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Authors' Affiliation:

1. Govt.
College Women University,
Faisalabad - Pakistan
2. Department of Botany,
University of the Punjab,
Lahore - Pakistan

*Corresponding Author:

Arneeb Tariq
Email:
arneebtariq@gcwuf.edu.pk

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In vitro Micropropagation of *Citrullus colocynthis* (L.) Schrad: an endangered medicinal plant

Arneeb Tariq^{1*}, Humera Afrasiab², Fozia Farhat¹

Abstract

Background: The experiment describes the establishment of *in vitro* conditions for seed germination, micropropagation, callogenesis, organogenesis and acclimatization of *Citrullus colocynthis* (L.) Schrad, of family Cucurbitaceae.

Methods: *In vitro* grown seedlings from decontaminated seeds were micropropagated in basal MS medium at 23±2°C temperature and light intensity of 3000 Lux for 16 hours in culture room. *In vitro* grown nodal explants were supplemented with BAP (6-Benzylaminopurine) and NAA (Naphthalene acetic acid) with basal MS (Murashige and Skoog) medium to induce multiple shoots. Indole butyric acid (IBA; 0.1 to 2.0 mg/L) was supplemented to MS medium to develop roots of micropropagated shoots. Internodes and leaves of micropropagated shoots used to induce callus in MS medium enriched with varying concentration of 2, 4-dichlorophenoxyacetic acid (2, 4 D; 0 to 2.0mg/L) and kinetin (KIN; 0 to 1.0mg/L). Shoot initiation from callus was tested by adding 2, 4-D (0.1 to 2.0 mg/L) and BAP (1.0 to 1.5 mg/L) in basal MS medium. Conditions were carefully monitored during the experiment. After hardening, the micropropagated plantlets were placed in open filed environment in pots filled with sand and peat moss (3:1).

Result: Surface sterilized seeds of *Citrullus colocynthis* (L.) showed 100% germination in regulator free medium. Significantly mature shoots (75%) from nodal explant recorded in BAP (2.0 mg/L) and NAA (1.0 mg/L) augmented MS medium. Highest number (90%) of roots per shoot explant were observed in IBA (2.0 mg/L). Leaf explants showed better response to form callus with a combination of 2, 4-D (1.0 mg/L) and KIN (1.0 mg/L) and further rise in 2, 4-D concentration caused a sharp decrease in callus formation. Shoot induction from callus cultures observed in MS medium containing 2, 4-D (2.0 mg/L) and BAP (1.5 mg/L), producing an average of 10 shoots per culture. Plants were effectively transplanted in open environment with survival rate of 85%.

Conclusion: Results indicate the successful establishment of the growth room conditions for *in vitro* micropropagation of the endangered medicinal plant, *Citrullus colocynthis*.



Introduction

Citrullus colocynthis is a member of genus *Citrullus* belonging to Cucurbitaceae family also known as bitter apple, bitter gourd, wild gourd and in India and Pakistan commonly called "Tumba" [1]. It is an ever-growing prostrate herb local to tropical countries and warmer parts of Asia, Egypt, Nigeria and Mediterranean region. In Pakistan, it is grown in sandy soil of Layyah, Muzaffargarh and Jhang [2].

About 70-80% of the population of the world relies upon traditional sources for their primary healthcare especially in the developing countries [3]. *C. colocynthis* is cultivated for its extremely bitter insect repellent pulp and seeds which are used to cure constipation, edema, diabetes, cancer and jaundice [4]. Other clinical studies also show its anti-inflammatory, anti-oxidant and antimicrobial properties and anti-microbial properties [5-6].

The seeds of *C. colocynthis* are a rich source of oil (53%), protein (28%), minerals and vitamins [7]. Its oil composition is similar to other edible oils such as safflower, sunflower, soybean, sesame and cottonseed and also contains high proportion of unsaturated fatty acids (80%) mainly linoleic acid and oleic acid [8]. Seeds are being used for biofuel production in Nigeria [9]. This plant can thrive in extreme arid environment by accumulating citrulline, an efficient ROS scavenger [10]. Its extract is rich in antioxidants and metabolites such as anthocyanin, cucurbitacin and flavonoids [11].

