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RESEARCH ARTICLE

MICRO-PROPAGATION, PLANT PROPAGATION USING FUNGUS PIRIFORMOSPORA INDICA OF *DIPCADI ERYTHRAEUM WEBB. & BERTHEL*

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ABSTRACT

In this universe all plants In this universe all plants have medicinal value for cure, prevention and treatment of various diseases due to rich source of secondary metabolites such as alkaloids, glycosides, steroids, tannins and flavonoids which is potential source of drugs. Thousands of phyto-constituents and their potential uses have been described so far and many of them have been isolated which are being used in modern medicinal system for the treatment of various diseases and disorders. The major problems which occurs during use of medicinal active constituents from plants is very less yield, which ultimately makes the whole process and also the medicine very high in terms of cost and expenses. Therefore a large number of plant species (especially medicinal) are under the threat of extinction because of their over exploitation. To overcome this problem, various techniques have been employed including plant tissue culture, genetic manipulation, UV radiation exposure, use of fungus Piriformospora indica with jaggery solution etc. to get promising result. *Dipcadi erythraeum* is also known as Jangali Dungari comes under the Rare, Endangered and Threatened species by IUCN and WCMC. *Dipcadi erythraeum* plants have a long history of traditional uses in treatments of wide ranges of diseases. It has been proved by traditionally uses that *Dipcadi erythraeum* has great folk medicinal value such as leaves are used as laxatives; ointments for wounds, bulb and capsules are edible during famine, while whole plant is used for cough, biliousness, diabetes and urinary discharge. Chopped bulbs are fed to animal against stomach pain, scorpion stinging, sweating and Bulbs are also used as remedy for bronchial troubles, cardiac trouble. So the plant was propagated using fungus Piriformospora indica and in vitro micropropagation (plant tissue culture). But overall result showed that there is no possibility of growth of plant *Dipcadi erythraeum*. So it should be conserved through natural conservation like plant protection by over grazing, collection of seeds etc.

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INTRODUCTION

In this universe there is no plant which has non medicinal value and which cannot be made use of for many purpose and by many modes. This definition suggests us that in principle all plants have a potential pharmacological and medicinal value. All medicinal plants have been known as important therapeutic aid for alleviating ailments of humankind. [(1)]In spite of huge developments in the field of allopathic medicines during the 20th century, plants still remain prime sources of drugs in modern as well as in traditional system of medicines. [(2)] These all are broadly used due to their potency, greater availability, affordable and comparatively being very less toxic effects. The medicinal plants are rich source of secondary metabolites such as alkaloids, glycosides, steroids, tannins and flavonoids which are potential source of drugs. There are thousands of phyto-constituents and their potential uses have been described so far and many of them have been isolated which are being used in modern medicinal system for the treatment of various diseases and disorders such as digitoxin obtained from Foxglove leaves (*Digitalis purpurea*), is reported to have cardio-tonic activity, morphine from Opium poppy (*Papaver Somniferum*) is used as narcotic, analgesic and hyoscine obtained from Datura leaves (*Datura metel*) is used as an anticholinergic agent. [(2, 3)] The major problems which occurs during use of medicinal active constituents from plants is very less yield, which ultimately makes the whole process and also the medicine very high in terms of cost and expenses.[(4)] At present there are many well established herbal and plant medicines which are popular in many parts of the world. The World Health Organisation (WHO) reported that 80% of people in the developing world use medicinal plants for their primary health care. Therefore a large number of plant species (especially medicinal) are under the threat of extinction because of their over exploitation. To overcome this problem, various techniques have been employed including plant tissue culture, genetic manipulation,

UV radiation exposure, use of fungus *Piriformospora indica* with jaggery solution etc. to get promising result. [5]

Tissue Culture Techniques: Tissue culture is *in vitro* cultivation of plant cell or tissue under aseptic and controlled environmental conditions, in liquid or on semisolid well-defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant. This technique affords alternative solution to problems arising due to current rate of extinction and decimation of flora and ecosystem. Gottlieb Haberland (1854-1945), a German Botanist is considered as the father of plant tissue culture, was the first to separate and culture plant cells on Knop's salt solution in 1898. [6]

The whole process requires a well-equipped culture laboratory and nutrient medium. This process includes various steps viz. preparation of nutrient medium involving inorganic and organic salts, enriched with vitamins, plant growth hormones, amino acids, Sterilization (free from micro-organism) of explants (source of plant tissue), glass wares, other accessories (flasks, slants, forceps, distilled water) inoculation (transfer of tissue to culture media) and incubation (storage of culture media containing plant tissue at optimum temperature and light). [7] Now a day, Plant tissue culture technique (including tissue culture of cells, tissues or organs of plant) has become a dominant technique in plant research. This plant tissue culture technique has been made practical and has been applied for commercial use.

