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# Phenolic compound production and biological activities from *in vitro* regenerated plants of gherkin (*Cucumis anguria* L.)

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ABSTRACT

*Background:* The effect of polyamines (PAs) along with cytokinins (TDZ and BAP) and auxin (IBA) was induced by the multiple shoot regeneration from leaf explants of gherkin (*Cucumis anguria* L.). The polyphenolic content, antioxidant and antibacterial potential were studied from *in vitro* regenerated and *in vivo* plants.
*Results:* Murashige and Skoog (MS) medium supplemented with 3% sucrose containing a combination of 3.0 μM TDZ, 1.0 μM IBA and 75 μM spermidine induced maximum number of shoots (45 shoots per explant) was achieved. Regenerated shoots elongated in shoot elongation medium containing 1.5 μM $GA_3$ and 50 μM spermine. The well-developed shoots were transferred to root induction medium containing 1.0 μM IBA and 50 μM putrescine. Rooted plants were hardened and successfully established in soil with a 95% survival rate. Twenty-five phenolic compounds were identified by ultra-performance liquid chromatography (UPLC) analysis The individual polyphenolic compounds, total phenolic and flavonoid contents, antioxidant and antibacterial potential were significantly higher with *in vitro* regenerated plants than *in vivo* plants.
*Conclusions:* Plant growth regulators (PGRs) and PAs had a significant effect on *in vitro* plant regeneration and also a biochemical accumulation of flavonols, hydroxybenzoic and hydroxycinnamic acid derivatives in *C. anguria*. Due to these metabolic variations, the antioxidant and antibacterial activities were increased with *in vitro* regenerated plants than *in vivo* plants. This is the first report describing the production of phenolic compounds and biological activities from *in vitro* and *in vivo* regenerated plants of *C. anguria*.



## 1. Introduction

*Cucumis anguria* L. commonly known as bur cucumber, bur gherkin, and West Indian gherkin is an important cucurbit vegetable and conventional medicinal crop. It is mainly cultivated and used in Africa, Brazil, Cuba, India, United States and Zimbabwe [1]. The fruits and leaves of gherkin are consumed as boiled, fried, stewed, pickled and also used as fresh in salads and hamburgers [2]. Additionally, the fruits, roots and seeds of gherkins are used for traditional medicine to treat stomach ache, jaundice, hemorrhoids and preventing stone formation in the kidney. Since there is a growing worldwide demand for pickled gherkins, many food companies have started to explore opportunities for producing gherkins [2]. Phytochemists have reported a number of important medicinal components of cucurbitacin B, cucurbitacin D and cucurbitacin G from *C. anguria*; the cucurbitacin B have been used for cancer prevention [2]. Gherkin consists of many useful compounds such as flavonoids, tannins, alkaloids, saponins and steroids that contained a high level of antioxidant activity. Anthraquinones and saponins that are present in *C. anguria* are used for antibacterial and antifungal activity [3]. Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals. Plant growth regulators (PGRs) are one of the most important factors affecting cell growth, differentiation and metabolite formation [4]. The type and concentration of PGRs especially auxins and/or cytokinins have been shown to modify the shikimate/phenylpropanoid pathways in the biochemical synthesis of phenolic acids, probably by regulating the enzyme activity of phenylalanine ammonia lyase and chalcone synthase [5]. The capacity for plant cell, tissue and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized since the inception of *in vitro* technology. Previously, many valuable compounds are produced by *in vitro* plant cell cultures [6,7,8].

Polyamines (PAs), a diamine putrescine, a triamine spermidine and a tetraamine spermine, are ubiquitous in the animal and plant kingdom. PAs have been associated with many important cellular processes such as cell division, protein synthesis, DNA replication, response to abiotic stress, regulation of rhizogenesis, embryogenesis, senescence, floral development and fruit ripening [9]. The exogenous application of PAs has shown a positive effect in the micropropagation

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of several species such as *Withania somnifera* [10] and *Glycine max* [11]. PAs have been successfully used in a variety of morphogenic processes in horticultural crops [12]. Very few studies reported in cucurbits the effect of exogenous PAs have been enhanced for somatic embryogenesis in *Momordica dioica* [9], multiple shoot regeneration from leaf explants of *Momordica charantia* [13] and shoot tip explants of *Cucumis sativus* [14]. An efficient plant regeneration protocol is a prerequisite for biotechnological breeding of economically important crops like cucurbits. Recently, we established the plant regeneration from petiole derived callus [15], and direct somatic embryogenesis from hypocotyl and leaf explants of gherkin [2]. However, there is no report describing the effect of PGRs and PAs on *in vitro* regeneration in gherkin. The objective of this investigation was to evaluate the synergistic effect of PAs (spermidine, spermine, and putrescine) with different PGRs for high-frequency multiple shoot regeneration systematically, *via* direct organogenesis using leaf explants. In addition, the variations of total polyphenolic content, individual polyphenols, antioxidant and antibacterial activities on *in vitro* and *in vivo* regenerated plants of *C. anguria*.