Citrullus colocynthis is susceptible to a number of fungi including *Colletotrichum bryoniae*, *Erysiphe cichoracearum*, *E. semitectum*, *Fusarium oxysporum*, and *Puccinia citrulli* considerably reducing its yield [12]. Its population is decreasing drastically due to overexploitation and rapid urbanization so current scenario demands protection of this endangered species. An alternative approach to overcome these problems is implementation of tissue culture techniques to produce disease-free plants with better yield and also for the conservation of germplasm of *C. colocynthis*. In other plants of family Cucurbitaceae, like muskmelon, watermelon, squash and cucumber, efficient protocol of plant tissue culture has been established. Very limited research work has been carried out on tissue culturing of *C. colocynthis*. Therefore, this study work was planned to optimize the growth conditions for the micropropagation of this medicinally significant plant.

Methods

Explant sterilization and medium preparation:

Seeds of *C. colocynthis* were taken from Punjab University Seed Center, Lahore. After washing with running water, the seeds were sterilized with alcohol (70%), followed by 3% sodium hypochlorite (NaOCl) solution and rinsed with autoclaved distilled water under sterile conditions. Seed coat was removed before inoculation in MS medium [13].

Seed germination and plantlet production:

Seeds were allowed to germinate for 4 days in MS basal medium at 23±2°C, at light intensity of 3000 Lux for 16

hours in culture room. Plantlets produced were micropropagated by culturing apices and nodes in fresh medium for further growth.

Multiple shoot induction

Excised nodes of micropropagated plants were incubated in MS medium having varying concentrations of BAP (0.1 to 2.0 mg/L) with NAA (1.0 mg/L). Multiple shoots arising from nodes were sub-cultured in medium under same hormonal concentrations in culture room at 23±2°C, at light intensity of 3000 Lux for 16 hours. For root formation, varying levels of IBA (0.1 to 2.0 mg/L) were tested.

Callus induction

For callogenic response 1-1.5 cm pieces of internodes and leaves were incubated in test tubes on medium supplemented with varying levels of 2, 4-D (0.5 to 2.0 mg/L) with constant level of KIN (1.0 mg/L). Cultures were kept at 3000 Lux light intensity for 16 hour photoperiod at 23±2°C temperature.

Organogenesis

Proliferative, healthy looking 30-40 days old calli were transferred on MS medium enriched with (0.5 to 2.0 mg/L) and BAP (1.0 to 1.5 mg/L) for regeneration of shoots at 23±2°C and 3000 Lux for 16 hours in culture room.

Acclimatization

From culture tubes, the micropropagated plants were shifted to pots containing sand and peat moss in 3:1. The pots were placed under optimized conditions of culture room for hardening. After 3-4 weeks, the pots containing plants were placed under field conditions for further acclimatization.

Statistical analysis

The experimental trials were performed in triplicates each with ten replicates. Data was tabulated and statistically evaluated using SPSS (21.0) software and significant results were recorded at $p \leq 0.05$. Variations between mean values were analyzed by Tukey's test.

Results

Decoated seeds inoculated on basal MS medium showed 100% germination response (Fig. 1a). Shoots from apices and nodes initiated after 7 and 10 days respectively in basal MS medium (Fig.1b, 1c). In MS basal medium devoid of any phytohormone, only 4% root induction was recorded from *in vitro* shoots (Fig. 1d). Multiple shoot formation was achieved through medium modified by different levels of BAP with NAA (1.0 mg/L). A slight swelling prior to shoot emergence was visible in incubated nodes. In all concentrations of phytohormones tried, MS medium containing BAP 2.0 mg/L in combination with 1.0 mg/L NAA, resulted highest number of shoots (4.93±1.45) per node after 15 days of inoculation. A direct and positive correlation (0.98) was found between the shoot number/length and multiple shoot induction to BAP compared to NAA (Fig 2c-2d; Table 1). For root initiation, micropropagated shoots were shifted on basal MS medium augmented

