Callus production and Micropropagation and Phytochemical Investigation of Corallocarpus epigaeus (Arn.) Cl. – A Potential Antidiabetic Medicinal Plant

Kaushik H Nakum
Sardar Patel University
Vipul B Vaja
Sardar Patel University
Kalpesh B Ishnava
kalpeshishnava@spuvvn.edu

Sardar Patel University

Article

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Abstract

The present study deals with the *in vitro* callus induction, micropropagation and phytochemical analysis of *Corallocarpus epigaeus* (Arn.) Cl. The tuber, node and leaf were used as explants and cultured on MS medium with NAA, 2,4-D, BAP and KN alone and different combinations for callus induction and micropropagation. The best callus formation of tuber explants was obtained on BAP: NAA after 20 days of inoculation while the nodal explants show best callus formation on BA: NAA after 10 days. Leaf explants not show callus induction in any combination. The nodal explants were selected for shoot multiplication and inoculated on MS media supplemented with the combination (BAP and IBA) of PGR shows the positive results of shoot multiplication and maximum shoot obtained after 15 days of inoculation. NAA of PGR shows the positive results of root production and maximum root and length obtained after 25 days of inoculation. After 25-day callus of tuber explants was collected and subjected to phytochemical investigation which indicates the presence of alkaloids, flavonoids, Phenols, steroids and saponins. The yield of alkaloids, flavonoids and Phenols are 4.71%, 0.23% and 0.013% respectively. Further study required for the large-scale production of secondary metabolites through callus production and also large-scale production of propagules required the standardization.

Introduction

The proof about utilization of therapeutic plants for human wellbeing can be seen in numerous old records, for instance records in ayurveda reports have numerous treatment methodology for various sicknesses [1, 2, 3]. These days there are many notable plant-based prescriptions are accessible in the market which has capability of relieving savage infections are famous all around the globe. As per world wellbeing association (WHO), around 80% of total populace relies upon the restorative plants for essential social insurance [4]. The plant-based medicines are growing nowadays worldwide, 25% peoples of United Kingdom (UK) use the plant-based medicines for curing the diseases[4]. The substance amalgamation of some pharmaceutical mixes is beyond the realm of imagination or not financially practical in this way around 40% compound utilized in assembling of prescriptions are gotten from plants [5, 6, 7].

Plant tissue culture is viewed as the most proficient innovation and it has a few favorable circumstances over conventional strategy for spread like uniting, through seed and cutting. Micropropagation method for the most part utilized and through this prevalent nature of plant is produce which has better ailment opposition and stress resilience limits, micropropagation system is utilized for the protection and duplication of yield plant like organic products, vegetables, grains, restorative, fancy and woodland, has made new open doors for ranchers, nursery proprietors, in worldwide exchanging and furthermore supportive for rustic advancement [8, 9]. Additionally the micropropagation techniques are employed for culturing the medicinal plants to produce important compounds for herbal and pharmaceutical industries. Micropropagation techniques are also used for conservation of threatened medicinal plants [10].

The *Corallocarpus epigaeus* (Arn.) Cl. is a rare important medicinal plant which belongs to the family Cucurbitaceae. It is commonly known as aakashguddah, jangalee suran, rakashguddah etc. It is a monoecious, deciduous and perennial tendril climber with a large turnip- shaped root and distributed in tropical Africa, Persian Gulf region and India. Monoecious, climber with tuberous root. Stem angular-sulcate, glabrous to finely pubescent. Leaves sinuately 3 lobed, hairy on both surfaces. Male peduncle 4–6 cm long, 5–15 flowered. Calyx lobes lanceolate. Corolla greenish yellow color and ovate. Female flowers often solitary; pedicel 1–5 mm long,

thickened in fruit; calyx-tube campanulate, petals 1.5–2.5 mm long, 1-1.5 mm broad, reflexed. Fruit ovoid or ellipsoid, beaked, glabrous, smooth, red except for the greenish base and portion of beak which is 2.5-5 mm long. Seeds asymmetrically pyriform, smooth, yellowish, turgid. Flowers and fruiting in June to October [11, 12].

Phytochemical screening of tubers reveals the presence of phytonutrients and medicinally valuable secondary metabolites including flavonoids, alkaloids, phenolics, tannins, saponins and steroids [13, 14]. Phytochemicals perhaps impart potent pharmacological properties to the great rhizomes that used in the controlling the diabetic disease [15, 16, 17].

Because of exploitative collecting of tubers for exchange and denudation of backwoods, the normal populace of *Corallocarpus epigaeus* has declined to such a degree, that it is presently viewed as uncommon and undermined in its common natural surroundings. A few creators put it under imperiled class since normal recovery of this species is very poor by virtue of long seed lethargy, failed seeds, amazingly poor germination, and deficient accessibility of tuberous roots as propagules attributable to exploitative gathering. Consequently, they unequivocally anticipate misgiving of its annihilation soon if sufficient preservation measures are not embraced earnestly.