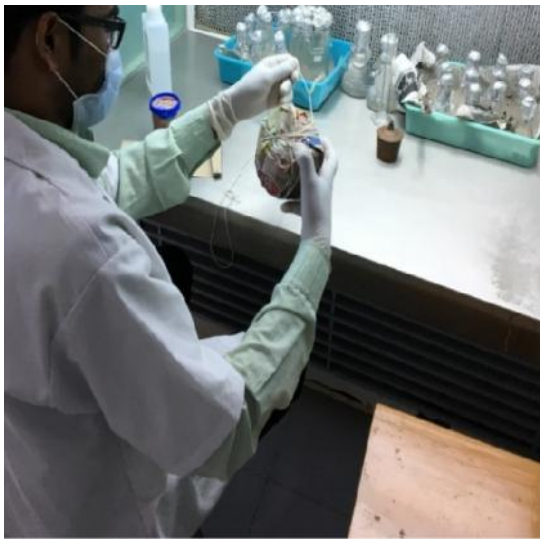

Figure 1. Plant tissue culture

Micro Propagation: Plant tissue culture is also called micro propagation, which was a technique consisting of taking a piece of plant (such as a stem tip node, meristem, embryo, or even a seed) and placing it in a sterile nutrient medium where it multiplies. Plant tissue can be described as plasticity or totipotency. Plasticity, allows plants to alter their metabolism, growth and development to best suit their environment. While totipotency can be defined as the ability of each living cell of a multicellular organism to develop independently if provided with proper external conditions. A totipotent cell is one that is capable of developing by regeneration into whole organisms, and this term was probably coined by Morgan (1901). [8]

Objectives of Micropropagation [9]: The objectives of micropropagation includes Production of virus free stock, to multiply plants whose multiplication rate is very low, to produce progenesis which are genetically identical to their parents, to obtain genetic variability, Recovery of distant hybrid,Germplasm conservation, Germplasm exchange and Genetic transformation (addition or deletion of gene). In conventional cultivation many plants do not germinate, flower, fruits and seeds produced under certain climatic conditions or have long periods of growth and multiplication. Micropropagation insures a good regular supply of medicinal plants, using minimum space and time.

Advantages: Various advantages of in vitro micropropagation includes like higher rate of multiplication, Environment can be controlled or altered to meet specific needs of the plants, Plant available all year round (independent of regional or seasonal variation), Identification and production of clones with desired characteristics, Production of secondary metabolites, New and improved genetically engineered plant can be produced, Conservation of threatened plant species, In vitro germ plasm preservation.

Culture Media: Nutritious (nutritional) condition for optimal growth of a plant tissue culture may depend upon plant species. Even tissues from different parts of a plant may have different requirements for proper satisfactory growth. There is no single medium which can be suggested for the satisfactory growth of all types of plant tissues and organs. Before starting with a new system, it is necessary to work out a medium that would fulfil the specific requirement of that tissue. List of several culture media developed by scientists to culture diverse tissues and organs are Gautheret (1942), White (1943), Haberland *et al* (1946), Haller (1953), Nitsch and Nitsch (1956), Murashige and Skoog (1962), Eriksson (1965) and B5 (Gamberg *et al* 1968). The composition of culture media included macronutrients and micronutrients (Inorganic nutrients), a separate iron supplement, vitamins, a carbon source (Organic nutrients) and usually plant growth regulators. Amino acids and various nitrogenous compounds may be present in vitamin mixture. Macronutrients were nitrogen, phosphorus, potassium, calcium, magnesium and sulphur, these were essential elements required in relatively large amounts. Micronutrients were traces of certain elements required by all plant cells. Micronutrients included iron, manganese, zinc, boron, copper, molybdenum, iodine, cobalt, and chlorine. Meanwhile, vitamins have catalytic functions in enzyme systems and were required only in trace amounts.

Figure. 2. Culture media

In addition to the nutrients, it is generally necessary to add one or more growth hormone such as auxin, cytokinins and gibberellins to support better growth of tissues and organs. However, the requirement for these substances varies considerably with tissue.

Auxins: Hormones of this group includes elongation of stem and internodes, apical dominance, tropism, abscission, and rooting etc. In plant tissue culture technique auxins promotescell division and root formation/differentiation. Both natural IAA (Indole-3-Acetic Acid) (quickly deactivated by certain environmental factors e.g.light) and Synthetic (relatively stable) IBA (Indole-3-Butyric Acid), NAA (Naphthalene Acetic Acid), NOA (Napth Oxyacetic Acid), 2,4-D (2,4-Dichlorophenoxy acetic acid) and 2,4,5-T (2,4,5-Trichlorophenoxy acetic acid) are utilized. In auxins, Indole-3-Butyric Acid and Naphthalene Acetic Acid are extensivelyutilized for rooting. In combination with cytokinins, the auxins are utilized for shoot proliferation. 2, 4-Dichlorophenoxy acetic acid and 2, 4, 5-Trichlorophenoxy acetic acid are capable for the induction and growth of callus. Indole-3-Butyric Acid prevent the bud formation and also play an important role in embryogenesis. Auxins are usually dissolved in ethanol or dilute Sodium Hydroxide.

