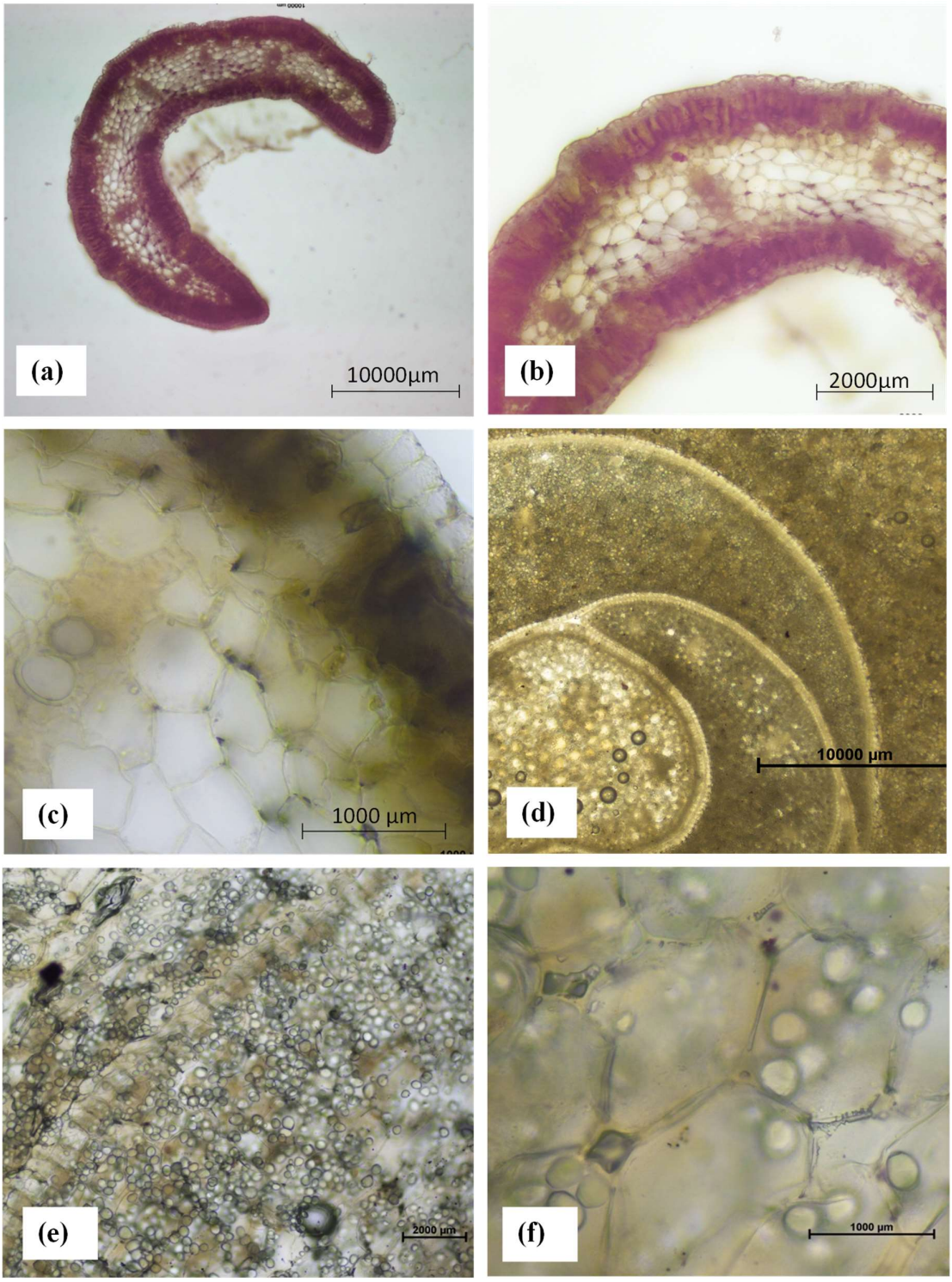

Plate 4.11: T.S of leaf and bulb of *Dipcadi goaense* showing localization of Phenols in, (a) T.S of leaf (4X), (b) T.S of leaf (10X), (c) T.S of leaf (40X), (d) T.S of bulb (4X), (e) T.S of bulb (10X), (f) T.S of bulb (40X).

4.4. PHYTOCHEMICAL ANALYSIS

4.4.1. Thin layer chromatography (TLC) analysis

a. TLC isolation of Alkaloids

The TLC plate was prepared and run using a specific solvent system **(Table 3.1)**. A total of 6 distinct bands were obtained. Out of which 4 bands were observed in T1 (ethanolic leaf extract) and 2 band was observed in T2 (ethanolic bulb extract). The bands were identified by dipping into Dragendorff's reagent which showed orange-colour bands for the alkaloid. compounds **(Table 4.1), (Plate 4.12)**.

b. TLC isolation of Phenols

The TLC plate was prepared and run using a specific solvent system (**Table 3.1**). A total of 5 distinct bands were obtained. Out of which 4 bands were observed in T1 (ethanolic leaf extract) and 1 band was observed in T2 (ethanolic bulb extract). The bands were identified by dipping into a 10% Ferric chloride solution which showed Grey or black spots under the visible light of phenols **(Table 4.2), (Plate 4.13)**.

c. TLC isolation of Steroids

The TLC plate was prepared and run using a specific solvent system (**Table 3.1**). A total of 2 distinct bands were obtained. Out of which 1 band was observed in T1 (ethanolic leaf extract) and 1 band was observed in T2 (ethanolic bulb extract). The bands were identified by dipping into Anisaldehyde sulphuric acid reagent which showed maroon bands of Steroids under long UV light **(Table 4.3), (Plate 4.14).**

d. TLC isolation of Flavonoids

The TLC plate was prepared and run using a specific solvent system **(Table 3.1)**. A total of 4 distinct bands were obtained. Out of which 3 band was observed in T1 (ethanolic leaf extract) and 1 band was observed in T2 (ethanolic bulb extract). The bands were

identified by dipping into a 10% Methanolic sulphuric acid reagent which showed fluorescent bands of Flavonoids under long UV light **(Table 4.4), (Plate 4.15).**

Table 4.1 TLC isolation of Alkaloids

Solvent system	Sample	Number of distinct bands	Rf value	Colour of band after reagent dipping	Possible compound
Toluene: Ethyl acetate: Methanol: Ammonia 25% (30:30:15:1) v/v/v/v (9: 1) v/v	T1 (Ethanolic leaf extract)	4	0.68	Dark blue	Unknown
			0.77	Dark green	Unknown
			0.89	Light green	Unknown
			1.01	Orange	Alkaloids
	T2 (Ethanolic bulb extract	2	0.06	Orange	Alkaloids
			1.01	Orange	Alkaloids

Table 4.2 TLC isolation of Phenols

Solvent system	Sample	Number of distinct bands	Rf value	Colour of band after reagent dipping	Possible compound
Tetrahydrofuran : toluene: formic acid: water (16:8:2:1) v/v/v/v	T1 (Ethanolic leaf extract)	4	0.13	Light green	Unknown
			0.82	yellow	Unknown
			0.84	Blue	Phenols
			1	Brownish black	Unknown
	T2 (Ethanolic bulb extract	1	0.85	Dark blue	Phenols

Table 4.3 TLC isolation of Steroids

Solvent system	Sample	Number of distinct bands	Rf value	Colour of band after reagent dipping	Possible compound
n-Butanol: Methanol: Water (3:1:1) v/v/v	T1 (Ethanolic leaf extract)	1	1	Maroon	Steroids
	T2 (Ethanolic bulb extract	1	0.50	White	Unknown

Table 4.4 TLC isolation of Flavonoid compounds

Solvent system	Sample	Number of distinct bands	Rf value	Colour of band after reagent dipping	Possible compound
Tetrahydrofuran: toluene: formic acid: water (16:8:2:1) v/v/v/v	T1 (Ethanolic leaf extract)	3	0.98	Fluorescent red	Flavonoids
			0.82	White	Unknown
			0.69	White	Unknown
	T2 (Ethanolic bulb extract	1	0.38	White	Unknown