Arrangement lies in mediations of biotechnological approaches for *in vitro* recovery which offers quick and boundless accessibility of planting materials consistently. Biotechnological mediations for recovery utilizing *in vitro* culture method have been effectively utilized for some yields and a few reports are likewise accessible for comparable wild Cucurbitaceae taxa. There has been no report accessible so far for *in vitro* callus induction and micropropagation and support of *in vitro* biodiversity for conservation of the germplasm for the specie *Corallocarpus.* Hence, into account the rising biological basic for restoration of species of *Corallocarpus epigaeus* for conservation for human benefit.

Material and Methods

2.1 Collection of seeds and explants

The seed, tuber and whole plant of *Corallocarpus epigaeus* were collected from surrounding area of Jalaram temple, Virpur, Gujarat during the month of June 2023 (Figure1). The plant specimen was identified by Dr. Kalpesh Ishnava (Plant Taxonomist) at Sardar Patel University, Vallabh Vidyanagar, Gujarat. The voucher specimen (SPU-2023-02) were deposited in the herbarium of P. G. Department of Biosciences, Sardar Patel University.

2.2 Seed germination study

2.2.1 *In vivo* seed germination (Effect of GA3 on seed germination)

Petriplates was taken and the same size of filter papers was placed into it. Then 2 to 3ml of different stock solutions of GA_3 was added to each plate and 1 seed per each plate was inoculated. Control was also prepared by inoculating 1 seed in distilled water. All the petridishes provided the normal light (16hr) and proper nutrition for growth and development. Seed germination rate was observed after 5 days.

2.2.2 *In vitro* seed germination and effect of GA3 on seed germination

The viability of the seed was determined by soaking the seeds in distilled water for 24 h. The non-viable seeds were observed at the surface of water and were discarded immediately. The surface sterilization of seeds was carried out in 3 different steps. Seeds were immersed in lab wash containing beaker under running tap water for about 30 minutes. Then immerse into surface sterilization 0.1% HgCl2 solution for approximately 3 min [18].

2.2.3 Culture medium

Seeds were then washed with sterile distilled water 3 times and inoculated on MS medium[19] with sucrose (3%) and pH 5.7-5.8 and also use of different concentration of hormones of GA3 (0.1ppm to 0.5ppm). After transfers the seed in semisolid medium inoculated and all tube transfers in the culture room in temperature 25^0C. Before the inoculation the Laminar Air Flow was subjected for UV light transmission for about 30 – 45 minutes. The inoculation of seeds was carried out under aseptic conditions under Horizontal Laminar Air Flow.

2.2.4 Surface sterilization

The surface sterilization of seed was carried out in different steps. The seeds were placed in different beaker and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface, then surface sterilized by immersion in 75% alcohol for 30 seconds, followed by one rinse of 3 min in sterile distilled water, additionally immersed in 0.1% mercuric chloride ($HgCl_2$) solution for 4 min followed by five times rinses of 5 min each in distilled water. The surface sterilized different seeds were blotted dry on sterile filter paper.

2.2.5 Inoculation of seeds

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench. The chamber was then sterilized with U.V. rays continuously on for one hour. Hands and arms which were to be used inside the inoculation chamber were scrubbed with alcohol before inoculation. The rims of the test tubes and the sides of the plugs were flame sterilized. Instruments (like forceps, scalpels, spatula etc.) were all sterilized by dipping in the alcohol and flaming a number of times. Care was taken to cool the instruments before putting into operation. After sterilization of seeds, seed were inoculated in culture tubes aseptically. For inoculation explants were transferred to large sterile glass petriplate or glass plate with the help of sterile forceps under strict aseptic conditions. Some Seeds transferred to culture tubes containing MS medium and some in to MS media with GA3 (0.1 % to 0.5%). After inoculating the seed in culture tube, the mouth of tube is quick flamed and tubes are tightly closed by cotton plug. After proper labeling clearly mentioning media, date of inoculation etc. the tubes were transferred to growth room.

2.2.6 Culture Condition

The tubes were shifted to culture room with controlled facility of diffused light (2000 lux) for 8 hrs. and rest of the period dark condition daily at 28 ± 2°C temperatures and 50 to 60% relative humidity.