Cytokinins: It is Phytohormones naturally present in plant, which have potent effect on cell division (cytokinesis). Chemically cytokinins are adenine derivatives and have been utilized in plant tissue culture to induce the formation of adventitious buds and shoots from undifferentiated cells. In cell cultures they have been shown to promote the biosynthesis of berberine, condensed tannins and rhodoxanthin. Cytokinin (adenine or kinetin) in the medium encourage bud differentiation and development. The potency of Kinetin is 30,000 times more than adenine, while kinetin is only seldom used for callus induction aside from specific experimental purpose. Other cytokinins which influence the induction of shoot buds include 6-Benzyl amino purine (BAP) or 6-Benzyl Adenine (BA), 6-y-y-Dimethyl amino purine (2-iP), 6-tetrahydropyrane-adenine and zeatin. Zeatin and 2-iP are naturally occurring cytokinins while BA (6-Benzyl Adenine) and kinetin are synthetically derived. BAP (6-Benzyl amino purine) and zeatin are very commonly used to promote and maintain growth of callus and cell suspension cultures. Cytokinins are generally dissolved in dilute Hydrochloric Acid or Sodium Hydroxide.

Gibberellins: There are over 20 identified gibberellins.Out of these, GA_3 is extensively employed to increase the shoot elongation in plant tissue culture. In comparison to auxins and cytokinins, gibberellins are used hardly. It is reported to stimulate normal development of plantlets from in vitro formed adventives embryos. GA is readily soluble in cold water up to 1000mg per litre.

Solidifying Agents for Solidification of the Medium: There are various solidifying agents used for solidification of the media such as Agar, Alginates, Carrageenan, Gelatin, Hydroxyethyl cellulose, Polyacrylamide, Starch and Silica Gel.

pH of The Culture Medium: In general, pH of the medium is maintained between 5.0 and 6.0 before sterilization. If pH of the medium higher than 6.0, it give fairly hard medium and if the pH of medium is below 5.0, it does not allow satisfactory gelling of the Agar.

Techniques of Plant Tissue Culture: There are various techniques of plant tissue culture like Callus culture/Static culture (growth of callus masses on solidified media), Suspension culture (growth in liquid media consisting of mixture of single cells or cell aggregates), Protoplast culture (protoplast culture can be grown as Callus culture/static tissue culture or Suspension culture [8, 9,)]

Application of Tissue Culture Technology: Tissue culture technology is used in the Production of Phyto-pharmaceuticals, Industrial production of secondary metabolites, Biochemical conversion (Biotransformation), Clonal propagation (Micropropagation), Soma clonal variation and Immobilization of plant cells.[10]

General Information of *Piriformospora indica*

Botanical name: *Piriformospora indica*

Piriformospora indica was discovered by Prof. (Dr.) Ajit Varma and his colleagues from Thar Deserts of Western India in 1992 from the root system of several xerophytic plants. Way baxk in 1997, the properties of the fungus *Piriformospora indica* were patented in Germany (European Patent Office, Muenchen, Germany. Patent No. 97121440.8-2105, Nov. 1998). So far, more than 148 plants have been interacted with the fungus *Piriformospora indica* and data was documented. The results are positive. Subsequently a new family *Sebacinaceae* and new order Sebacinales were erected. [11, 12]

Morphological Characters of *Piriformospora indica:* The fungus has very simple morphology containing hyphae and pear shaped large spores. The fungus can be cultivated on simple defined medium both in solid and liquid broth. Optimum conditions for growth are temperature 27°C ± 2, pH 6.8 and carbon energy 1.5% glucose. Incubation is done on the rotatory shaker (120 rpm). Best growth is obtained after 7 days incubation where colonies can be large or small. [13]

Figure. 3. Amorphous powder form of fungus Piriformospora indica

Hyphal Cells: The hyphal cells of Piriformospora indica are found as thin walled, and they are always hyaline and not pigmented. They have an obvious less diameter in comparison to other hyphal types of the Basidiomycota. Dimension of the hyphae strongly depend up on the culture conditions. In water agar or under less nutrient conditions, the hyphal cells are very long and extremely small. Cultivation of the *Piriformosporaindica* in complex media, the hyphal diameter ranges from 0.6 mm to 3.5 mm. The length of hyphal in Momagar is 10-20 mm but in water agar the hyphal cells can be a 50mm or more. The hyphae is found in tubular form but very often the hyphae are found to be strongly moniliform. The hyphae are frequently separated but the number of nuclei per

cell is not stable. The hyphal cells are multinucleate. There is no development of clamp connection at the septa. Sometimes the hyphae are collected in many layers. It is not rare that hyphae are interconnected by several anastomoses. Within the hyphal cells, maximum number of observed nuclei was upto 8, but in the most case, 2-6 nuclei per cell only. The single nucleus is always distributed within the hyphal cells. [14]