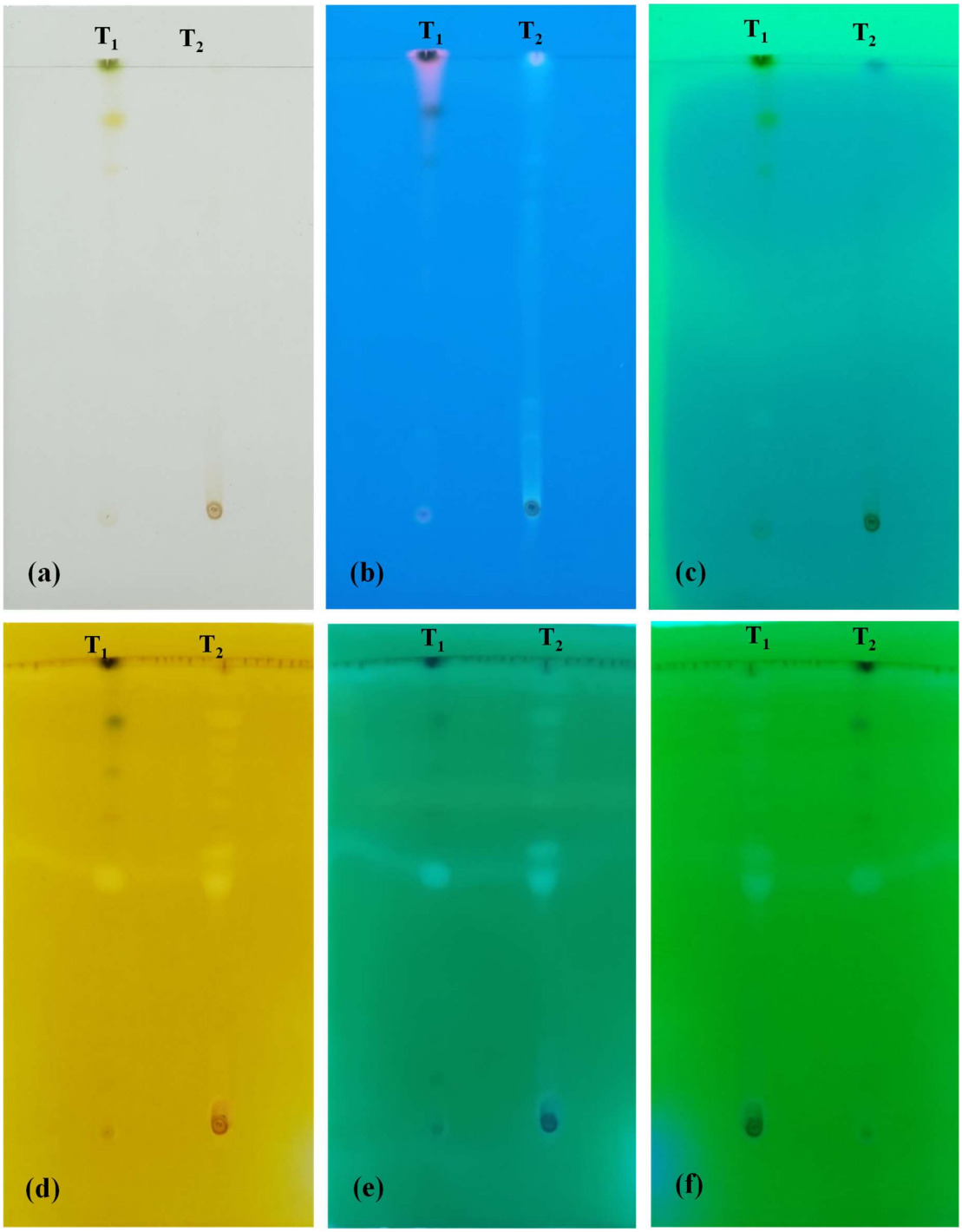

Plate 4.12: TLC isolation of alkaloids before and after derivatization a) under visible light before, b) under long uv before, c) short uv before d) under visible light after, e) under long uv after, f) under short uv after (T1= Ethanolic leaf extract, T2= Ethanolic bulb extract).

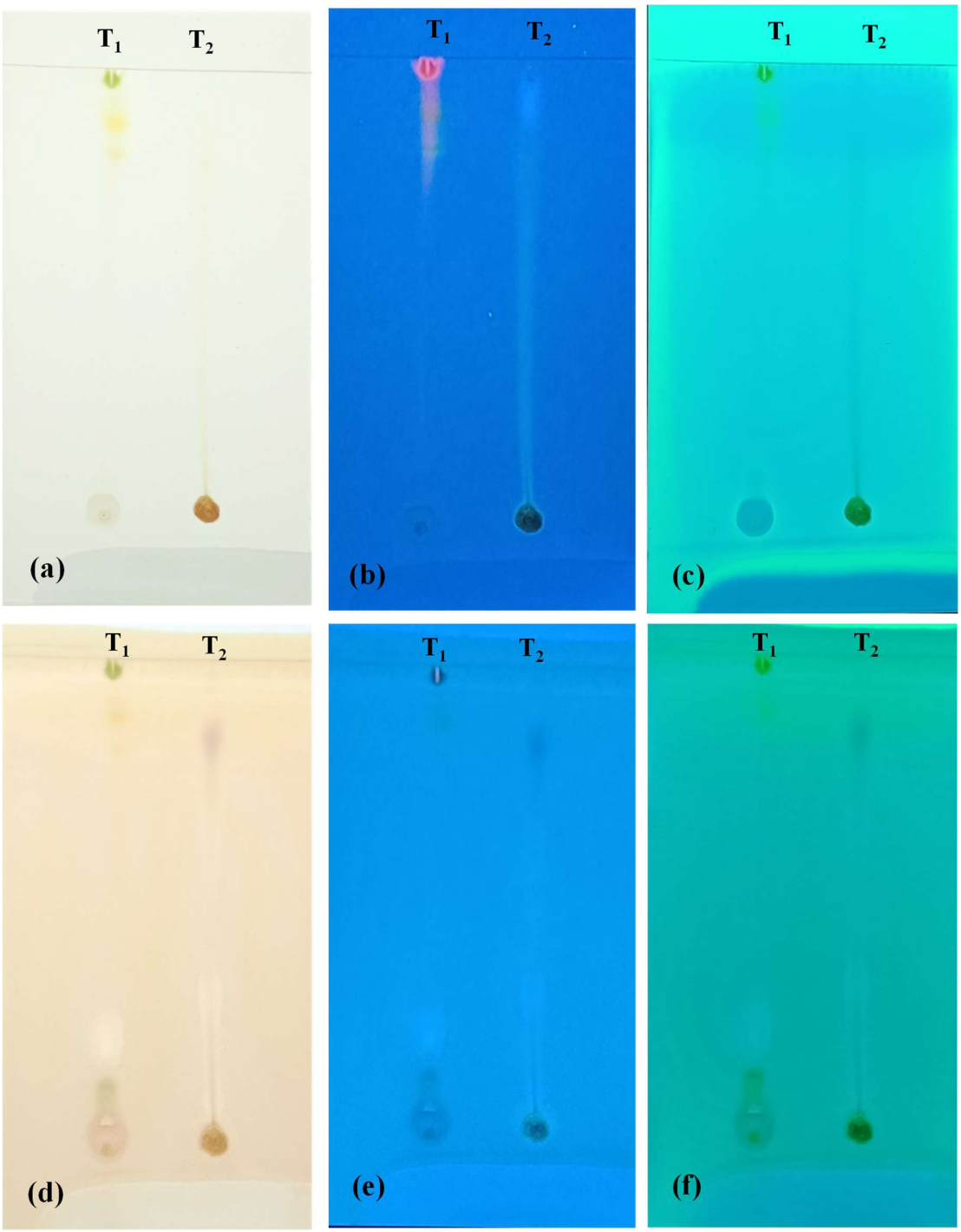

Plate 13: TLC isolation of phenolics before and after derivatization a) under visible light before, b) under long uv before, c) short uv before, d) under visible light after, e) under long uv after, f) under short uv after (T1= Ethanolic leaf extract, T2= Ethanolic bulb extract).