## 2. Materials and methods

### 2.1. Plant material

Seeds of gherkin (*C. anguria* L.) were obtained from Nunhems Seeds Pvt. Ltd. (Bangalore, India). Seeds were surface sterilized following our previous reports [2], and were inoculated with the hormone free MS [16] basal salt mixture + B5 vitamins [17] ($MSB_5$) supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose. The seedlings were grown under white fluorescent light (40 $\mu moL \cdot m^{-2} \cdot s^{-1}$) at a photoperiod of 16/8 h of light/dark and temperature 25 ± 2°C. The leaf explants were trimmed into appropriate sizes (0.5 $cm^2$) to obtain 10 d old *in vitro* seedlings.

### 2.2. Multiple shoot induction

The shoot bud initiation media which consisted of $MSB_5$ medium with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar supplemented with different concentrations (1.0–4.0 μM) of thiadiazuron (TDZ) or 6-benzylaminopurine (BAP) either separately or in combination with 1.0–3.0 μM of indole butyric acid (IBA) were tested for shoot bud induction. The growth regulator combination is composed of different concentrations (25–100 μM) of PAs such as putrescine, spermidine and spermine (Sigma-Aldrich, USA) to study their synergistic effect in improving shoot bud regeneration. The medium was adjusted to pH 5.8 prior to autoclaving at 121°C for 15 min. The cultures were maintained at 25 ± 2°C under 16 h light and 8 h dark photoperiod, with a light intensity of 40 $\mu moL \cdot m^{-2} \cdot s^{-1}$ as three subculture were made at intervals of 11 d in the same induction medium.

### 2.3. Shoot elongation and root induction

The regenerated shoots were cultured in a test tube containing 15 mL of $MSB_5$ medium augmented with different concentrations (0.5–2.0 μM) of gibberellic acid ($GA_3$) and combination with various concentrations (25–100 μM) of PAs (putrescine, spermidine and spermine) for shoot elongation. Elongated shoots were transferred to root induction medium containing half $MSB_5$ supplemented with different concentrations (0.5–2.0 μM) of indole 3-butyric acid (IBA) or indole-3-acetic acid (IAA) and combination with different concentrations (25–100 μM) of PAs (putrescine, spermidine and spermine). The cultures were maintained above mentioned growth conditions.

### 2.4. Transplantation and acclimatization

Rooted plantlets were washed thoroughly with tap water to remove agar and transplanted to plastic pots containing a mixture of autoclaved sand, red soil, and vermiculite (1:1:1 v/v/v). Potted plants were grown in a growth chamber (Sanyo, Tokyo, Japan) at 85% relative humidity for 2–3 weeks, and then moved to a greenhouse for 3 weeks before transferring to the field. The survival percentage was calculated after 4 weeks in the greenhouse. After, 4 weeks of acclimatization, plants were transplanted to field.

### 2.5. Sample preparation

Nine-week-old leaf derived from *in vitro* regenerated plants and *in vivo*-grown seed-derived from plants (one-month-old) of *C. anguria* were oven dried at 50°C to constant weight. The dried fine powder samples (0.1 g) were extracted with 10 mL 80% methanol in a sonication bath for 30 min. The methanolic extracts were then filtered under vacuum through Whatman No. 1 filter paper, and the filtrates were dried under vacuum using Rotary evaporator of crude extract. The extracts obtained were dissolved in the methanol immediately used for the determination of total phenolics, flavonoids, antioxidant and antibacterial activity.

### 2.6. Determination of total phenolic (TPC) and flavonoid (TFC) contents

The TPC and TFC contents of the samples were analyzed by following our previous procedure [18]. The concentration of the TPC and TFC was determined as mg of gallic acid and quercetin equivalent respectively.

### 2.7. Extraction of phenolic compounds and analysis by ultra-performance liquid chromatography (UPLC)

One gram of dried plant powder was extracted as described in the procedure [8]. The filtrate was used for analysis using a Thermo Accela UPLC (Thermo, New York, USA) system. The separation was achieved using a HALO C18 (2.7 μm, 2.1 × 100 mm) column and the absorbance was measured at 280 nm. The mobile phases were 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The gradient procedure described earlier [8]. Solutions of pure gallic acid, homogentisic acid, protocatechuic acid, gentisic acid, β-resorcylic acid, veratric acid, vanillic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid, vanillin, ρ-coumaric acid, salicylic acid, chlorogenic acid, ferulic acid, rutin, *t*-cinnamic acid, *o*-coumaric acid, myricetin, catechin, quercetin, naringenin, kaempferol, biochanin A and hesperidin were used as standards. The individual standards (25, 50, 100, and 150 μg/mL) purchased from Sigma-Aldrich (St. Louis, MO, USA) were dissolved in methanol and analyzed the samples. Phenolic compounds of *in vivo* and *in vitro* plant extracts were identified based on the retention time and UV spectra of authentic standards while the quantitative data were calculated based on the calibration curves of the individual standards. Results were expressed as μg/g of each compound from the total phenolic compounds.