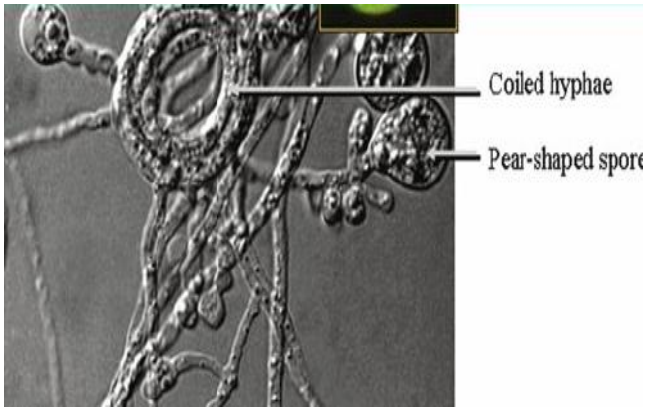

Figure. 4. Microscopic view of fungus *Piriformospora indica*showing hyphae

Mycelium: *Piriformospora indica* can be cultured (cultivated) on several synthetic and complex media; it develops under solid and liquid culture conditions. The morphology/anatomy of the mycelium strongly varies depending on the constitutions of the nutrients of the culture medium and the condition of cultivation (solid or liquid media). Most of the mycelium of *Piriformospora indica* develop under the surface of the agar media. Using solid culture media, only few aerial hyphae are formed. The mycelium grows concentric and covers the agar media homogenously. In few cases the mycelium forms rhythmic rings in the petri dishes. Young mycelium cultures are white but as the ageincrease, the colour changed to cream yellow. Structure of mycelium is homogenous, no specialized hyphae could be observed, but few hyphae are arranged in coils. [14]

Chlamydospore: The hyphae produce colourless Chlamydospore in culture. The Chlamydospore appears from terminal hyphal tips. Their formation begins with a swollen thin-walled vesicle differentiated by a cell wall. The mature spores (fully grown spores) are almost globular, but one side is little bit smaller and they look like a pear shaped. Size of the mature Chlamydospore differ between 14-33 mm in length and 20 mm in width. The Chlamydospore producing hyphae are unevenly swollen near the point of the first spore formation and a little bit inflated. Young (immature) developing spores are clavately. Sequentially some more Chlamydospore are developed at the end of less branching hyphae. Therefore, the Chlamydospore is usually aggregate in groups [14, 15]. Traditionally, fungi have been regarded as pathogens by agronomists. However, in recent years, symbiotic fungi providing benefits to crop plants have become an additional focus on research. In addition to the *Arbuscular mycorrhizal* fungi (Amf) that constitute a distinct fungal phylum, one particular endophyte, *Piriformosporaindica*, a member of the *Sebacinales* order of the Basidiomycota, recently has received some attention. Originally this fungus was recovered from the rhizopore of shrubs growing in the Thar Desert of Rajasthan, India. The root endophyte fungus *Piriformospora indica* a novel identified cultivable endophyte that colonizes monocot as well as Dicot roots and it functions like a bio-regulator, bio-fertilizer and bio-protector against root pathogens, it defeat water stress (dehydration), acidity, drought and heavy metal toxicity, it secure from pests, delay the wilting of the leaves, extend aging of callus tissues, it improve secondary metabolites production and the nutritional value of the plant. [11]

Inoculation with the fungus *Piriformospora indica* and application of fungal culture filtrate promotes plant growth and biomass production. Due to its ease of culture, this fungus provides a model organism for the study of beneficial plant-microbe interactions and a new tool for improving plant production systems. Fungus interactions are characterized by a more efficient nutrient uptake from the soil due to a better hyphae penetration into the soil compared to penetration of the thicker root hair. The plant deliver phosphor-assimilates to the fungus. Mycorrhizal associated plant acquire phosphate from the extensive network of fine extra radical hyphae of fungus, which extend beyond root depletion zones to mine new regions of the soil. [13]

Activities of *Piriformospora indica:* Several studies have indicated that the interaction, alter the pathway for nitrogen metabolism where by transferring more nitrogen nutrients to the plants because of the presence of hyphal cells that penetrates into the soil. *Piriformospora indica* influence the sulphate reduction leading to formation of sulphur protein and glutathione contents. This in turn, influences the resistance against water deficiency and drought. [16] The supply of the fungus with carbon sources and the faster growth of colonized plants require the background of starch which is deposited in the root amyloplasts. Thus it is not surprising that one of the major starch degrading enzymes, the glucon water dikinase is activated by the fungus. [16]

General Information of *Dipcadi erythraeum*

Botanical Name: *Dipcadi erythraeum* Webb. & Berthel.