with varying concentration of IBA (0.1 to 2.0 mg/L) and kept in optimized conditions at 23±2°C for 7 to 10 days. Medium fortified with 2.0 mg/L IBA, showed 90% root induction in *C. colocynthis* after 7 days of inoculation. Thickness, number and diameter of roots were directly proportional to IBA concentration tried (Fig. 1g-1h; Table 2).

To generate callus, internodes and leaves taken from 14 days old micropropagated plants were inoculated in a medium containing varying concentrations of 2, 4-D (0.5 to 2.0 mg/L) with KIN (1.0 mg/L). Leaf explants exhibited better callogenic frequency (90.21%) in MS medium complemented with 2,4-D (1.0 mg/L) and KIN (1.0 mg/L) than internodal explants which showed 50.62% callogenic frequency in medium containing higher level of 2, 4-D (1.5 mg/L) with the same concentration of KIN after 14 days of culturing. Compact green callus formation started from green base of internodes and whole explant turned into callus mass (Fig. 2a-2b; Table 3).

Calli produced from internodes and leaves were further propagated in medium enriched with combination of two phytohormones 2, 4-D and BAP for shoot initiation. Significant results were obtained in medium fortified with BAP (1.5 mg/L) and 2, 4-D (2.0 mg/L) regarding the length of shoots (Fig. 2c, 2d; Table 4). Root induction was achieved on medium containing IBA at a concentration of 1.0 mg/L. Under field conditions 85% survival rate of the regenerated plants was recorded (Fig. 3a and 3b).

| | BAP | NAA | Shoot formation | No. of Shoots | Shoot length (cm) |
|--------------------------|-------|-------|-----------------|---------------|-------------------|
| BAP | 1.000 | - | - | - | - |
| NAA | 0.523 | 1.000 | - | - | - |
| Multiple Shoot induction | 0.984 | 0.552 | 1.000 | - | - |
| Shoots per explant | 0.945 | 0.457 | 0.936 | 1.000 | - |
| Shoot length (cm) | 0.981 | 0.557 | 0.999 | 0.931 | 1 |

Table 1: Correlation of 2, 4-D and BAP with multiple shoot formation, number and length of shoots per explant.

| Concentration (mg/L) | | Multiple Shoot induction (%) | Shoots per nodal explant | Shoot length (cm) | Concentration (mg/L) IBA | Root induction (%) |
|----------------------|-----|------------------------------|--------------------------|-------------------|--------------------------|--------------------|
| BAP | NAA | | | | | |
| 0.0 | 0.0 | 00±00 | 0.00±0.00 | 0.0±00 | 0.0 | 00±00 |
| 0.1 | 1.0 | 00±00 | 0.00±0.00 | 0.0±00 | 0.1 | 30.32±1.69 |
| 0.5 | 1.0 | 30±00 | 1.37±0.24 | 1.5±02 | 0.5 | 59.80±1.93 |
| 1.0 | 1.0 | 44±21 | 1.73±0.89 | 2.0±01 | 1.0 | 61.62±1.49 |
| 1.5 | 1.0 | 58±67 | 2.15±0.82 | 2.8±04 | 1.5 | 74.89±1.33 |
| 2.0 | 1.0 | 75±33 | 4.93±1.45 | 3.5±02 | 2.0 | 90.75±1.11 |

Table 2: Effect of BAP for multiple shoot induction and effect of IBA for root induction in MS medium.