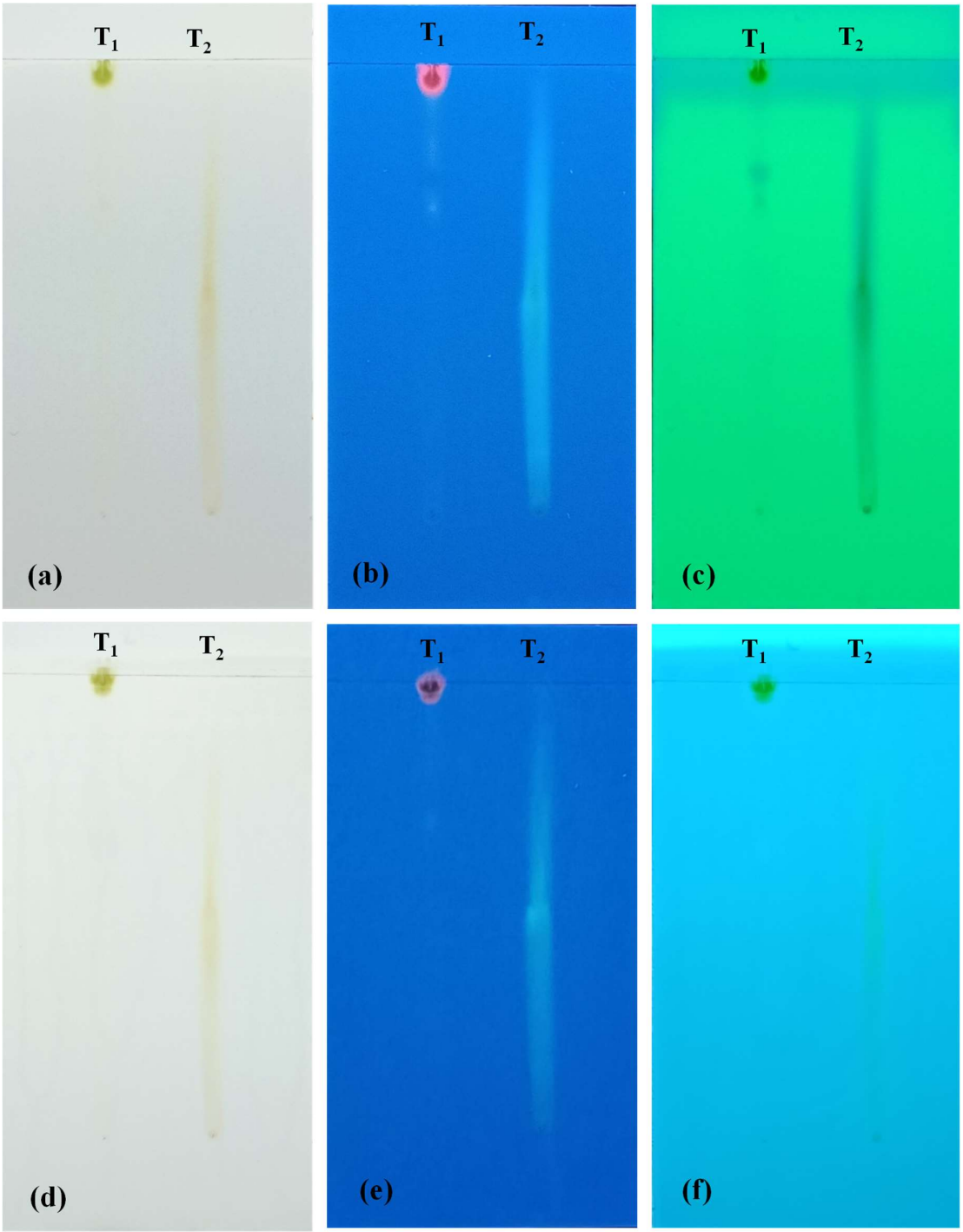

Plate 4.14: TLC isolation of steroids before and after derivatization a) under visible light before, b) under long uv before, c) short uv before d) under visible light after, e) under long uv after, f) under short uv after (T1= Ethanolic leaf extract, T2= Ethanolic bulb extract).

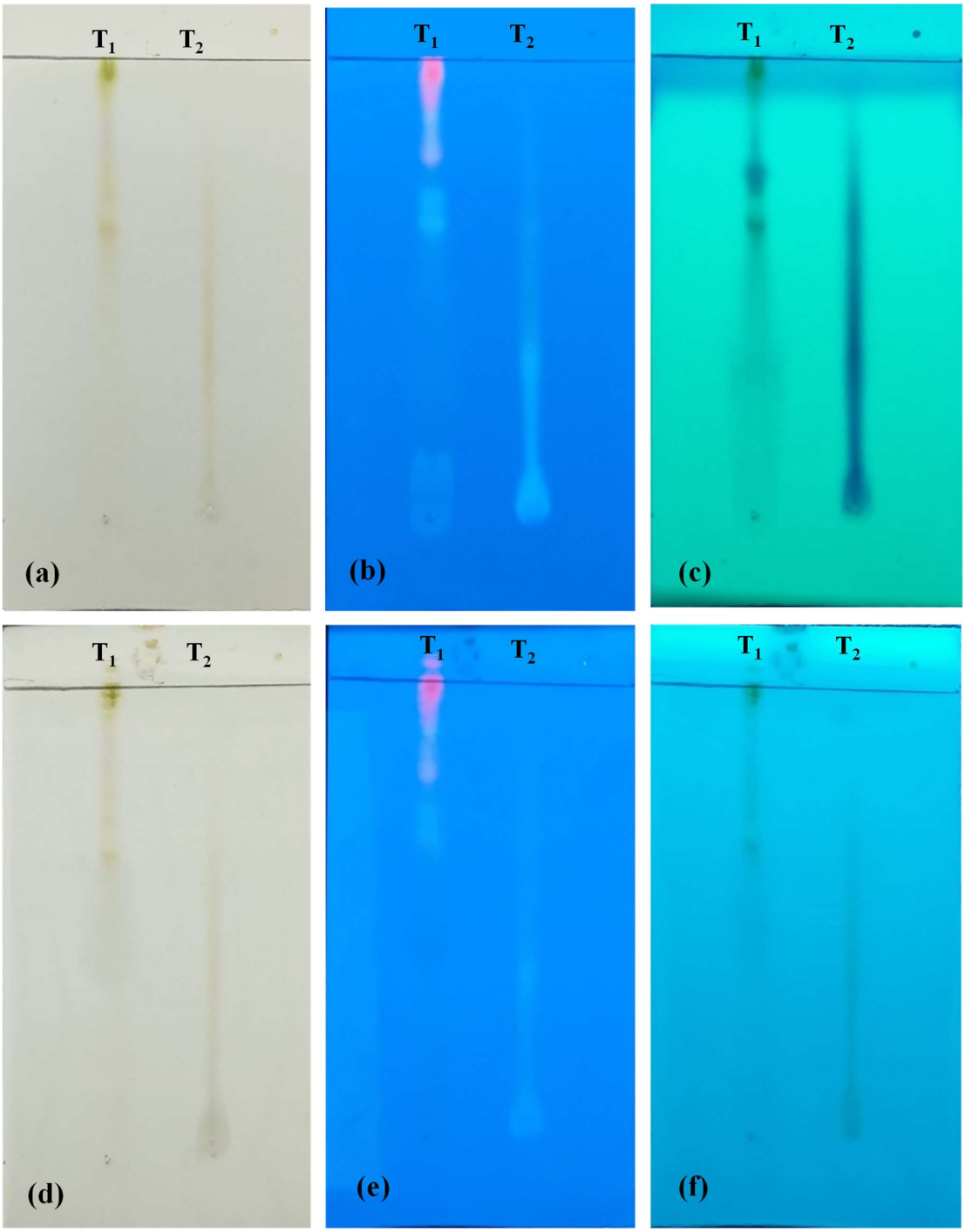

Plate 4.15: TLC isolation of flavonoids before and after derivatization a) under visible light before, b) under long uv before, c) short uv before d) under visible light after, e) under long uv after, f) under short uv after (T1= Ethanolic leaf extract, T2= Ethanolic bulb extract).

4.4.2. Phytochemical Estimation of Secondary metabolites

4.4.2.a. Determination of Phenolic Contents

The total phenol content (TPC) of leaf and bulb extracts of *Dipcadi goaense* was determined using the Folin Ciocalteu's method, employing different solvent combinations. The results, depicted in **Fig. 4.1** and **Table 4.5**, revealed varying TPC values across the extracts. Specifically, the ethanolic extracts exhibited the highest TPC values for both leaf (64.33 ± 0.0005) and bulb (53.45 ± 0.004) extracts, followed by the methanolic extracts with values of 56.08 ± 0.004 for leaf and 42.83 ± 0.0005 for bulb. The TPC values were comparatively lower in the distilled water extracts, with values of 47.29 ± 0.0005 for leaf and 25.54 ± 0.001 for bulb.

These findings indicate that ethanol serves as a more effective solvent for extracting phenolic compounds from *Dipcadi goaense* compared to methanol and distilled water. This observation aligns with previous studies, including the research conducted by Adly *et al*. in 2015, further supporting the efficacy of ethanol in extracting phenolic compounds from plant materials.