Synonym:*Dipcadi unicolor, Ornithogalum erythraeum, Uropetalon erythraeum, Uropetalon unicolor, Uropetalum unicolor.* [17]

Other Names: Jangli Dungri, Jangali Bussur. [18]

Habitat: It comprised about 40 species, distributed in Southern Europe, most area of Africa, the Middle East to South Asia, Qatar, and Pakistan. The greatest diversity is found in South Africa (13 species) and India (9 species). In Egypt, the genus *Dipcadi* is a member of family *Hyacinthaceae* and is represented by two species: *Dipcadi erythraeum* (*D. erythraeum*) and *Dipcadi unifolium*. In India, it is very rare plant, found on the rocky substratum, drier parts of Rajasthan and Gujarat (in Indian Thar Desert). In Rajasthan it is observed on rocky substratum near guptaganga, sursagar, Jodhpur and Barmer after first few rains. [19, 24]

Morphological Character/Description of Plant: It is a bulbous, scapigerous herb, 15-18cm high, bulb tunicated, and 13-20mm in diameter. Leaves 15-20x4-5mm, narrow linear, scape up to 20cm long, Flowers greenish in a lax raceme, bracts 1-2cm long, gradually smaller upwards, ovate finely acuminate, pedicle 4-6mm long. Perianth campanulate, 15mm long, outer lobes 9x3mm, elliptic oblong, obtuse with tips recurved from the middle, 7 nerved inner lobes, 5 nerved,

reflexed from the tips only. Ovary elliptic-ovoid, 6mm long, sessile. Fruit a capsule, 12-15mm long, slightly narrowed at base, orbicular, flat, black. Flowering and fruiting occurs in the month of August to September. [19, 20]

Figure. 5 Whole plant of Dipcadi erythraeum

Figure. 6. Bulbous part of Dipcadi erythraeum

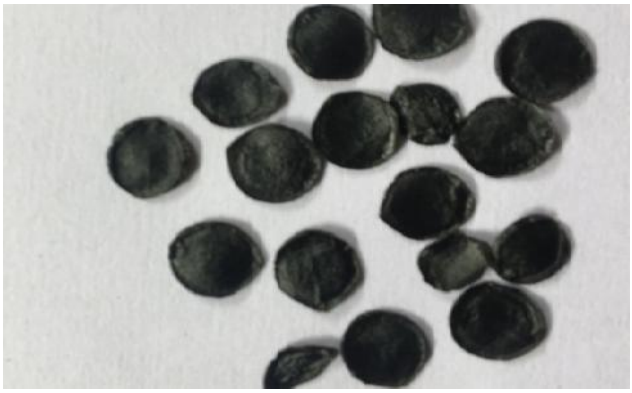

Figure. 7. Seeds of Dipcadi erythraeum

Phytoconstituents of *Dipcadi erythraeum:* The phytochemical screening of aqueous methanol extract of *Dipcadi erythraeum* was analysed by LC-ESI-MS system (High Performance Liquid Chromatography and Mass Spectroscopy) revealed the presence of alkaloids, tannins, saponins and flavonoids.

The *Dipcadi erythraeum* bulb extract revealed 22 phenolic compounds characterized for the first time, fourteen of them were identified as C-glycosyl flavonoids. The phenolic compound were identified as Gluconic acid, Shikimic acid hexoside, Coumaric acid di hexoside, Caffeic acid di hexoside, Quinic acid, Malonyl coumaroyl quinic acid, Ferulic acid hexoside, Isoorientin-7-O-β-glucopyranoside (lutonarin), Apigenin 6,8-di-C-rhamnoside, Apigenin 6,8-di-C-glucoside, Isoorientin X''-O-acetyl, Apigenin 6-C-pentoside-8-C-hexoside X''-O-acetyl, Delphinidin-O-hexoside X''-O-acetyl-O-pentoside, Luteolin 6-C-β-glucopyranoside-8-C-α-arabinopyranoside (Carlinoside), Apigenin 6-C-β-glucopyranoside-8-C-α-rhamnopyranoside (Violanthin), Apigenin 6,8-di-C-pentoside-O-rhamnoside, Apigenin 6-C-hexoside-8-C-pentoside, Luteolin-O-methyl ether 6-C-pentoside-8-C-hexoside, Apigenin 6-C-pentoside-8-C-rhamnoside, Apigenin 6-C-pentoside-8-C-rhamnose-O-rhamnoside, Apigenin 6-C-α-rhamnopyranoside-8-C-β-glucopyranoside (isoviolanthin), Apigenin-O-methyl ether 6-C-hexoside-8-C-rhamnoside-O-hexoside. It also contains kaempferol, quercetin, quercetin-3-O-(6''-a-rhamnopyranosyl B-glucopyranoside-7-O-α-rhamnopyranoside, vitexin, isovitexin, orientin and isoorientin.[23] The highest values of total chlorophylls and carotenoids were observed during flowering stage which was ranged from 0.473 -0.860 and 0.000261-0.000426 mg/gm freshly weighted respectively. The proline values ranged from 0.406 to 1.213 µg g-1 freshly weighted during three stages, being maximum in vegetative stage. Total sugar values ranged from 27.28 to 38.24 mg g-1 dried weight during three phases, being maximum in vegetative stage. Crude protein was observed highest (4.882% dried weight) during vegetative phase followed by flowering (4.650) and lowest at fruiting phase (3.022% dried weight). The phosphorus content was highest during vegetative stage followed by flowering and minimum at fruiting stage. Total alkaloid and phenol contents were found to be maximum during flowering stage and values ranged from 2.1 to 3.7 % dried weight and 976.66 to 1198 mg 100 g-1 dried weight, respectively. [25, 26]