| Medium | Concentration (mg/L) | | Callus induction from leaf explants (%) | Callus induction from internodal explants (%) |
|--------|----------------------|-----|---|---|
| | 2,4-D | KIN | | |
| 1. | 0.0 | 0.0 | 0.00±0.00 | 0.00±0.00 |
| 2. | 0.1 | 1.0 | 59.37±1.27 | 22.25±2.14 |
| 3. | 0.5 | 1.0 | 66.21±1.99 | 33.25±2.79 |
| 4. | 1.0 | 1.0 | 90.21±1.43 | 40.62±2.40 |
| 5. | 1.5 | 1.0 | 50.21±1.17 | 50.62±1.96 |
| 6. | 2.0 | 1.0 | 39.79±1.14 | 30.91±1.44 |

Table 3: Callus induction from leaf and internodal explants in MS medium supplemented with 2,4-D and KIN.

| Medium | Concentrations (mg/L) | | Number of shoots per callus |
|--------|-----------------------|------|-----------------------------|
| | 2, 4-D | BAP | |
| 1 | 0.00 | 0.00 | 0.00±0.00 |
| 2 | 0.5 | 1.0 | 1.32±0.11 |
| 3 | 1.0 | 1.5 | 5.19±0.35 |
| 4 | 1.5 | 1.0 | 8.93±0.67 |
| 5 | 2.0 | 1.5 | 10.43±0.45 |

Note: Data were recorded for multiple shoot initiation after 35 days of inoculation. Mean ± SE were subjected to Tukey's test ($P \leq 0.05$).

Table 4: Effect of 2, 4-D and BAP on shoot regeneration from callus cultures.

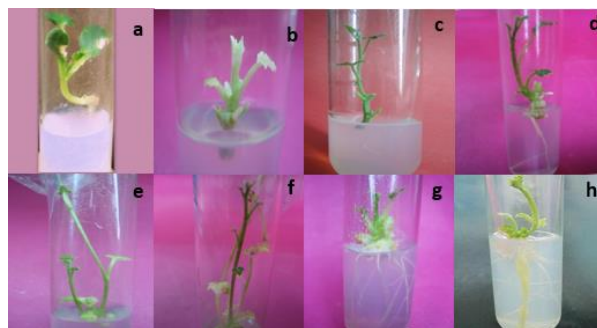


Figure 1: (a) Seed germination, (b) shoot formation from apices, (c) shoot initiation from nodal explants, (d) *in vitro* root induction of micropropagated shoots, (e, f) multiple shoot proliferation (g, h) root induction from shoots.

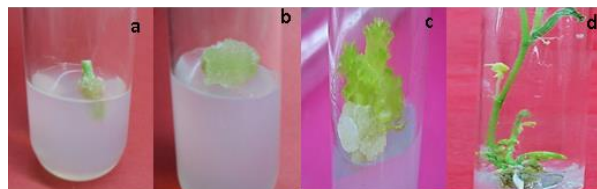


Figure 2: Callus raised by (a) internode (b) leaf (c) multiple shoot formation and (d) elongated shoots after 14 days of culture.

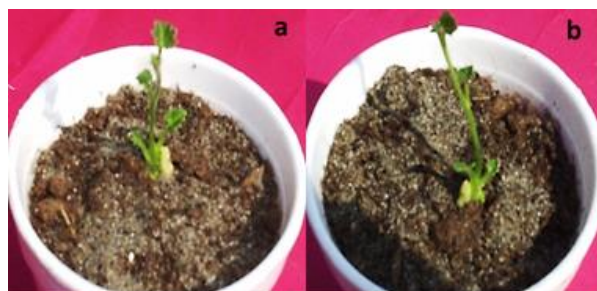


Figure 3: (a, b). *In vitro* grown plants under field conditions.

Discussion

In *in vitro* germination, chances of seed germination and survival of plantlets increase under sterile conditions. Decoating of seeds supported germination as more moisture is available to the seeds as compared to hard seed coat [14]. MS basal medium has been utilized by many workers for *in vitro* seed germination for different plants [15], while others working on different compositions of MS media found 1/2 strength MS media efficient enough for germination percentage, root and shoot length [16, 17].