### 2.8. Antioxidant activity and antimicrobial activity

For the antioxidant studies, the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity, reductive potential and phospho-molybdenum method were analyzed by following the procedure [18]. The pathogenic microorganisms *Staphylococcus aureus* (KACC 13257), *Pseudomonas aeruginosa* (KACC 10259), and *Escherichia coli* (KACC 13821) were used to test for antibacterial activity. The pure bacterial strains were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, South Korea. The methanolic extracts of *in vitro* regenerated and *in vivo* plants (100 mg/mL) were tested for

antibacterial activity. Antibacterial tests were carried out by the NCCLS disc diffusion method was previously described [8]. For the positive control, paper discs were impregnated with 50 μL of chloramphenicol.

### 2.9. Statistical analysis

All the experiments were performed in triplicates and each experiment was repeated twice. The data was expressed as means ± standard deviations (SD). One-way ANOVA analysis followed by the Duncan's test was used to determine significant ($P \leq 0.05$) differences. All the statistical analyzes were done by using SPSS Ver. 20 (SPSS Inc., Chicago, IL, USA) statistical software package.

## 3. Results

### 3.1. Plantlet regeneration from leaf explants

The cytokinins TDZ and BAP were used in a range of 1.0–4.0 μM in combination with IBA (1.0 μM) to determine its ability to induce shoot bud initiation from leaf explants of *C. anguria*. Shoot bud induction on $MSB_5$ medium supplemented with 3.0 μM TDZ combined with 1.0 μM IBA resulted in high frequency (27.5 shoots per explants) of shoot regeneration (Table 1). The $MSB_5$ medium supplemented with 3.0 μM BAP and 1.0 μM IBA produced 15.5 shoots from leaf explants (Table 1). In our study, TDZ was found to be more efficient in shoot regeneration as compared to BAP. The present investigation proved that a TDZ and IBA combination significantly enhanced shoots from leaf explants. The regenerated shoots, when cultured in MSB5 medium containing 1.5 μM GA3, favored the shoot elongation after one week culture (Table 1). The elongated shoots were transferred to $MSB_5$ medium containing different concentrations of IAA and IBA. Three weeks after inoculation, root formation was observed from the cut portion of the shoot. Of the two auxins used, IBA (1.5 μM) was the best for root induction (Table 1).

**Table 1**
Effect of growth regulators on shoot bud initiation, shoot elongation and root induction from leaf explants of *C. anguria*.

| Growth regulators (μM) | | Mean number of shoots/explant | Mean shoot length (cm) | Mean number of roots/shoot |
|---|---|---|---|---|
| TDZ | IBA | | | |
| 1.0 | 1.0 | $7.0 \pm 0.5^{e}$ | | |
| 2.0 | 1.0 | $16.7 \pm 1.0^{c}$ | | |
| 3.0 | 1.0 | $27.5 \pm 1.2^{a}$ | | |
| 4.0 | 1.0 | $20.1 \pm 1.0^{b}$ | | |
| BAP | IBA | | | |
| 1.0 | 1.0 | $3.5 \pm 0.5^{f}$ | | |
| 2.0 | 1.0 | $9.4 \pm 0.5^{d}$ | | |
| 3.0 | 1.0 | $15.5 \pm 1.2^{c}$ | | |
| 4.0 | 1.0 | $7.9 \pm 0.6^{e}$ | | |
| $GA_3$ | | | | |
| 0.5 | | | $2.0 \pm 0.2^{c}$ | |
| 1.0 | | | $3.5 \pm 0.4^{b}$ | |
| 1.5 | | | $4.1 \pm 0.5^{a}$ | |
| 2.0 | | | $3.0 \pm 0.2^{bc}$ | |
| IBA | | | | |
| 0.5 | | | | $2.4 \pm 0.3^{d}$ |
| 1.0 | | | | $3.8 \pm 0.2^{b}$ |
| 1.5 | | | | $5.5 \pm 0.5^{a}$ |
| 2.0 | | | | $3.1 \pm 0.5^{c}$ |
| IAA | | | | |
| 0.5 | | | | $2.1 \pm 0.2^{e}$ |
| 1.0 | | | | $3.0 \pm 0.4^{c}$ |
| 1.5 | | | | $3.4 \pm 0.5^{bc}$ |
| 2.0 | | | | $2.9 \pm 0.5^{c}$ |