Table 4.5. Total amount of Phenolic content (TPC) in Distilled water, Methanolic and Ethanolic leaf and bulb extract of *Dipcadi goaense*. Data represented mean value ± Standard deviation (N=3)

Plant part used	***Concentration of TPC mg of Gallic Acid equivalents (GAE)***		
	Distilled water	**Methanol**	**Ethanol**
Leaf	47.29 ± 0.0005	56.08 ± 0.004	64.33 ± 0.0005
Bulb	25.54 ± 0.001	42.83 ± 0.0005	53.45 ± 0.004

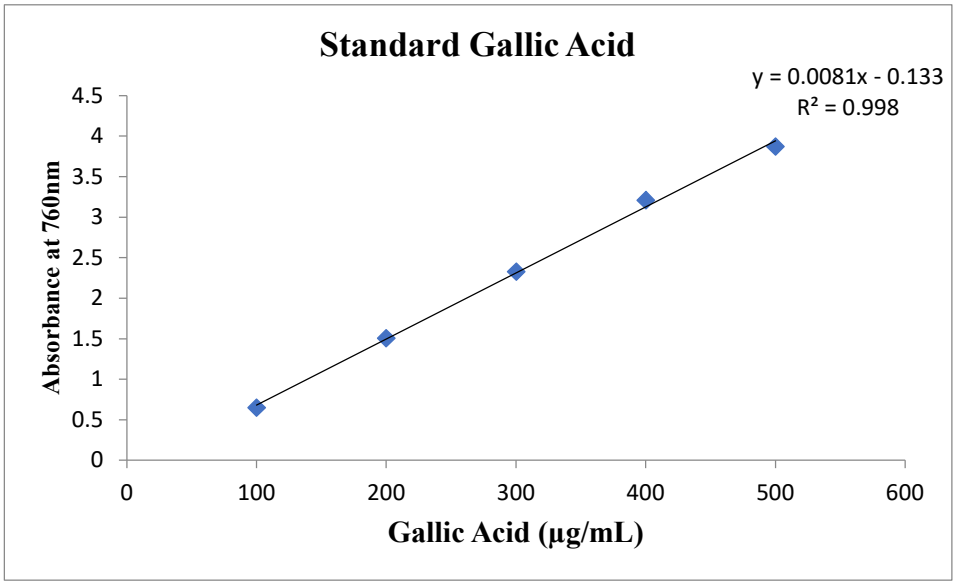

Fig. 4.1 Calibration curve for Gallic acid for determination of total Phenolic content (mg of Gallic acid equivalent (GAE)/gm of extract) at varying concentrations.

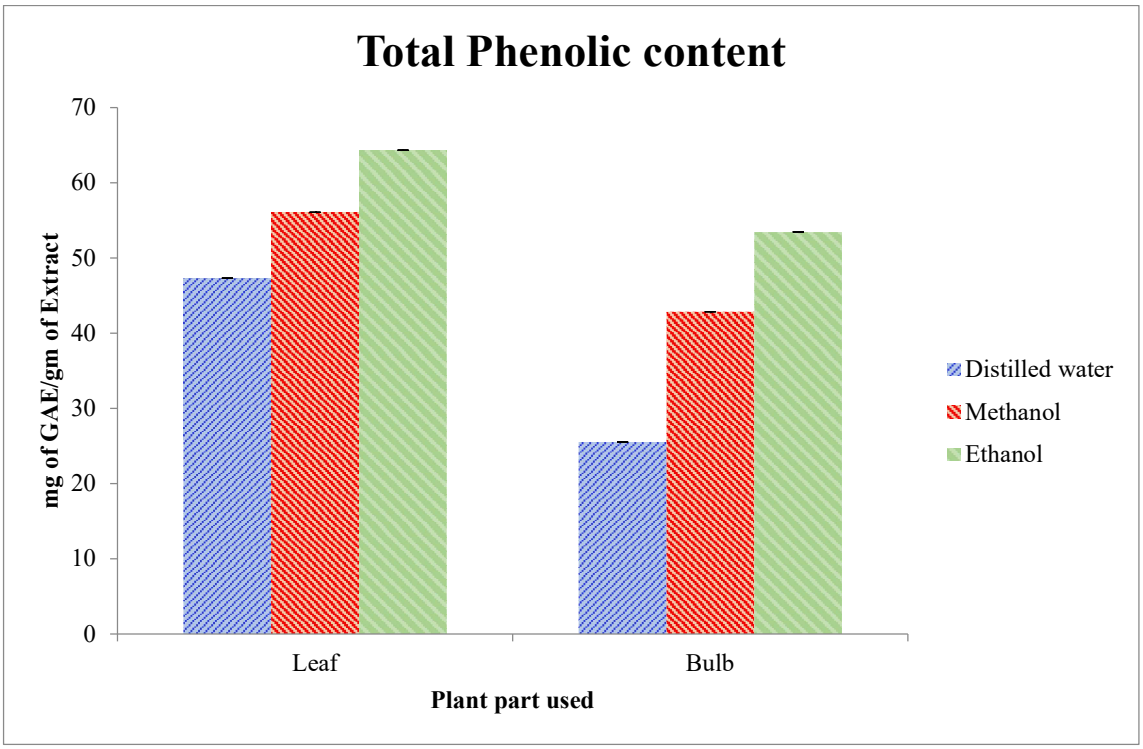

Fig. 4.2 Determination of Total Phenolic content (TPC) in mg of Gallic Acid equivalent (GAE)/g of *Dipcadi goaense* leaf and bulb extract.

4.5. TISSUE CULTURE STUDIES

Tissue culture studies play a crucial role in the conservation and propagation efforts of *Dipcadi* species, given their ecological and pharmacological significance. Several *Dipcadi* species, including *D. goaense*, face imminent threats of extinction due to habitat degradation, human activities, and inadequate conservation measures. To address these challenges, urgent scientific attention, thorough botanical documentation, and effective conservation interventions are required to preserve the rich biodiversity of the Western Ghats. *In situ* conservation methods have been employed to conserve *Dipcadi* species, including *D. goaense*, along with other endangered plant species (Joshi *et al*., 2012). Additionally, *ex-situ* germplasm conservation efforts have been undertaken for species like *D. saxorum*, utilizing seeds as propagation material (Srinivasa *et al*., 2009). Radhika *et al*. (2020) conducted research on multiple shoot induction from bulb explants of *D. montanum* using various concentrations of auxins, contributing to the development of propagation protocols for this species. These tissue culture studies are vital for the conservation and propagation of *Dipcadi* species, offering hope for their survival and continued existence in the face of mounting threats and environmental challenges.

In the *in vitro* study of shoot induction in *D. goaense*, bulb explants were utilized on Murashige and Skoog (M.S) media supplemented with various concentrations and combinations of auxins and cytokinins, including 2,4-D + BAP, IBA + BAP, and NAA + BAP (at concentrations of 3, 3.5, and 4 mg/mL + 1 mg/mL). The response of shoot induction was assessed, and the results were compared across different combinations **(Table 3.4).**

Analysis of the data revealed significant differences in the percentage of shoot response among the various concentration combinations of auxins and cytokinins. Notably,

the combination of 4 mg/mL 2,4-D + 1 mg/mL BAP exhibited the highest percentage of shoot response, with a remarkable 100% response rate. Following closely behind were the combinations of 3 mg/mL 2,4-D + 1 mg/mL BAP and 3.5 mg/mL 2,4-D + 1 mg/mL BAP, which demonstrated response rates of 96.29 ± 0.57% **(Table 4.6), (Plate 4.16-4.19)**.

Further investigation involved the use of cut bulbs treated with higher concentrations of 2,4-D + 1 mg/mL BAP (5, 5.5, and 6 mg/mL). Among these, only the concentrations of 5 and 6 mg/mL 2,4-D + 1 mg/mL BAP showed any response **(Plate 4.20, 4.21).**

These findings underscore the importance of auxin and cytokinin concentrations in influencing shoot induction in *D. goaense*. The results suggest that specific concentration combinations, particularly those containing higher levels of 2,4-D and BAP, are more effective in promoting shoot development from bulb explants. These insights are crucial for optimizing tissue culture protocols and enhancing the efficiency of micropropagation techniques for the conservation and propagation of *D. goaense*.

Table 4.6 Effect of auxin and cytokinin combination on shoot induction from bulb explant of *Dipcadi goaense*.

PGR combination mg/mL Auxins + Cytokinins		***% of response***
Combination No.	***Combination***	
C 1	*M.S Medium + 3 mg/mL 2,4-D + 1 mg/mL BAP*	96.29 ± 0.577
C 2	*M.S Medium + 3.5 mg/mL 2,4-D + 1 mg/mL BAP*	96.29 ± 0.577
C 3	*M.S Medium + 4 mg/mL 2,4-D + 1 mg/mL BAP*	100
C 4	*M.S Medium + 3 mg/mL IBA + 1 mg/mL BAP*	92.59. ± 0.577
C 5	*M.S Medium + 3.5 mg/mL IBA + 1 mg/mL BAP*	70.37 ± 0.577
C 6	*M.S Medium + 4 mg/mL IBA + 1 mg/mL BAP*	0
C 7	*M.S Medium + 3 mg/mL NAA + 1 mg/mL BAP*	0
C 8	*M.S Medium + 3.5 mg/mL NAA + 1 mg/mL BAP*	77.77 ± 1
C 9	*M.S Medium + 4 mg/mL NAA + 1 mg/mL BAP*	70.37 ± 0.577