2.3 Callus induction study

2.3.1 Preparation of culture medium

MS medium was used for callus of *Corallocarpus epigaeus*. Murashige and Skoog (MS) medium supplemented with different concentrations of PGRs such as α-naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D), benzyl amino purine (BAP) alone and BAP in combinations with NAA, Benzyl adenine (BA) in combinations with NAA, 2,4-D in combinations with Kinetin (KN) for callus induction. The medium was supplemented with 3% sucrose, gelled with 0.8% agar and the pH was adjusted to 5.7-5.8. Around 20ml of medium was used for each culture tubes. Then media was autoclaved at 121°C and 15 psi pressure for 15 minutes.

2.3.2 Callus induction

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench. After sterilization of explants (Nodal, tuber and leaf), explants were inoculated in culture tubes aseptically. For inoculation explants as per standard protocol. The culture tubes were transferred to growth room [20].

2.3.3 Culture Condition

The tubes and bottles were shifted to culture room with controlled facility of diffused light (3000 lux) for 10 hrs. and rest of time dark condition daily at 28 ± 2°C temperatures and 50 to 60% relative humidity.

2.3.4 Collection of calluses

The calluses were collected after 25 days. Calluses were washed with distilled water to remove all adhering particles. After that calluses were allowed to dry at room temperature. This callus was use for different analysis for study. Good quality callus was sub cultured and again collected after more 30 days and subjected to further analysis.

2.4 Micropropagation

2.4.1 Preparation of culture medium

MS medium was used for micropropagation of *Corallocarpus epigaeus*. Murashige and Skoog (MS) medium supplemented with different concentrations of PGRs such as α-naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D), benzyl amino purine (BAP) alone and 2,4-D in combinations with Kinetin (KN), IBA in combination with BAP for shooting And IBA in combination with BAP for rooting. The medium was supplemented with 3% sucrose, gelled with 0.8% agar and the pH was adjusted to 5.7-5.8. Around 40ml of medium was used for each culture bottles. Then media was autoclaved at 121°C and 15 Psi pressure for 15 minutes.

2.4.2 Explants sterilization

The surface sterilization of nodal explants was carried out in different steps. First off, all cut the leaves of isolated nodal segment. Explants were further soaked in fungicide (1gm/lit Bavistin) for 5 min and then antibiotic Streptomycin (50mg/lit) for 3 min then surface sterilized by immersion in 75% alcohol for 30 seconds, followed by one rinse of 3 min in sterile distilled water, additionally immersed in 0.1% mercuric chloride ($HgCl_2$) solution for 3 min followed by five rinses of 5 min each in distilled water. The surface sterilized nodal explants were blotted dry on sterile filter paper.

2.4.3 Inoculation of explants

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench. After sterilization of explants, explants were inoculated in culture tubes aseptically. For inoculation explants were transferred to large sterile glass petriplate or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed to make them in suitable sizes. Trimming was removed with sterile scalpel blade. After cutting explants into suitable size (for Node 1-2 cm), explants are transferred to culture tubes and bottles containing MS medium with different hormone concentration. After vertically inoculating the explants in culture tube the mouth of tubes is quick flamed and tubes are tightly capped with cotton plug. After proper labeling clearly mentioning media, date of inoculation etc. the bottles were transferred to growth room.

2.4.4 Multiplications of shoot stage

After 15 days of inoculation culture showing spouting were transferred to full length MS media supplemented with different combination of growth hormones concentration for multiplication of shoot regeneration.

2.4.5 Rooting stage

Newly formed shoots measuring 2.0-3.0 cm in length were excised individually from the parents and explants and transferred to new MS medium with different concentration of IAA and NAA hormone.

2.4.6 Culture Condition

The tubes and bottles were shifted to culture room with controlled facility of diffused light (3000 lux) for 10 hrs. and rest of time dark condition daily at 28 ± 2°C temperatures and 50 to 60% relative humidity.

2.4.7 Hardening

In vitro rooted plantlets with 2 to 3 nodes and having at least 2 to 4 roots of 2 to 4 cm length were washed carefully with water to remove traces of agar and then transferred to the pots containing different sterile soil mixtures viz. Cocopeat + Sand + Soil (1:1:1). The pots were covered with tight plastic covers (With holes) to prevent desiccation and to avoid rapid changes in environment and acclimatized in the mist house at 25-30 °C temperature and 14 hrs. illumination. During the hardening procedure, plastic covers were gradually perforated after one week and after second week they were removed and the plants were maintained in the mist house conditions for 15 more days.

2.5 Phytochemical analysis of callus

2.5.1 Extraction of callus

Dried sample of callus from *in vitro* grown plant were grinded with mortar and pestle. The dry sample was extracted using 20 ml of methanol in Erlenmeyer flask placed on shaker at 100 rpm for overnight at room temperature. The crude extract filtered with filter paper (Whatman No. 1). The filtrate was collected and allowed to solvent evaporation. After evaporation the remaining material was collected and different stocks were prepared by dissolving in methanol.