Ethno medicinal Uses: *Dipcadi erythraeum* is a wild medicinal plant which have significant folk medicinal use. Its bulb and capsule are edible (consumed) especially in Pakistan. In Bahrain, the leaves are consumed as a laxative and as an ointment for wounds. While whole plant is consumed for cough, biliousness, diabetes and urinary discharge. Chopped bulbs are nourished to animal against stomach pain, scorpion stinging & sweating. Bulbs are also used as remedy for bronchial troubles, cardiac trouble and anticancer [22]

Pharmacological Activity: *Dipcadi erythraeum* plant has not been studied extensively; despite of the various medicinal values, only anticancer activity of phytoconstituents present in *Dipcadi erythraeum* has been evaluated. [24]

MATERIALS AND METHODS

Identification, Collection and Authentication of Plant *Dipcadi erythraeum:* Seeds and one Plant of *Dipcadi erythraeum* were initially collected in the month of August (rainy season) from CAZRI, Jodhpur and one plant was collected from rocks nears Kaylana lake. It was then allowed to grow and then leaves dried properly.

Herbarium sheets of plant were then prepared for identification and authentication purpose. Authentication of plant was then done by Botanical Survey of India (BSI), Jodhpur. The seeds and bulb from the plant were used for further experimental work.

Figure. 8. Collected Plant of Dipcadi erythraeum from CAZRI, Jodhpur

Figure. 9. Collected Seeds of Dipcadi erythraeum from CAZRI, Jodhpur

Plant propagation of *Dipcadi erythraeum:* The seeds were propagated without treating with fungus *Piriformospora indica* (control seeds) and using fungus *Piriformospora indica* (test seeds).

Plant propagation of *Dipcadi erythraeum* without treating with fungus *Piriformospora indica* (control seeds); Initially the weeds were removed from field and the field was planned for sowing of seeds. Collected seeds (control seeds/fungus untreated seeds) were sown in the planed field. Watered the plants regularly and observed for growth of plants.

Plant propagation of *Dipcadi erythraeum* using fungus *Piriformospora indica* (test seeds): Initially, Jaggery Solution was prepared by dissolving 20gm jaggery in 100ml water which worked as adhesive for inoculation of seeds with fungus and also provided nutrients for plant growth. Seeds were then inoculated with the fungus in Petri dishes for 1 hour, which allowed complete interaction of the seeds and fungus and then the test seeds (fungus treated) were sown into the cleaned and planed field. Watered the plants regularly and observed for growth of plants.

Figure. 10. Sown seeds of plant *Dipcadi erythraeum*(control seeds) and watered the field

Figure. 11. Jaggery solution

Figure. 12. Seeds of Plant Dipcadi erythraeum treated with Fungus Piriformospora indica and Jaggery solution

In vitro* Micropropagation of *Dipcadi erythraeum

Sterilization of culture room/growth chamber room and glassware: Sterilization of culture room and growth chamber room was done with disinfectant (Sufficient amount of $KMnO_4$ was allowed to contact with 40% Formalin solution) for 48

hours, and then with UV light for 8 minute. Sterilization of glass wares were done in hot air oven at 105$^{\alpha}$C for 45 min.

Preparation of MS media: All the micronutrients, macronutrients and other nutrients were taken in separate 100 ml conical flask as per table no. 1, and all components were dissolved using distilled water. The entire dissolved component contained in flask was mixed in 1000 ml beaker and then 30 gm of sugar (as source of carbon) was added to it. The mixture was allowed to stirring on the magnetic stirrer with the help of magnetic bead. 16 gm of agar (solidifying agent) was added slowly and volume was made up to 1000 ml using distilled water.

The culture media was allowed to continue stirring for proper mixing of all components. The prepared culture media was transferred in various conical flasks and slants. All the flasks and slants contained culture media were cotton plugged and packed with the help of aluminium foil and allowed to sterilization.

Figure. 13. Prepared culture media

Sterilization of culture media and glassware: The culture media, forceps and petri dishes were sterilized in autoclave at 15psi pressure and 121$^{\alpha}$C temperature for 30 minutes. After completion of 30 minutes the autoclave was switched off and the temperature was allowed to settle down at room temperature.

All the flasks and slants contained culture media, forceps and petri dishes were removed from autoclave and transferred to aseptic room for inoculation of seeds and explants of *Dipcadi erythraeum.*

Sterilization of laminar air flow bench and blade: Laminar air flow bench was sterilized by ethanol and then with UV light for 2 minutes. The blade used to cut explant was sterilized on Bunsen burner.