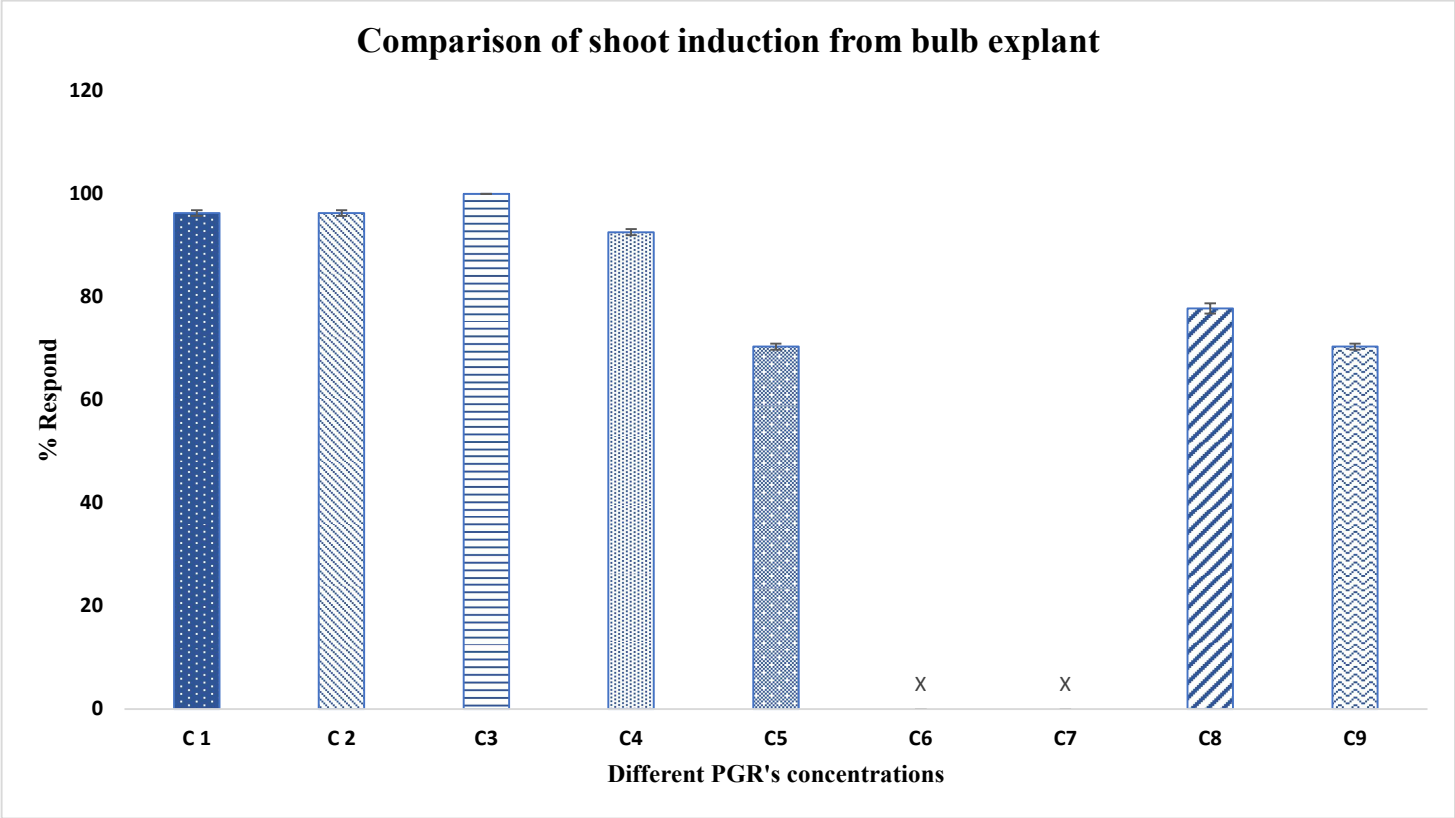

Fig.4.3 Comparison of shoot induction from bulb explant of *D. goaense*in M.S supplemented with different combination of PGR's.

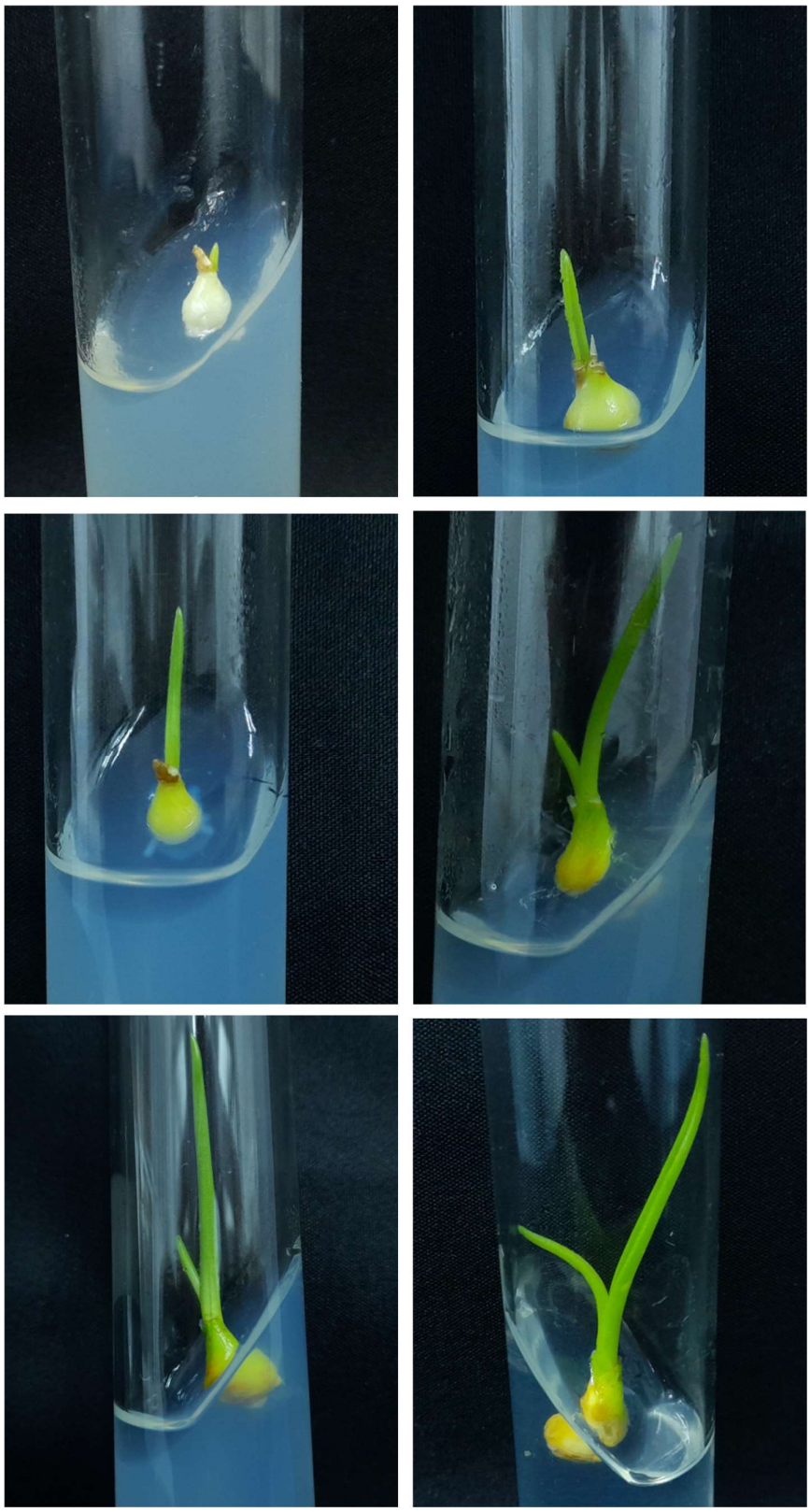

Plate 4.16: Shoot induction of bulb explant of *Dipcadi goaense* after 6 weeks of inoculation on MS Media supplemented with growth hormones in combination of: 3 mg/mL 2,4 –D + 1 mg/mL BAP.