Means ± standard deviation within a column followed by the same letter are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

### 3.2. Effects of PAs on shoot bud initiation and multiplication

The leaf explants were transferred to $MSB_5$ medium supplemented with 3.0 μM TDZ, 1.0 μM IBA and four concentrations of polyamines (Put, Spd, Spm; 25, 50, 75 and 100 μM) for shoot multiplication (Table 2). The combination of 3.0 μM TDZ, 1.0 μM IBA and 75 μM Spd produced a greater number shoots (45 shoots per explant) (Fig. 1a, Fig. 1b). In this investigation, compared to individual treatment of TDZ, IBA and combination with spermidine exhibited a higher percentage of shoot induction followed by TDZ, IBA with spermine, and TDZ, IBA with putrescine (Table 2). However, in the present study spermidine rather than putrescine or spermine, was most effective in multiple shoot induction from the leaf explants of *C. anguria*. It was assumed that spermidine provided a nitrogen source and exhibited a synergistic effect with TDZ and IBA to enhance shoot differentiation in leaf explants of *C. anguria*.

### 3.3. Effects of PAs on shoot elongation and root induction

The various concentrations of PAs (Put, Spd, Spm; 25, 50, 75 and 100 μM) were combined into shoot elongation medium containing optimal concentration of $GA_3$ (1.5 μM) to further optimize shoot elongation (Table 2). The present investigation, maximum response to shoot elongation was shown by a combination of $GA_3$ (1.5 μM) and spermine (50 μM) (Table 2), where shoot length after 15 d of culture was increased with an average shoot length of 9.5 cm (Table 2). The elongated shoots were transferred to the $MSB_5$ medium supplemented with different concentrations (0.5 to 2.0 μM) of IBA and combination with various concentrations of polyamines (Put, Spd, Spm; 25, 50, 75 and 100 μM) for root induction (Table 2). The maximum number of roots (12) produced 1.5 μM IBA and 50 μM Put (Fig. 1c). Other two PAs, spermidine and spermine produced a lower number of roots.

### 3.4. Transplantation and acclimatization

The rooted plants were gently removed from the vessels and transferred to plastic cups containing a sterile soil, sand, and vermiculite mixture (Fig. 1d), and after 2 weeks, they were transferred to pots. We had a 95% survival rate of plants derived from leaf explants when rooted plantlets were transferred from pots to field conditions.

**Table 2**
Effect of PAs (spermidine, spermine, and putrescine) on shoot bud initiation, shoot elongation and root induction from leaf explants of *C. anguria*.

| PAs (μM) | Mean number of shoots/explant* | Mean shoot length (cm)** | Mean number of roots/shoot*** |
|---|---|---|---|
| *Spermidine* | | | |
| 25 | $31.5 \pm 1.0^{d}$ | $7.3 \pm 0.2^{bc}$ | $7.0 \pm 0.5^{e}$ |
| 50 | $39.6 \pm 1.2^{b}$ | $8.0 \pm 0.5^{b}$ | $9.0 \pm 1.0^{c}$ |
| 75 | $45.0 \pm 1.0^{a}$ | $7.0 \pm 1.0^{d}$ | $6.0 \pm 0.5^{f}$ |
| 100 | $36.8 \pm 1.2^{c}$ | $6.3 \pm 0.3^{e}$ | $4.0 \pm 0.2^{h}$ |
| *Spermine* | | | |
| 25 | $29.1 \pm 0.9^{de}$ | $8.0 \pm 0.5^{b}$ | $8.2 \pm 0.3^{d}$ |
| 50 | $34.4 \pm 1.1^{cd}$ | $9.5 \pm 1.0^{a}$ | $10.5 \pm 0.5^{b}$ |
| 75 | $39.5 \pm 1.5^{b}$ | $7.7 \pm 0.4^{b}$ | $7.1 \pm 0.4^{e}$ |
| 100 | $31.0 \pm 1.0^{d}$ | $6.9 \pm 0.2^{d}$ | $4.5 \pm 0.5^{gh}$ |
| *Putrescine* | | | |
| 25 | $27.6 \pm 1.2^{e}$ | $7.0 \pm 0.5^{d}$ | $9.0 \pm 0.5^{c}$ |
| 50 | $30.5 \pm 1.5^{d}$ | $7.5 \pm 0.5^{c}$ | $12.0 \pm 1.0^{a}$ |
| 75 | $34.2 \pm 1.0^{cd}$ | $6.6 \pm 0.4^{de}$ | $8.0 \pm 0.8^{d}$ |
| 100 | $29.5 \pm 0.5^{de}$ | $5.0 \pm 0.5^{f}$ | $5.0 \pm 0.5^{g}$ |

Means ± standard deviation within a column followed by the same letter are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

* MS medium supplemented with 3.0 μM TDZ, 1.0 μM IBA and various concentrations of PAs.