Collection and Sterilization of explant and seeds of *Dipcadi erythraeum:* Explant and seeds of *Dipcadi erythraeum* collected from CAZRI, Jodhpur were washed properly with distilled water and sterilized with 0.1% mercuric chloride solution.

Figure. 14. Sterilization of explant of *Dipcadi erythraeum*

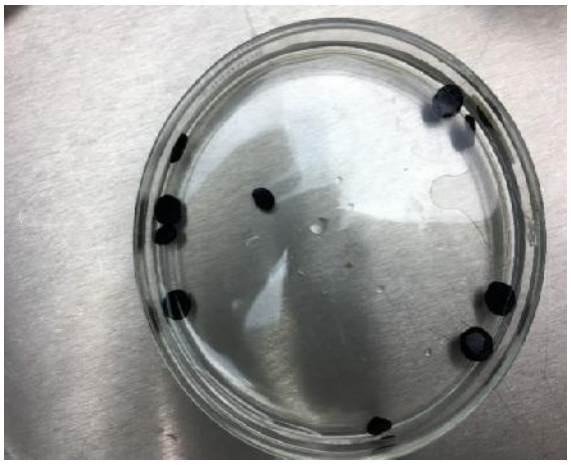

Figure. 15. Sterilization of seeds of *Dipcadi erythraeum*

Inoculation of seeds and bulb: The inoculation was done in laminar air flow bench by sterilizing hands with ethanol. The sterilized seeds and bulbs rinsed 5 times with distilled water were taken one by one with the help of forceps and then dropped into each flask and slants containing sterilized MS media by opening cotton lid of flask near flame and then recapped the cotton lid to flasks.

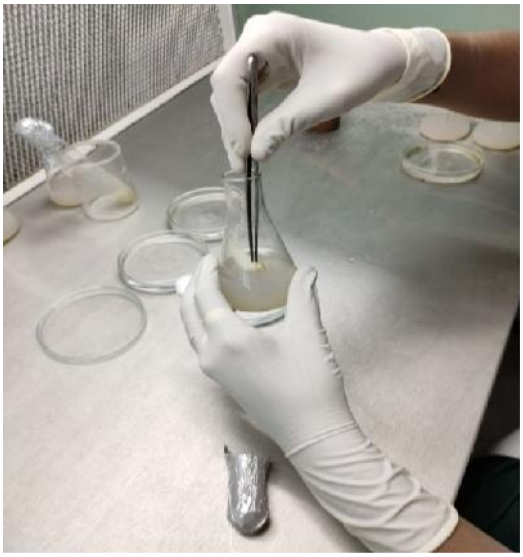

Figure. 16. Inoculating explant of *Dipcadi erythraeum*

Figure. 17. Inoculating seeds of plant *Dipcadi erythraeum*

Incubation of Flask and slants containing Seeds and Explant of *Dipcadi erythraeum:* The recapped flasks and slants were placed in growth chamber maintained at temperature 25±2 $^{\alpha}$C under 7/5/12 hours/day i.e. 7h/day photoperiod from cool while fluorescent tube light, 5h/day dim-light, and 12h/day in dark. Then culture was observed for growth.

Figure. 18. Incubation of flasks containing Seeds of *Dipcadi erythraeum*in MS media

Figure. 19. Incubation of flasks containing explants of *Dipcadi erythraeum*in MS media

Inoculation of Explants: After 21 days, the shoots grown of above culture of bulbs were used as explant. The bulbs were cut with sterilized blade into small pieces inside laminar air flow chamber; each piece must contain 2-3 nodal portions.

Explant culture were established in solid basal MS medium supplemented with different concentration of kinetin and Indole Acetic Acid for shoot proliferation and multification. Then the flasks were placed in growth chamber maintained at temperature 25±2 $^{\alpha}$C under 7/5/12 hours/day i.e. 7h/day photoperiod from cool while fluorescent tube light, 5h/day dim-light, and 12h/day in dark. Then culture was observed for growth.

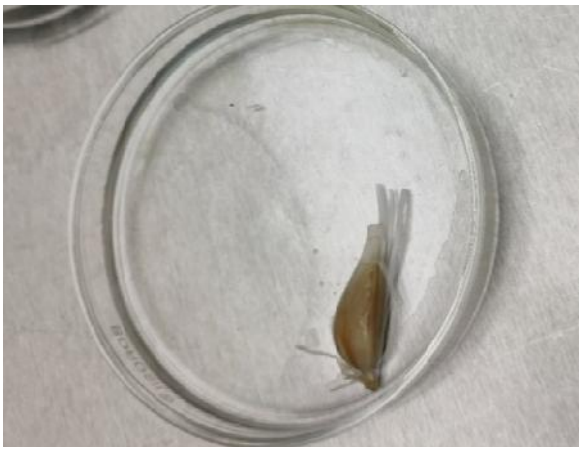

Figure. 20. Sterilization of explant of *Dipcadi erythraeum*

Figure 21. Incubation of grown explant of *Dipcadi erythraeum*

RESULTS

The growth of plants was not observed in fungal treated seeds (test) and fungal untreated seeds (control) of *Dipcadi erythraeum* plant in field. The growth of plants were not observed in the flasks and slants containing seeds in MS media but slightly growth of nodal part were observed in the flasks containing Bulb as nodal part in the MS media.