In tissue culture, the ability of callus formation depends upon various factors including explant type, culture conditions, medium and concentration of phytohormones used. In the current work, 2, 4-D and KIN at similar level i.e. 1.0 mg/L, showed maximum callogenic frequency (90.21%) from leaf explants. Similar results were reported for calli formation of *C. colocynthis* with same concentrations and combination of growth regulators in MS medium [18, 19]. Other workers reported 66% callus induction response from both leaf and internode of *C. colocynthis* in basal MS medium containing 2.0 mg/L BAP and NAA each which is higher as compared to present work [20]. In the present study, a higher concentration of 2, 4-D (1.5 mg/L) with KIN (1.0 mg/L) produced best response to callus induction from internodal explants, while other workers reported that medium augmented with higher concentration of KIN than 2, 4-D showed better callogenic response from different explants of *C. colocynthis* [21].

In the current study, multiple shoots raised from nodes in MS medium augmented with BAP (2.0 mg/L) and NAA (1.0 mg/L) is favored by the previous studies on different plants like Banana [22]; *Buchanania lanzan* [23]; *Musa sapientum* [24] and *Dendrobium nobile* [25]. This shows the conversion of growth hormones to metabolic signals and morphological responses by plant [26]. MS medium added with NAA (0.5 mg/L) and BAP (1.5 mg/L) also reported same findings from stem explants, which are lower concentrations as compared to our results [27]. In contrast to this work, BAP alone is also found efficient enough by some researchers regarding multiple shoot initiation from nodes and shoot tips of *C. colocynthis* [28].

In current findings, medium supplemented with IBA (2.0 mg/L) showed maximum thickness and percentage of root induction (90.75%) from micropropagated shoots. Contrary to present study, other workers obtained rhizogenic response at a higher concentration of IBA (4.0 mg/L) [29] while other scientific experts obtained root induction from other explants of *C. colocynthis* at a very low (0.1 mg/L) concentration of IBA [30]. The discrete behavior of same plant at different concentrations of same growth regulator is not unusual in plant tissue culture studies [31]. Previous studies also reported similar role of IBA in root induction in several medicinal plant species like *Mentha piperita* [32]; *Cyphomandra betacea* [33]; *Azadirachta indica* [34] and *Pluchea lanceolata* [35].

Shoots emerged from callus in different concentrations of 2, 4-D along with BAP but only medium containing BAP (1.5 mg/L) along with 2, 4-D (2.0 mg/L) produced more thick and stout shoots. Presence of BAP is a critical factor in organogenesis in cucurbit plants [36]. BAP at the level of 1.0 mg/L resulted in shooting directly via epidermal cells in watermelon [37]. Overall, results of this trial helped to establish a protocol for callus production, root and multiple shoot induction and regeneration of endangered medicinal cucurbit plant, *C. colocynthis*.

Stimulatory effect of different hormones was checked on callus production, multiple shoot induction, root induction and regeneration from callus. It was found that 2, 4-D along with combination of other hormones (BAP, KIN) leads to effective establishment of *in vitro*

propagation of *C. colocynthis* (L.) in the form of shoot and root induction. Moreover, successful establishment of micropropagated plants was also observed in an open field. This study is a door way for the scientists to further explore variation of phytochemicals and secondary metabolites by growing it under *in vitro* condition.

Author Contributions

Being principle author, done passionate work in the laboratory to make this research possible. I have carried out research on *C. colocynthis* (L.) under the kind supervision of Dr. Humera Afrasiab (Assistant Professor, PU, Lahore). It was not possible to make this work available for other workers without guidance and technical assistance of Dr. Humera Afrasiab. I also want to pay my gratitude to Dr. Fozia Farhat who helped in analysis of data and compilation of manuscript.

Conflict of Interest Statement

There is no conflict of interest regarding the publication of this paper.

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