2.5.2 Phytochemical screening

Qualitative assay for the presence of plant phytoconstituents such as Alkaloids, Glycosides, Flavonoids, Phenol, Tannins and Saponins and quantitative assay were carried out on alkaloids, total phenol and total flavonoidsfollowing standard procedure [20, 21].

Results and Discussion

Due to exploitative harvesting of tubers for trade and denudation of forests, the natural population of *Corallocarpus epigaeus* has declined to such an extent that it is now considered rare and threatened in its natural habitats. Hence, they explicitly predict apprehension of its extinction in the near future if adequate conservation measures are not adopted urgently. So here in the present study we have initiated and developed the callus and micropropagation of the leaf, node and tuber of *Corallocarpus epigaeus* and then evaluated the phytochemical analysis of methanolic extract of tuber callus.

3.1 Seed germination study (*In vivo* and *in vitro*)

In vivo seeds germination of *Corallocarpus epigaeus* was done usingGA_3 at different concentration (1ppm to 5ppm). There is no germination was observed in any concentration of GA_3 after 25 days. The seed germination of this plant is very poor because of seeds having long dormant period. Seed coat of this plant is very hard and also the embryo abortion observed during seed germination. *In vitro* seeds germination attempt of *Corallocarpus epigaeus* was made byusing MS media supplemented with different concentrations of GA_3 (1ppm to 5ppm). No seed germination was observed in MS medium (*in vitro*).

3.2 Callus Production

Callus induction from leaf explants of *Corallocarpus epigaeus* using MS basal medium supplemented with different plant growth regulators such as NAA, 2,4-D and BAP alone and KN in combination with 2,4-D is mentioned in Table 1. The NAA, 2,4-D and BAP alone do not show any response in the leaf explants. The leaf explants inoculated in MS media supplemented with KN (0.3mg/lit) + 2,4-D (3mg/lit) and KN (0.5mg/lit) + 2,4-D (5mg/lit) shows the callus induction after 6 days of inoculation. The callus produced is green and yellow respectively.

Callus induction from nodal explants of *Corallocarpus epigaeus* using MS basal medium supplemented with different concentrations of BAP and NAA is mentioned in Table 1 and Figure 2A. Brownish callus was produced after 6 days of incubation on MS media supplemented with BAP (4ppm). Brownish, White and Green callus was produced in the nodal explants inoculated in MS media supplemented with NAA (1ppm to 5pmm) after 6-7 days of inoculation (Figure 2B). Callus induction from nodal explants of *Corallocarpus epigaeus* using MS basal medium supplemented with different concentrations of 2,4-D is mentioned in Table 1. Brownish callus was produced in the nodal explants inoculated in MS media supplemented with 2,4-D (0.2 mg/lit.), 2,4-D (1.5 mg/lit.) and 2,4-D (5 mg/lit.) after 15 and 6 days of inoculation respectively. Callus induction in the nodal explants was observed in the different combinations of NAA and BAP (Table 1). Brownish callus was produced in the nodal explants inoculated in MS media supplemented with different combinations of hormones BAP (0.5mg/lit) + NAA (5mg/lit), BAP (1mg/lit) + NAA (4.5mg/lit) and BAP (3.5mg/lit) + NAA (2mg/lit) after 6-8 days of inoculation (Figure 2B). Mehul and Kalpesh 2015 reported that a combination of BAP and NAA was more suitable

combination for callus induction of *M. dioica* [22]. Hoque et al., (1995) found that a combination of 1.5mg/l BAP and 0.1 mg/l NAA was more suitable combination for adventitious multiple shoots formation of *M. dioica* [23]. Where present investigated BAP + NAA combination is most suitable for callus induction. Devendra et al. (2012) reported that the callus initiation started from nodal base on 10th day 2.0 mg/l NAA, 2.0 mg/l BAP auxin or cytokinin either alone or in combinations were efficient for the induction of callus depending on the varied concentrations of the growth regulators [24]. The different combinations of KN and 2,4-D shows callus induction in the nodal explants (Table 1). Off white and brownish callus was produced in the nodal explants inoculated in MS media supplemented with different combinations of hormones KN(1mg/lit) + 2,4-D(1mg/lit), KN(0.1mg/lit) + 2,4-D(1mg/lit), KN(2mg/lit) + 2,4-D(0.2mg/lit) and KN(0.2mg/lit) + 2,4-D(2mg/lit) after 4-5 days of inoculation (Figure 2B). Salvador et al., (2009) reported the *Alternanthera tenella*, an amaranthaceae family, MS basal medium was supplemented with 1.0 mg/lit 2, 4-D and 1.0 mg/lit KN and 2.5 mg/lit NAA and 1.0 mg/lit BA for callus induction [25]. The different combinations of NAA and BA show callus induction in the nodal explants (Table 1). Green callus was produced in the nodal explants inoculated in MS media supplemented with different combinations of hormones BA (2mg/lit) + NAA (1mg/lit), BA (1mg/lit) + NAA (1mg/lit), BA (0.5mg/lit) + NAA (1mg/lit) after 4-5 days of inoculation.