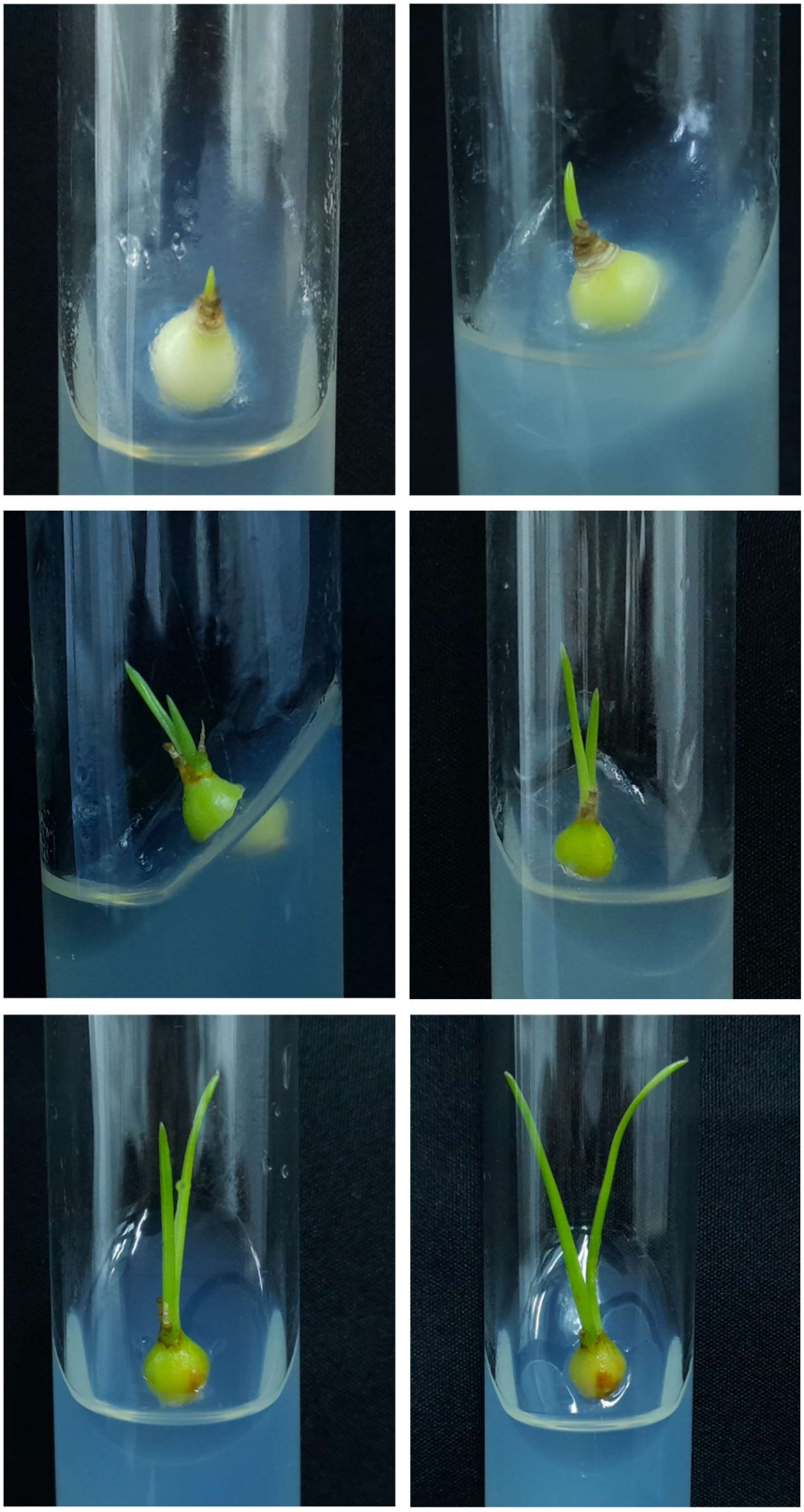

Plate 4.17: Shoot induction of bulb explant of *Dipcadi goaense* after 6 weeks of inoculation on MS Media supplemented with growth hormones in combination of: 3.5 mg/mL 2,4 – D + 1 mg/mL BAP.

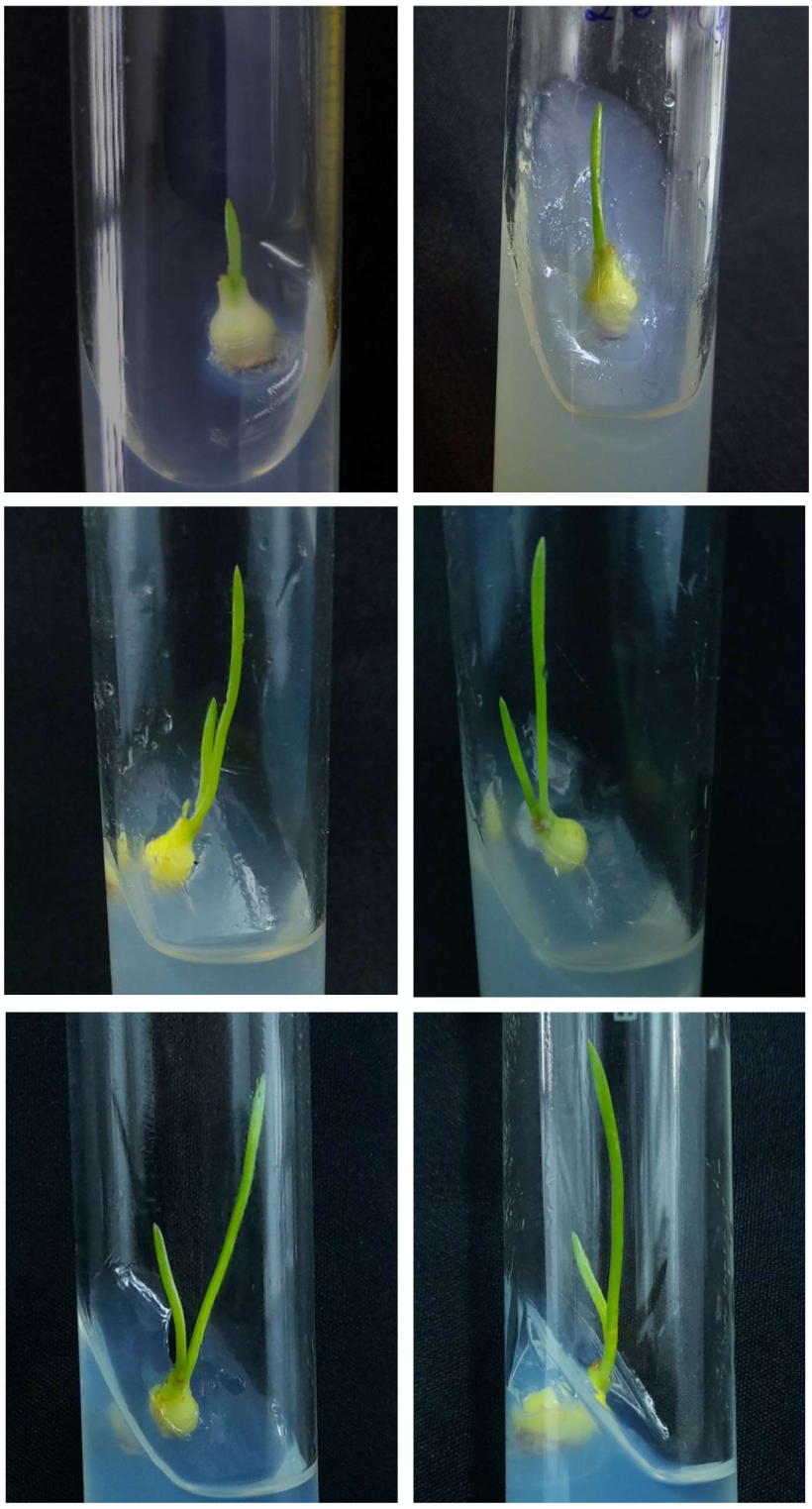

Plate 4.18: Shoot induction of bulb explant of *Dipcadi goaense* after 6 weeks of inoculation on MS Media supplemented with growth hormones in combination of: 4 mg/mL 2,4 – D + 11 mg/mL BAP.