** MS medium supplemented with 1.5 μM $GA_3$ and various concentrations of PAs.

*** MS medium supplemented with 1.5 μM IBA and various concentrations of PAs.

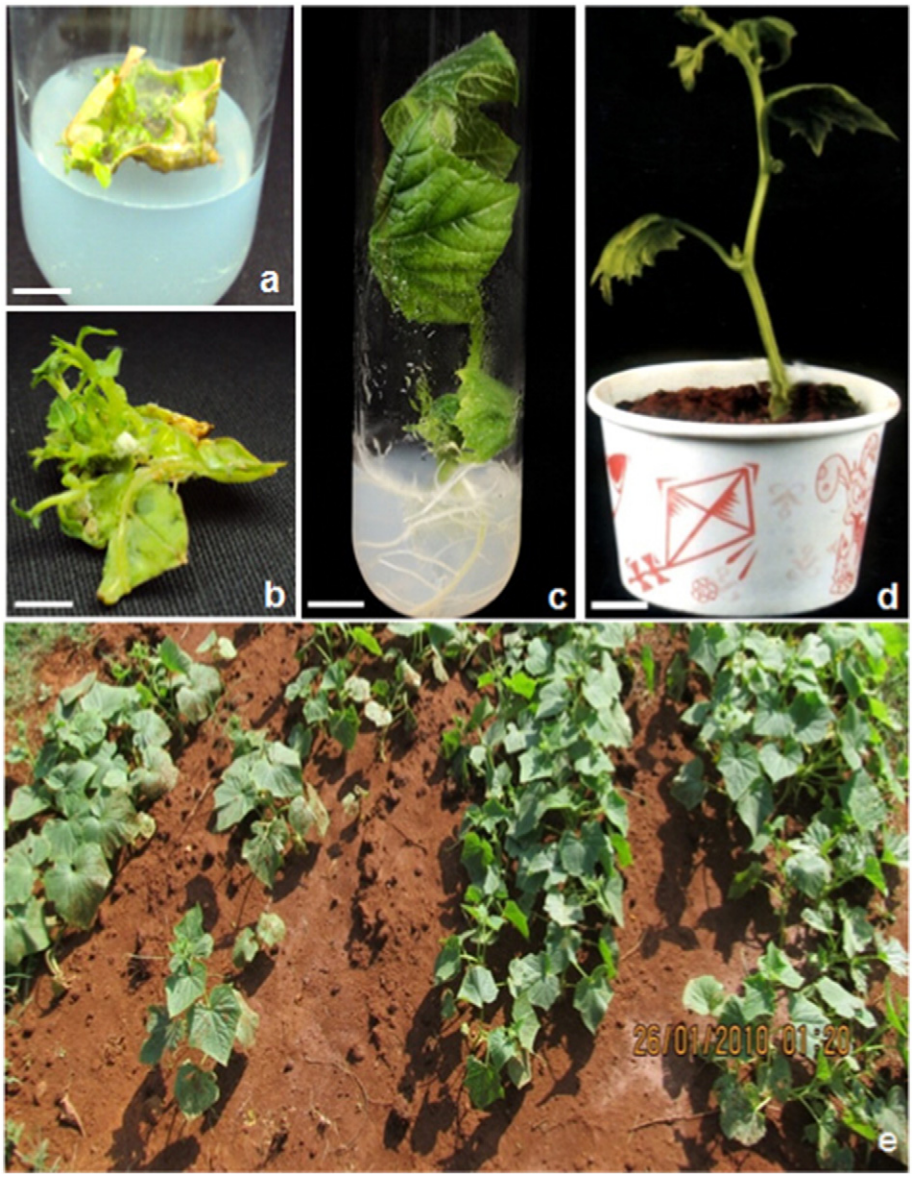


**Fig. 1.** The influence of polyamines and plant growth regulators on direct plant regeneration from leaf explants of *C. anguria*. a) Shoot bud initiation; b) multiple shoot regeneration from leaf explants, Bar: 10 mm; c) elongated shoots with rooted plants, Bar: 10 mm; d) hardened plants, Bar: 100 mm; e) hardened plants transferred to field.

Regenerated plants transferred to the field became fully established and grew well and were similar to the parental plants in their morphology (Fig. 1e).