Callus induction from tuber explants of *Corallocarpus epigaeus* using MS basal medium supplemented with different concentrations of BAP in combination with NAA is mentioned in Table 1 and Figure 2F. The different combinations of NAA and BAP show callus induction in the tuber explants. Brownish callus was produced in the tuber explants inoculated in MS media supplemented with different combinations of hormones BAP (0.5mg/lit) + NAA (5mg/lit), BAP (1mg/lit) + NAA (4.5mg/lit) and BAP (1.5mg/lit) + NAA (4.0mg/lit) after 7 days of inoculation (Figure 2I, 2J, 2K, 2L). Castillo *et al*., (1998) reported that auxin 2,4-D by itself or in combination with cytokinin has been widely used to enhance callus induction and maintenance [26]. Moreover, many researchers observed 2,4-D as the best auxin for callus induction as common as in monocot and even in dicot [27, 28, 29, 30, 31]. This combination is more suitable for more production of the biomass for secondary metabolites. Callus induction from tuber explants of *C. epigaeus* using MS basal medium supplemented with different concentrations of BAP is mentioned in Table 1. Brownish, green and yellow callus was produced after 6 days of incubation on MS media supplemented with BAP (1 to 5 mg/lit). Callus induction from tuber explants of *C. epigaeus* using MS basal medium supplemented with different concentrations of NAA is mentioned in Table 1. Brownish, yellow and green callus was produced in the tuber explants inoculated in MS media supplemented with NAA (3 and 5 mg/lit) after 9 days of inoculation (Figure 2H). Callus induction from tuber explants of *C. epigaeus* using MS basal medium supplemented with different concentrations of 2,4-D is mentioned in Table 1. Brownish callus was produced in the tuber explants inoculated in MS media supplemented with 2,4-D (0.1mg/lit to 5mg/lit) after 9 days of inoculation (Figure 2H). Callus induction from tuber explants of *C. epigaeus* using MS basal medium supplemented with different concentrations of BAP in combination of NAA is mentioned in Table 1. The different combinations of NAA and BAP show callus induction in the tuber explants. Cream white callus was produced in the nodal explants inoculated in MS media supplemented with different combinations of hormone KN (2mg/lit) + 2,4-D (0.2mg/lit) after 4-5 days of inoculation (Figure 2G). No callus was produced in the tuber explants inoculated in MS media supplemented with different combinations of hormones BA in combination with NAA after 40 days of inoculation.

3.3 Micropropagation

MS media supplemented with higher concentration of cytokinin along with lower concentration of auxins were used for the shoot multiplication. The nodal explants were selected for shoot multiplication and inoculated on MS media supplemented with higher concentration of BAP and lower concentration of IBA. The combination (BAP 1ppm : IBA 0.01ppm) of PGR shows the positive results of shoot multiplication and maximum shoot (3 shoot) obtained after 15 days of inoculation (Table 2 and Figure 2C). The *in vitro* raised shoots were inoculated on to MS supplemented with various combinations of auxin and cytokinin for root formation. Root formed in all combination and concentration of hormone but highest frequencies of rooting were observed on MS fortified with 1.0 mg/lit of IAA and 0.5 mg/lit NAA after 15 days of inoculation (Figure 2D). But prior to the root initiation callus was taking place (Figure 2B). The reduced rooting may be due to the imbalance between the endogenous auxin and exogenous auxin, IAA. In the present study, initially the rooting percentage was very low, these results are in accordance with those on *Withania somnifera* [32], when IAA was tested for rooting, there was not only decrease in the rooting response but also enhanced basal callusing from the *in vitro* raised shoots were observed. These results conform to those of Anand et al., 1997 in *Kaempfera rotunda* [33]and Ankita and Kalpesh, (2015) in *Coccinia grandis* (L.) Voigt. [34]. After shoot and root production transfer to primary and secondary hardening of the plants. After 3 week plants transfer to natural condition (Figure 2E).

3.4 Phytochemical analysis of callus

3.4.1 Qualitative analysis of callus from tuber

The callus obtained in different combination of growth hormones MS + BAP (0.5mg/lit) + NAA (5mg/lit), MS + BAP (0.5mg/lit) + NAA (5mg/lit) and MS + BAP (0.5mg/lit) + NAA (5mg/lit) was extracted by methanol. Methanolic extract of various calluses was subjected to qualitative analysis. The analysis shows the presence of alkaloids, flavonoids, saponins, phenols and steroids (Table 3). Kattamanchi et al., (2013) reported the ethanolic extract of *Corallocarpus epigaeus* rhizomes was found the presence of phenolic compounds, Flavonoids, terpenoids and phytosterols [15].