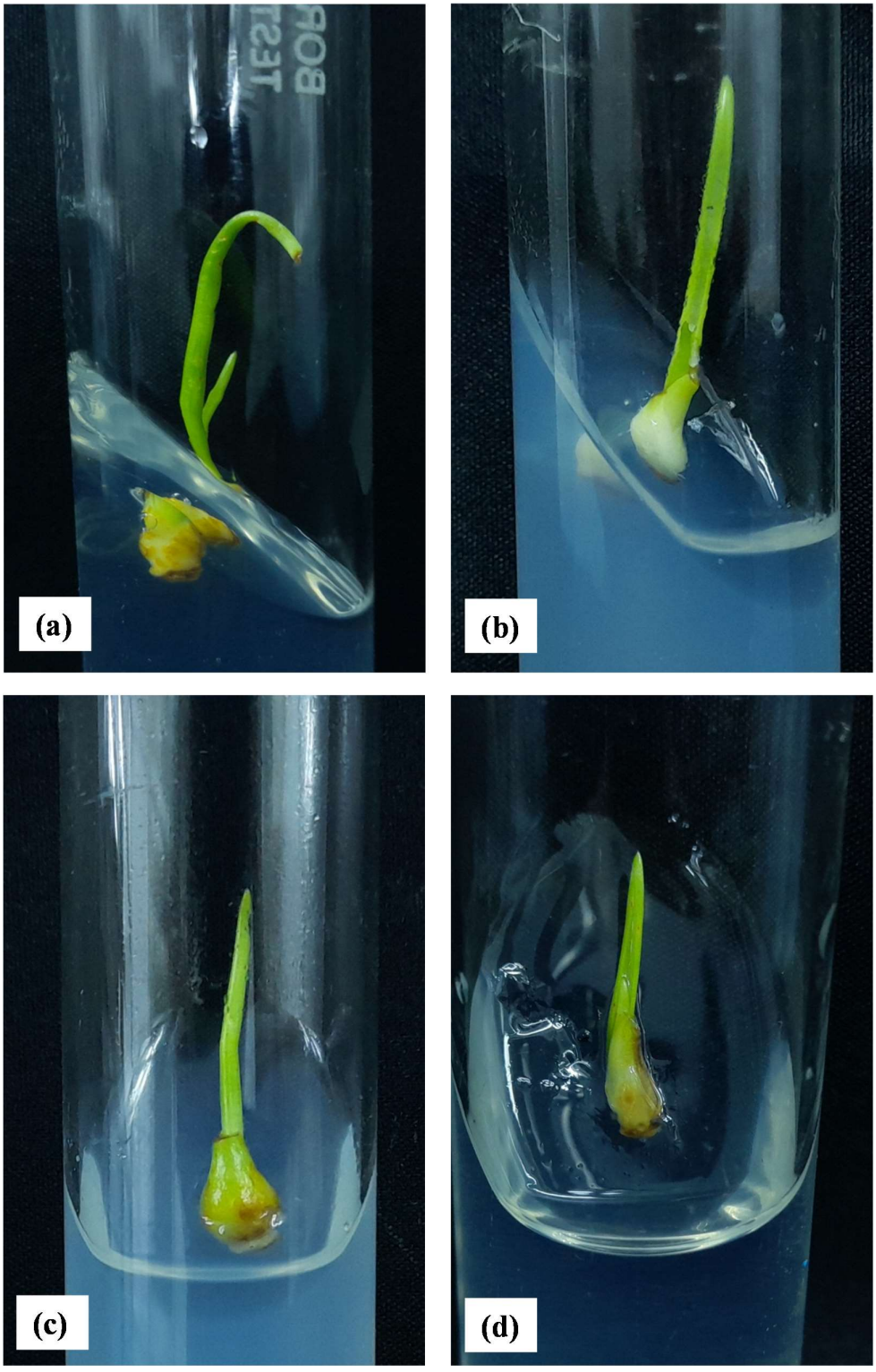

Plate 4.19: Shoot induction of bulb explant of *Dipcadi goaense* after 6 weeks of inoculation MS Media supplemented with growth hormones in combination of: a) 3 mg/mL IBA + 1 mg/mL BAP, b) 3.5 mg/mL IBA + 1 mg/mL BAP, c) 3.5 mg/mL NAA + 1 mg/mL BAP, d) 4 mg/mL NAA + 1 mg/mL BAP.

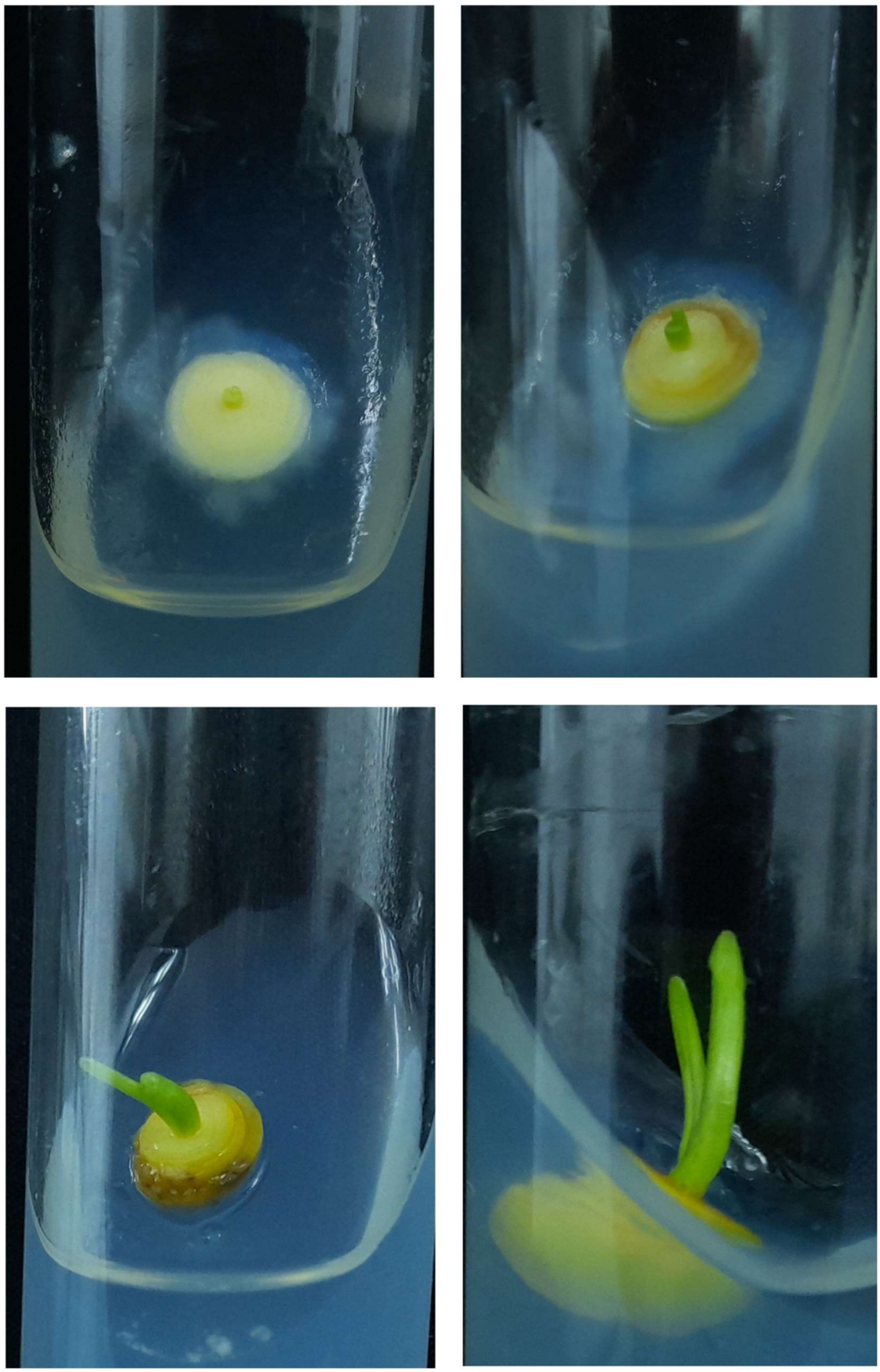

Plate 4.20: Shoot induction of cut bulb explant of *Dipcadi goaense* on MS Media supplemented with growth hormones in combination of: 5 mg/mL 2,4-D + 1 mg/mL BAP.