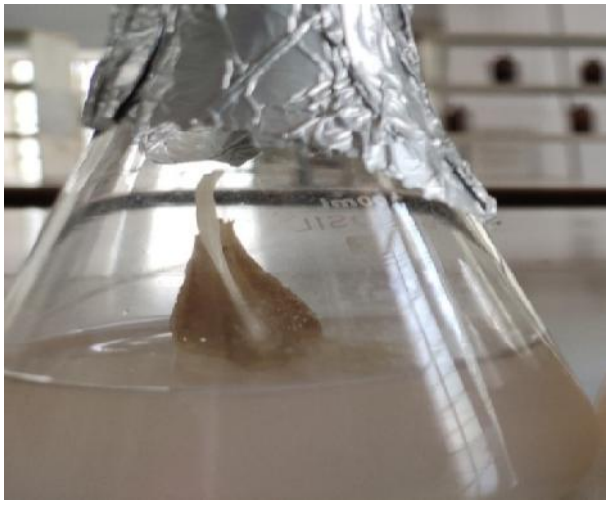

Figure. 22. Slightly growth observed in explant of *Dipcadi erythraeum*

Table 1. Composition Of Ms Media [8, 9]

Ingredients	Quantity
Macronutrients	
Ammonium Nitrate	1650 mg/L
Calcium Chloride	440 mg/L
Magnesium Sulphate	370 mg/L
Potassium Phosphate	170 mg/L
Potassium Nitrate	1900 mg/L
Micronutrients	
Boric Acid	6.2 mg/L
Cobalt Chloride	0.025 mg/L
Cupric Sulphate	0.025 mg/L
Ferrous Sulphate	27.8 mg/L
Manganese Sulphate	22.3 mg/L
Potassium Iodide	0.83 mg/L
Sodium Molybdate	0.25 mg/L
Zinc Sulphate	8.6 mg/L
Sodium Salt of EDTA	37.2 mg/L
Common Organic Compounds	
Myo-Inositol	100 mg/L
Niacin	0.5 mg/L
Pyridoxine.HCl	0.5 mg/L
Thiamine.HCL	0.1 mg/L
IAA	1-30 mg/L
Kinetin	0.04-10 mg/L
Glycine	2.0 mg/L
Sucrose	30 g/L
Agar	10 g/L

Table 2. Taxonomical Classification Of Fungus *Piriformospora Indica*[16]

Kingdom	Fungi
Division	Basidiomycota
Class	Agarimycota
Order	Sebacinales
Family	*Seacinaceae*
Genus	*Piriformospora*
Species	*Piriformospora indica*

Table 3. Taxonomical Classification of *Dipcadi Erythraeum* [23]

Kingdom	Plantae
Super division	Spermatophyta
Division	Angiospermae
Class	Monocotyledon
Family	Asparagaceae
Subfamily	Scilloideae
Genus	Dipcadi
Species	Dipcadi erythraeum

CONCLUSION

Dipcadi erythraeum popularly known as Jangli Dungri, is a shrub belonging to family Asparagaceae. Review of Literature survey claimed that Dipcadi erythraeum has great folk medicinal value including leaves as laxatives properties (relief from constipation), prepared ointment is used for wound healing, bulbous are eaten during famine in Pakistan, while whole plants have been used in cardiac trouble, urinary discharge, sweating (cause body cooling), stomach pain in animals, scorpions stinging and snake bite etc.. Anticancer activities have been reported recently. So, due to over exploitation, soil erosion, habitat loss, over grazing and less potency of seeds fertilization, International Union for Conservation of Nature and World Conservation Monitoring Centre marked this plant as rare, endangered and threatened plant of Thar Desert. Therefore Dipcadi erythraeum was selected for its conservation and preservation using plant propagation with fungus P. indica and in vitro Micropropagation. The seeds and plant of Dipcadi erythraeum were collected from CAZRI Jodhpur, Rajasthan. The plant species was identified and authenticated from Botanical Survey of India, Jodhpur. The seeds of Dipcadi erythraeum were treated with jaggery solution and fungus Piriformospora indica (test) and untreated seeds (control) were grown in the field and observed every week. No any growths were observed in test and control group of seeds of plants. The in vitro Micropropagation of seeds and bulbs (shoot) was developed by making MS media and by sterilization of MS media, seeds and nodal part these were transferred in MS media. Seeds don't reveal any growth in both, fields as well as in MS media while nodal parts (bulb) shown slightly growth in MS media. The effects on growth was compared in both field condition and in MS medium, no any growth were obtained which indicates infertility (less Potency) of seeds. The nodal portion revealed the growth of plants in in-vitro micropropagation that indicates the capability of nodal part and also concludes that nodal part can be multiplied using MS media.

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