### 3.5. Phytochemical properties of gherkin extracts

In our study, total phenolic and flavonoid contents were higher in the *in vitro* regenerated plants compared to the *in vivo* plants (Fig. 2a, Fig. 2b). The qualitative and quantitative analysis of phenolic compounds from *in vivo* and *in vitro* plant extracts were studied using UPLC (Table 3). The phenolic compounds in the plant extracts were identified by comparisons of the retention time, and UV spectra of authentic standards and the quantitative data were calculated from calibration curves. Both *in vivo* and *in vitro* plant extracts of *C. anguria* contained flavonols, hydroxycinnamic acid, hydroxybenzoic acid, and other phenolic compounds (Table 3). *In vitro* regenerated plants contained higher amounts of flavonols (2277.23 μg/g), hydroxycinnamic acid (1537.57 μg/g), and hydroxybenzoic acid (1386.62 μg/g) when compared to *in vivo* plants, which produced lower amounts of flavonols (2153.36 μg/g), hydroxycinnamic acid (1502.80 μg/g), and hydroxybenzoic acid (1281.33 μg/g).

### 3.6. Antioxidant and antibacterial activity of gherkin extracts

The antioxidant potential of *in vitro* and *in vivo* regenerated plants were determined using free radicals scavenging, reducing potential, and phospho-molybdenum assays. The high antioxidant activity was exhibited in *in vitro* regenerated plants (77.50%), compared with *in vivo* plants (62.00%) (Fig. 2c). The results of this study demonstrated that flavonol and phenolic acid levels increased in *in vitro* regenerated plants and directly influenced their antioxidant potential.

In the present study, reducing the capacity of extracts indicated that *in vitro* regenerated plants had more antioxidant potential than *in vivo* plants (Fig. 2d). Reducing properties are associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. The antioxidant capacity of *in vitro* and *in vivo* plant extracts was determined using the phospho-molybdenum method, which is based on the reduction of Mo(VI) to Mo(V) by the sample analyte, and the

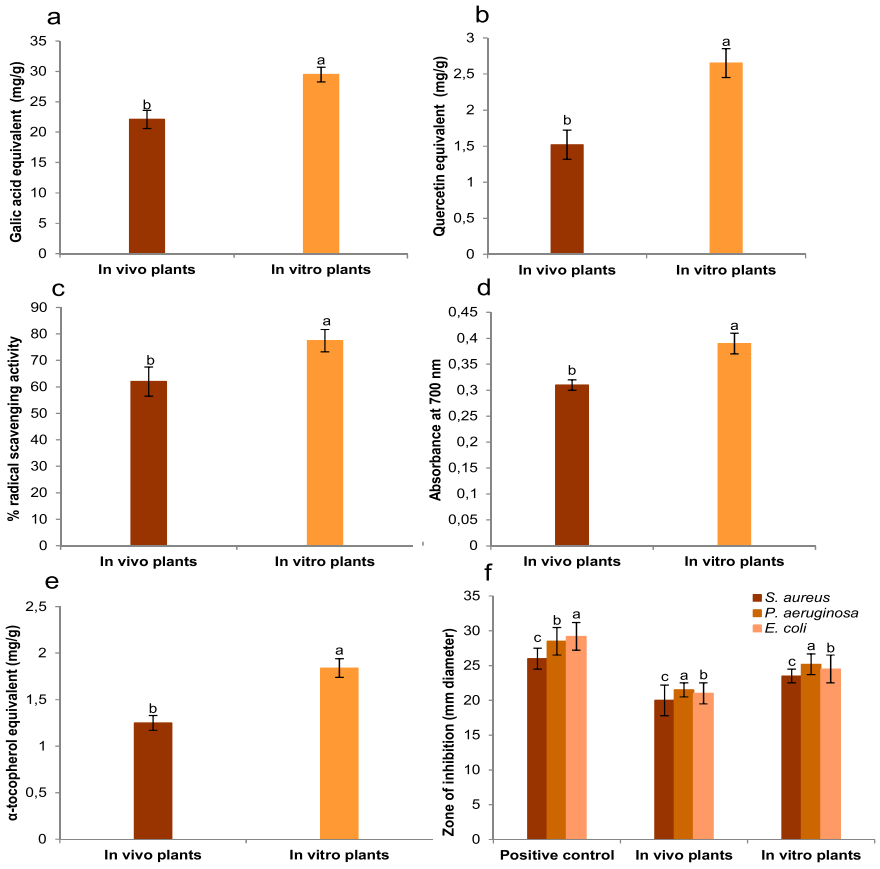


**Fig. 2.** Evaluation of total phenolic compound and flavonoid contents, as well as biological activities of *in vitro* regenerated and *in vivo* plants of *C. anguria*. a) Total phenolic content; b) total flavonoid content; c) free radical-scavenging activity by the DPPH method; d) reducing power, and e) the phospho- molybdenum method; f) antibacterial activity. Means ± standard deviation and same letter are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the *in vitro* regenerated plants was higher when compared with *in vivo* plant extracts (Fig. 2e).