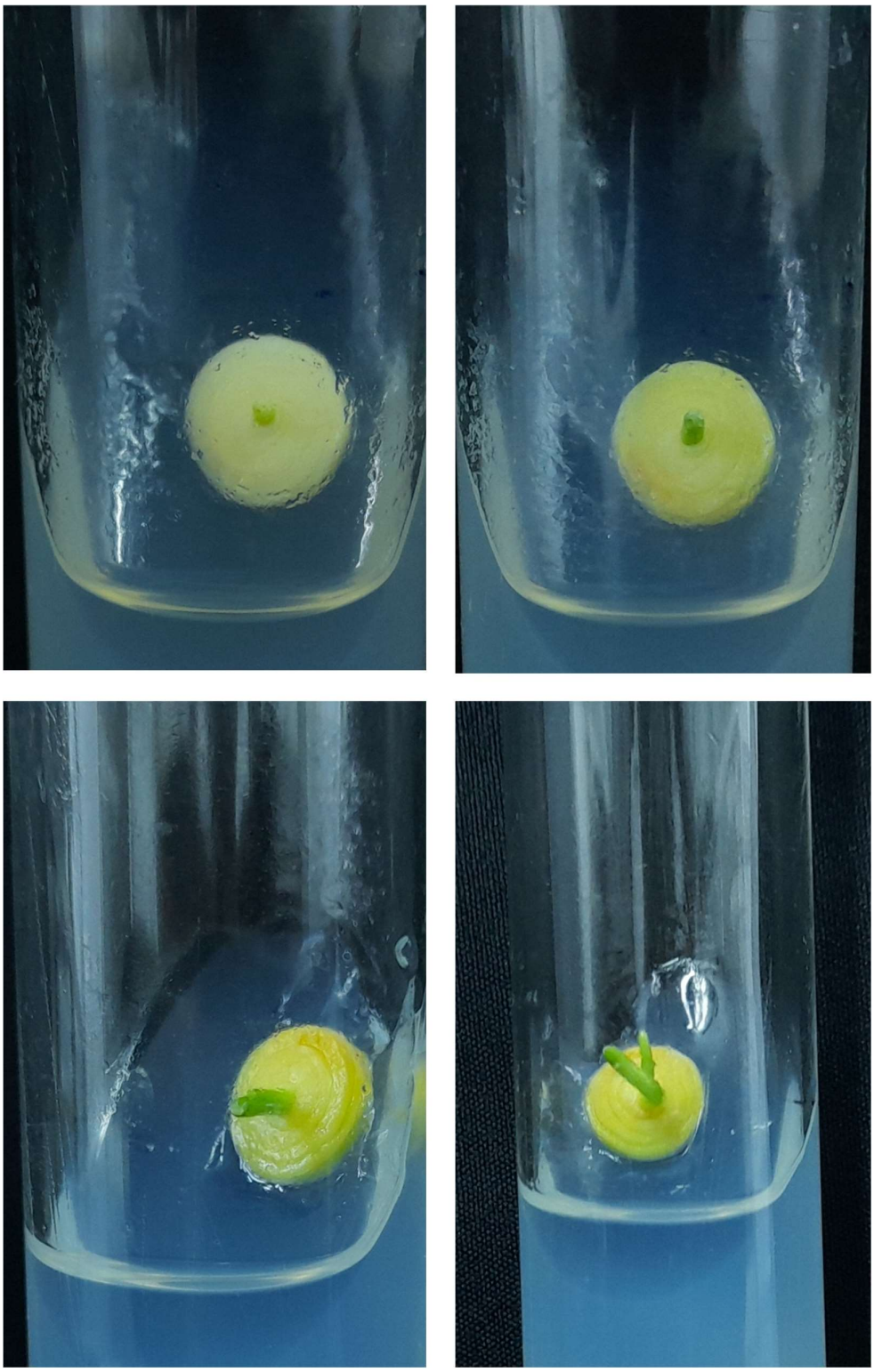

Plate 4.21: Shoot induction of cut bulb explant of *Dipcadi goaense* on MS Media supplemented with growth hormones in combination of: 6 mg/mL 2,4-D + 1 mg/mL BAP.

CHAPTER: 5 CONCLUSION

The collective findings of this dissertation have significant implications for the conservation and management of *Dipcadi goaense*. By elucidating its morphoanatomical characteristics, biochemical composition, and tissue culture responses, this study lays the groundwork for targeted conservation strategies, including in situ and ex situ conservation measures. Furthermore, the identification of pharmacologically active compounds underscores the potential medicinal value of *D. goaense*and calls for further research into its therapeutic applications.

Morphoanatomical Insights: The morphoanatomical investigation of *D. goaense*provided detailed insights into the structural organization of its various organs, including leaves, bulbs, seeds, and roots. Key observations include the presence of amphistomatic leaves with anomocytic stomata, the formation of starch grains and raphide crystals in bulbs, and the radial arrangement of vascular bundles in leaves. These observations enrich our understanding of the adaptive features and ecological adaptations of *D. goaense*, facilitating its taxonomic classification and ecological significance within its habitat.

Phyto-Histochemical Analysis: Phyto-histochemical studies revealed the distribution of essential biochemical compounds, including starch, lipids, proteins, alkaloids, phenolics, and other secondary metabolites, across different tissues of *D. goaense*. The localization of these compounds provides insights into the metabolic processes and defence mechanisms within the plant, highlighting its pharmacological potential and ecological interactions. Additionally, the presence of these compounds underscores the significance of *D. goaense*in traditional medicine and underscores the need for further pharmacological investigations.

***In Vitro* Culture Studies:** In vitro culture studies aimed at micropropagation and conservation of *D. goaense*explored the efficacy of different growth regulators and culture conditions for shoot induction from bulb explants. Optimal combinations of auxins and cytokinins were identified, leading to successful shoot regeneration. These findings have practical implications for the conservation and propagation of *D. goaense*, providing a valuable tool for ex situ conservation efforts and sustainable utilization of its genetic resources.

In conclusion, the multidisciplinary approach adopted in this study provides a holistic understanding of *Dipcadi goaense*, emphasizing its ecological, physiological, and pharmacological significance. By integrating morphoanatomical, phyto-histochemical, and in vitro culture studies, this dissertation contributes to the broader efforts aimed at conserving and harnessing the potential of this critically endangered plant species for the benefit of both biodiversity conservation and human well-being.

CHAPTER: 6 SUMMARY

Dipcadi goaense, a member of the Hyacinthaceae family, is predominantly comprised of bulbous plants known for their reported medicinal properties. This family has garnered attention in ethnobotanical practices and pharmaceutical research due to the presence of various phytochemicals such as tannins, alkaloids, flavonoids, and saponins. Taxonomically aligned with the genus *Dipcadi*, *Dipcadi goaense* is one of around 30 species within this genus.

Endemic to the region of Goa, India, *Dipcadi goaense* is primarily found in the Rivona area, situated at the foothills of the Western Ghats in South Goa. This unique geographical distribution underscores its ecological significance and highlights its vulnerability to habitat degradation and anthropogenic activities.

In the present study, morphoanatomical investigations were conducted on two distinct plant parts: the leaf and the bulb of *Dipcadi goaense*. The transverse section of the leaf revealed several distinctive features, including a thick cuticle layer on both upper and lower epidermis, amphistomatic characteristics with abundant anomocytic stomata, and a mesophyll tissue devoid of differentiation into palisade and spongy parenchyma. Furthermore, the collateral vascular bundles exhibited an oval or round shape, contributing to the leaf's structural integrity and functionality.

Similarly, the bulb of *Dipcadi goaense* exhibited unique anatomical characteristics, including a single layer of epidermal cells covered with a thin cuticle, oval-shaped parametric cells filled with needle-shaped calcium oxalate raphide crystals and starch granules, and collateral vascular bundles embedded within the parenchymatic tissue. These

anatomical features play crucial roles in the storage, nutrient absorption, and structural support functions of the bulb, contributing to the plant's overall fitness and survival.

Histochemical studies complemented the morphoanatomical investigations by revealing the presence of various plant primary metabolites, including starch, lipids, and proteins, as well as secondary metabolites such as alkaloids and phenols. These biochemical constituents not only influence the physiological processes within *Dipcadi goaense* but also hold potential pharmacological significance, warranting further exploration in pharmaceutical research and drug development.

Dipcadi goaense leaf and bulb extract were subjected to phytochemical investigations using chromatography techniques, isolated phytochemicals such as alkaloids, phenols, steroids and flavonoids, while using UV-Vis spectrophotometric method results revealed that Ethanolic leaf and bulb extracts contain more total phenolics then methanolic and distilled water leaf and bulb extract. *In vitro* tissue culture of *Dipcadi goaense* on M.S Medium supplemented with different concentrations of auxins 2,4-dichlorophenoxyacetic acid (2,4-D), Indole -3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) in combination with cytokines benzylamino purine (BAP) using bulb explant for shoot induction .The percent response for shoot induction from the bulb explant was determined in combinations, MS media supplemented with 2,4-D + BAP, IBA+ BAP, NAA+BAP (3,3.5,4 mg/mL + 1 mg/mL) and revealed that combination 4 mg/mL 2,4-D + 1mg/mL BAP showed highest % of shoot induction that is 100 % followed by 3 mg/mL 2,4-D + 1mg/mL BAP and 3.5 mg/mL 2,4-D + 1 mg/mL BAP both with 96.29 ± 0.57% response. The bulb were also plant in pots in lab which showed full growth after 7 weeks of planting which were further used for different studies like anatomical, histochemical and phytochemical.

Further research may be conducted on the antibacterial and antifungal activities for detailed pharmacological screening for exploration of effective and natural drug. The use of other plant parts of *Dipcadi goaense* other than bulb for tissue culture.

The phytochemical investigations of *Dipcadi goaense* leaf and bulb extracts revealed the presence of various bioactive compounds, including alkaloids, phenols, steroids, and flavonoids. UV-Vis spectrophotometric analysis indicated higher total phenolic content in ethanolic extracts compared to methanolic and distilled water extracts, highlighting the potential pharmacological significance of these compounds.

In vitro tissue culture studies using bulb explants demonstrated the efficacy of different combinations of auxins (2,4-D, IBA, NAA) and cytokinins (BAP) in promoting shoot induction. Among the tested combinations, 4 mg/mL 2,4-D + 1 mg/mL BAP exhibited the highest shoot induction response of 100%, followed by 3 mg/mL 2,4-D + 1 mg/mL BAP and 3.5 mg/mL 2,4-D + 1 mg/mL BAP, both showing 96.29 ± 0.57% response. These findings provide valuable insights into the optimization of tissue culture protocols for mass propagation of *Dipcadi goaense*.

Furthermore, the successful cultivation of *Dipcadi goaense* bulbs in laboratory pots, with full growth observed after 7 weeks, underscores the potential for controlled cultivation and further studies on anatomical, histochemical, and phytochemical aspects. These studies contribute to a deeper understanding of the plant's morphology, biochemical composition, and ecological adaptations.

Future research endeavours may focus on exploring the antibacterial and antifungal activities of *Dipcadi goaense* extracts, thereby providing insights into its potential as a natural source of antimicrobial agents. Additionally, investigating tissue culture protocols

using other plant parts beyond the bulb could expand the scope of cultivation and conservation efforts for this critically endangered species.

Overall, the comprehensive studies presented here pave the way for further exploration of *Dipcadi goaense* and its pharmacological properties, conservation strategies, and potential applications in pharmaceutical research and drug development.

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