3.4.2 Quantitative analysis of callus

Estimation of alkaloids *in vitro* callus obtained in the medium supplemented different combinations of PGRs (BAP-0.5mg/lit + NAA-5mg/lit; BAP-1mg/lit + NAA-4.5mg/lit; BAP-1.5mg/lit + NAA-4mg/lit) was done by Harborne method. The evaluation alkaloids of callus were done after 25 days of inoculation. Then remaining callus was subjected to sub culture on same medium and again analysis of alkaloids was done after sub culture. The percent yield of alkaloids in callus was calculated by back calculation and mentioned in Table 4. After 25 days maximum percentage yield present in BAP-1mg/lit + NAA-4.5mg/lit and after sub culture percentage yield of alkaloids was obtained in same concentration.

Estimation of phenols *in vitro* callus obtained in the medium supplemented different combination of PGRs (BAP-0.5mg/lit + NAA-5mg/lit; BAP-1mg/lit + NAA-4.5mg/lit; BAP-1.5mg/lit + NAA-4mg/lit) was done by Folin's method. The evaluation alkaloids of callus were done after 25 days of inoculation. Then remaining callus was subjected to sub culture on same medium and again analysis of alkaloids was done after sub culture. The percent yield of phenols in callus was calculated by back calculation and mentioned in Table 4.

Estimation of flavonoids *in vitro* callus obtained in the medium supplemented different combination of PGRs (BAP-0.5mg/lit + NAA-5mg/lit; BAP-1mg/lit + NAA-4.5mg/lit; BAP-1.5mg/lit + NAA-4mg/lit) was done by

aluminum chloride method. The evaluation flavonoids of callus were done after 25 days of inoculation. Then remaining callus was subjected to sub culture on same medium and again analysis of flavonoids was done after sub culture. The percent yield of flavonoids in callus was calculated by back calculation and mentioned in Table 4. Kattamanchi et al., (2013) reported the ethanolic extract of *Corallocarpus epigaeus* rhizomes possesses the antidiabetic action which is comparable with that of the standard Glibenclamide drug employed [15]. This work supports the traditional claim of the rhizomes for their use in diabetes. This ethanolic extract of *Corallocarpus epigaeus* presents the flavonoids. In our study show the in vitro callus induction more production of theflavonoids compares to the normal is less produce. This work supports the traditional claim of the rhizomes for secondary metabolites production through callus induction increases the biomass.

Conclusion

Callus initiation of nodal explants took place after 4-6 days of inoculation while the tuber explants shows callus initiation after 7-9 days of inoculation. The best degree for callus formation of tuber explants was obtained on MS medium supplemented with BAP:NAA (0.5mg/lit:5.0mg/lit, 1.0mg/lit:4.5mg/lit and 1.5mg/lit:4mg/lit) while the nodal explants shows highest degree for callus formation on MS medium supplemented with BA (2mg/lit) in combination with NAA (1mg/lit). The nodal explants were selected for shoot multiplication and inoculated on MS media supplemented with higher concentration of BAP and lower concentration of IBA. The combination (BAP 1ppm: IBA 0.01ppm) of PGR shows the positive results of shoot multiplication and maximum shoot (3 shoot) obtained after 15 days of inoculation. NAA (3ppm) of PGR shows the positive results of root production and maximum root (1 root) and length (4cm) obtained after 25 days of inoculation. The present study showed successfully micropropagation of *Corallocarpus epigaeus.* Phytochemical investigation the tuber callus indicated the presence of alkaloids, flavonoids, phenols, steroids and saponins. The yield of alkaloids, flavonoids and phenols are 4.71%, 0.23% and 0.013% respectively in 25-day old callus of tuber explants. Further study required for the large-scale production of secondary metabolites through callus production and also large-scale production of propagules required the standardization.

Declarations

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

All data include in the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest about this manuscript and research.

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Author Contribution

Kaushik Nakum and Vipul Vaja design the methodology and perform the experiments. Dr. Kalpesh Ishnava interpretation of experiment results and preparing the manuscript.