The *in vitro* regenerated and *in vivo* plants exhibited varying antimicrobial activity, as shown by the growth inhibition zones (Fig. 2f). The results from the disc diffusion method indicated that both *in vitro* regenerated and *in vivo* plant extracts had anti-bacterial (Gram-positive and Gram-negative bacteria) activities. *In vitro* regenerated plant extracts showed higher activity against antibacterial compared to *in vivo* plant extracts (Fig. 2f). These results were compared with chloramphenicol for positive control activity. The present investigation demonstrated that *C. anguria* contains biologically potent therapeutic phytochemicals with high antibacterial and antioxidant activities.

## 4. Discussion

In our investigation, TDZ with NAA was found to be more effective in shoot regeneration as compared to BAP with NAA. Similarly, TDZ with NAA produced a high frequency of shoot regeneration from leaf derived callus in *M. charantia* [19]. In our study, $GA_3$ was used for shoot elongation and IBA induced roots in *C. anguria*. Correspondingly, $GA_3$ showed a better response to shoot elongation in *M. charantia* [19]. The effectiveness of IBA in rooting has been reported in *Cucurbita* species [20] and *M. dioica* [21]. Although shoot development occurs quite frequently, shoot elongation and rooting are difficult in melon [22]. The present study, PGRs with PAs induced high frequency of *in vitro* plant regeneration of *C. anguria*. It was advocated that plant regeneration and differentiation could be drastically improved by the application of PAs [12]. PAs have been shown to interact with phytohormones, act as PGRs or hormonal secondary messengers, and as a reserve of carbon and nitrogen in culturing tissues [10,23]. It was suggested that regeneration and differentiation could be drastically improved by application of PAs in bitter melon [13]. Our study demonstrated that Spd was induced by shoot initiation, Spm was used for elongation and Put was induced by rooting in *C. anguria*. Consistently, Spd is essential for shoot multiplication in cucumber [24] and bitter melon [13]. In contrast, spermine enhances *in vitro* shoot formation from cotyledons of cucumber [24] and *Brassica rapa* [25]. Similar to our reports, the effect of $GA_3$ and Spm induced a maximum shoot elongation in soybean [11]. In many dicotyledonous species, PAs involvement particularly Put in the root development and root architecture [23]. Similarly, Put induced root induction in cucumber [24], *W. somnifera* [10] and soybean [11].

Polyphenolic compounds are commonly found in both edible and non-edible plants and that they have multiple biological effects, including antioxidant activity [8]. *In vitro* shoot cultures provide a viable option for the production of secondary metabolites, especially where the compounds are synthesized and/or stored in the aerial plant parts [6]. Regenerated plantlets can accumulate secondary metabolites similar to those found in the mother plant [26]. PGRs and PAs had a significant effect on individual polyphenols, total phenolic and flavonoid contents. Similarly, the effect of PAs and PGRs were increased the amount of bioactive compounds in W. somnifera *in vitro* regenerated plants compared to *in vivo* plants [10]. The present study, PGRs, and PAs had a significant effect on the biochemical production of phenolic acids in regenerated plants when compared to the *in vivo* plants of *C. anguria*. In particular, the highest concentration of *p*-coumaric acid, ferulic acid, chlorogenic acid, *p*-hydroxybenzoic acid,

**Table 3**
Major phenolic constituents identified from *in vitro* and *in vivo* regenerated plants of *C. anguria* by UPLC analysis.