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Tables

Table 1. Callus induction from tuber and nodal explants of *Corallocarpus epigaeus*

Nutrient media	Tuber Explants			Nodal explants		
MS- media + Growth hormones (mg/L)	% of explants showing callus	Duration of callus production	Responses	% of explants showing callus	Duration of callus production	Responses
BAP (1)	50	9 days	Light yellow	-	-	-
BAP (2)	100	11 days	Brownish	-	-	-
BAP (3)	50	6-7 days	Green	-	-	-
BAP (4)	-	-	No change	100	6 days	Brownish
BAP (5)	100	15 days	Brown green	-	-	-
NAA (1)	-	-	-	100	6-7 days	Brownish
NAA (2)	-	-	-	100	6-7 days	Brownish
NAA (3)	50	9 days	Light yellow	100	6-7 days	Brownish
NAA (4)	-	-	No change	100	5-6 days	White
NAA (5)	100	9 days	Brown green	100	6-7 days	Green
2,4-D (0.1)	50	9 days	Off white	-	-	-
2,4-D (0.2)	-	-	No change	100	15 days	Brownish
2,4-D (0.5)	50	9 days	Off white	-	-	No change
2,4-D (0.7)	50	22 days	Off white	-	-	No change
2,4-D (1)	100	15 days	Brownish	-	-	-
2,4-D (1.5)	100	22 days	Off white	100	6 days	Brownish
2,4-D (2)	50	22 days	Off white	-	-	-
2,4-D (3)	50	15 days	Off white	-	-	-
2,4-D (4)	50	9 days	Off white	-	-	No change
2,4-D (5)	50	15 days	Off white	100	6 days	Brownish
BAP (0.5) + NAA (5)	100	7 days	Light brown	100	6-7 days	Cream brown
BAP (1) + NAA (4.5)	100	7 days	Light brown	100	6-7 days	brown

BAP (1.5) + NAA (4)	100	7days	Light brown	-	-	No change
BAP (2) + NAA (3.5)	50	15 days	Light brown	-	-	-
BAP (2.5) + NAA (3)	50	15 days	Light brown	-	-	No change
BAP (3) + NAA (2.5)	50	11 days	Light brown	-	-	No change
BAP (3.5) + NAA (2)	50	9 days	Light green	100	8 days	Brownish
BAP (4) + NAA (1.5)	-	-	No change	-	-	No change
BAP (4.5) + NAA (1)	-	-	No change	-	-	-
BAP (5) + NAA (0.5)	-	-	-	-	-	-
BAP (3) + NAA (3)	100	9 days	Light green	-	-	-
KN (1) + 2,4-D (1)	-	-	No change	100	4-5 days	Off white
KN (0.1) + 2,4-D (1)	-	-	No change	100	4-5 days	Off white
KN (2) + 2,4-D (0.2)	50	15 days	Cream white	100	4-5 days	Off white
KN (2) + 2,4-D (2)	-	-	No change	-	-	-
KN (0.2) + 2,4-D (2)	-	-	No change	100	4-5 days	Brown
BA (1) + NAA (0.5)	-	-	No change	100	4-5 days	Green
BA (1) + NAA (1)	-	-	No change	-	-	-
BA (0.5) + NAA (1)	-	-	No change	100	4-5 days	Brown green
BA (2) + NAA (1)	-	-	No change	100	4-5 days	Light Green
BA (1) + NAA (0.5)	-	-	No change	100	4-5 days	Green

Table 2. Effect of hormones on shoot multiplication and root production

Sr. No.	MS medium + BAP : IBA	No. of shoots (in Days)			Length of shoot (cm)			No. of Roots (in Days)			Length of Root (cm)		
		5th	10th	15th	5th	10th	15th	5th	10th	15th	5th	10th	15th
1	1 ppm : 0.1 ppm							-	-	-	--	-	-
2	1 ppm : 0.01 ppm	1	3	3	1±0.2	4±0.5	6±0.4	-	-	-	--	-	-
3	2 ppm : 0.2 ppm	-	-	-	--	-	-	-	-	-	--	-	-
4	2 ppm : 0.02 ppm	-	-	-	--	-	-	-	-	-	--	-	-
5	3 ppm NAA	-	-	-	--	-	-	1	3	3	1±0.5	3±0.4	5±1

Table 3: Qualitative analysis of *Corallocarpus epigaeus* callus from tuber

Secondary Metabolite	MS + BAP (0.5mg/l) + NAA (5mg/l)	MS + BAP (1mg/l) + NAA (4.5mg/l)	MS + BAP (1.5mg/l) + NAA (4mg/l)
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Terpenoids	-	-	-
Phenols	+	+	+
Tannins	-	-	-
Glycosides	-	-	-
Steroids	+	+	+

Table 4: Quantity analysis of *Corallocarpus epigaeus* callus from tuber

Callus inoculated on MS-media + PGRs (mg/L)	Alkaloids			Phenols			Flavonoids		
	Wt. of callus (gm)	Wt. of alkaloids (mg)	% yield	Wt. of callus (gm)	Wt. of Phenols (mg)	% yield	Wt. of callus (gm)	Wt. of flavonoids (mg)	% yield
BAP (0.5) + NAA (5)	1.511	68	4.50	1.511	199.8	0.013	1.511	1.34	0.088
BAP (1) + NAA (4.5)	1.528	72	4.71	1.528	201.6	0.013	1.528	3.45	0.225
BAP (1.5) + NAA (4)	1.541	57	3.69	1.541	219.6	0.014	1.541	1.50	0.097

Figures

Figure 1

Explants (Leaf, Nodal and Tuber) of *Corallocarpus epigaeus*

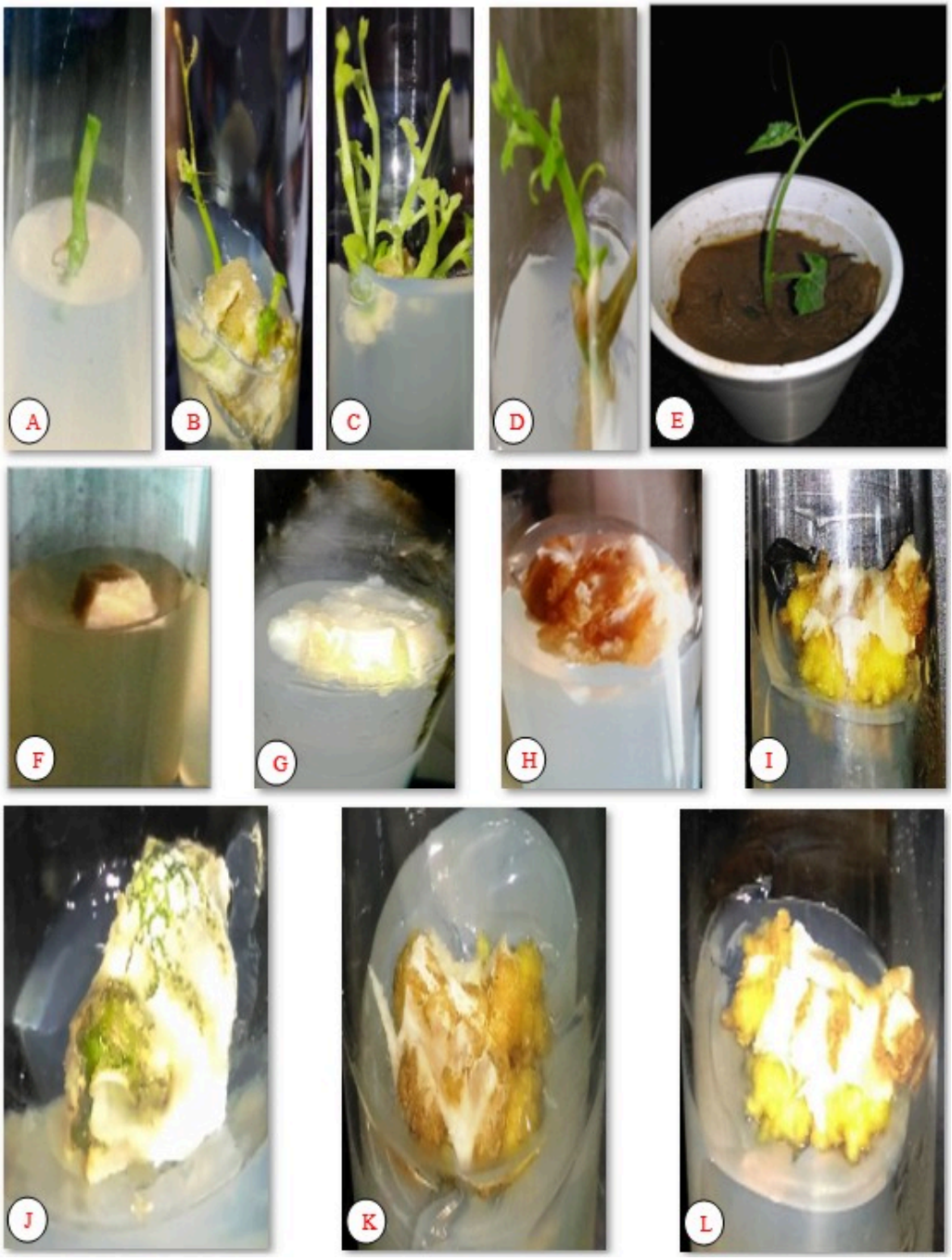

Figure 2

Callus induction and micropropagation of Corallocarpus *epigaeus*

A: Inoculation of nodal explants; B: Nodal explants produce callus; C: Shoot production; D: Shoot and root production; E: Harding the plants; F: Inoculation of tuber; G: Tuber explants produce white callus; H: Tuber explants produce brown callus; I: Tuber explants produce brownish green callus; J: Tuber explants produce green callus; K: Callus production after 25 days; L: Callus production after 35 days