| No | Compounds | Concentration (μg/g) | |
|---|---|---|---|
| | | *In vivo* plants | *In vitro* plants |
| | Flavonols | | |
| 1 | Myricetin | $674.15 \pm 25.22^{b}$ | $714.15 \pm 35.10^{a}$ |
| 2 | Quercetin | $599.15 \pm 3.00^{c}$ | $675.12 \pm 2.00^{b}$ |
| 3 | Kaempferol | $388.15 \pm 3.50^{f}$ | $390.25 \pm 2.50^{e}$ |
| 4 | Catechin | $132.12 \pm 14.45^{l}$ | $145.12 \pm 14.45^{k}$ |
| 5 | Rutin | $205.10 \pm 2.50^{h}$ | $205.10 \pm 2.50^{i}$ |
| 6 | Naringenin | $125.12 \pm 2.25^{lm}$ | $120.32 \pm 2.25^{l}$ |
| 7 | Biochanin A | $29.57 \pm 0.91^{p}$ | $27.17 \pm 0.51^{mn}$ |
| | Total | 2153.36 | 2277.23 |
| | Hydroxycinnamic acid | | |
| 8 | Caffeic acid | $695.55 \pm 4.30^{a}$ | $674.50 \pm 4.00^{bc}$ |
| 9 | *p*-coumaric acid | $185.10 \pm 6.25^{i}$ | $215.02 \pm 5.55^{h}$ |
| 10 | Ferulic acid | $164.05 \pm 4.25^{j}$ | $169.05 \pm 5.10^{j}$ |
| 11 | *o*-coumaric acid | $25.0 \pm 1.50^{q}$ | $18.00 \pm 2.00^{\circ}$ |
| 12 | Chlorogenic acid | $427.10 \pm 10.00^{e}$ | $465.00 \pm 7.25^{d}$ |
| 13 | *t*-cinnamic acid | $9.25 \pm 0.21^{s}$ | $9.25 \pm 0.21^{p}$ |
| | Total | 1502.80 | 1537.57 |
| | Hydroxybenzoic acid | | |
| 14 | *p*-hydroxybenzoic acid | $155.11 \pm 2.25^{k}$ | $175.50 \pm 4.00^{hi}$ |
| 15 | Gallic acid | $325.25 \pm 5.50^{g}$ | $350.25 \pm 10.00^{f}$ |
| 16 | Protocatechuic acid | $38.41 \pm 1.10^{n}$ | $38.41 \pm 1.10^{mn}$ |
| 17 | β-resorcylic acid | $22.75 \pm 2.00^{q}$ | $20.15 \pm 2.11^{n}$ |
| 18 | Vanillic acid | $30.00 \pm 2.50^{p}$ | $30.00 \pm 2.50^{m}$ |
| 19 | Syringic acid | $15.16 \pm 1.25^{r}$ | $15.16 \pm 1.25^{op}$ |
| 20 | Gentisic acid | $153.65 \pm 7.45^{kl}$ | $158.15 \pm 5.25^{j}$ |
| 21 | Salicylic acid | $541.00 \pm 7.52^{d}$ | $599.00 \pm 5.00^{c}$ |
| | Total | 1281.33 | 1386.62 |
| | Other Phenolic compounds | | |
| 22 | Vanillin | $65.21 \pm 1.51^{m}$ | $77.25 \pm 2.21^{l}$ |
| 23 | Veratric acid | $205.25 \pm 5.00^{h}$ | $226.14 \pm 4.00^{g}$ |
| 24 | Hesperidin | $47.58 \pm 1.00^{n}$ | $45.58 \pm 1.00^{m}$ |
| 25 | Homogentisic acid | $35.55 \pm 1.00^{np}$ | $33.75 \pm 1.10^{n}$ |
| | Total | 353.59 | 382.72 |

Means ± standard deviation within a column followed by the same letter are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

gallic acid, gentisic acid, salicylic acid, vanillin and veratric acid was presented in the regenerated shoots which cultured on MS medium supplemented with TDZ and PAs. Similarly, biochemical accumulation of hydroxybenzoic and hydroxycinnamic acid derivatives in organ cultures of *Hypoxis hemerocallidea* [5]. In our study, total phenolic and flavonoid contents were higher in *in vitro* regenerated plants than *in vivo* plants. It was demonstrated that the presence of cytokinins in MS medium was shown to induce the production of flavonoids in *Hypericum perforatum* [27]. Previously, it was reported that plants regenerated *in vitro* have a potential use for the synthesis of bioactive compounds from medicinal plants [6,7,28]. PGRs and PAs significantly increased the antioxidant activity of 9-week-old *in vitro*-grown regenerated plants in *C. anguria* when compared to the *in vivo* plants, as measured by DPPH, reducing potential, and phospho-molybdenum assays. Consistently, cytokinins significantly increased the antioxidant activity of *H. hemerocallidea in vitro* organ cultures than *in vivo* plants [5]. PGRs and PAs derived *in vitro* plant extracts exhibited high antibacterial activity against Gram-positive and Gram-negative bacteria. Similarly, *in vitro* plant extracts were higher antibacterial activity than *in vivo* plants of *Cichorium pumilum* [29] and *Coleonema pulchellum* [7].

## 5. Concluding remarks

This is the first report on the effects of PGRs and PAs on *in vitro* plantlet regeneration using leaf explants of *C. anguria*. In this study, we demonstrated highly efficient plant regeneration system using leaf explants of *C. anguria via* organogenesis. Our experiment suggests that exogenous application of spermidine with TDZ and IBA plays a synergistic role in enhancing the multiple shoot formation, followed by the use of spermine was elongated shoots and putrescine for root development, which ultimately resulted in better acclimatization and survival of regenerated shoots. With the utilization of this protocol, approximately 45 plants per leaf explant were obtained within a short culture period of 75 d. The contents of phenolic compounds, including flavonols, hydroxycinnamic acid, and hydroxybenzoic acid, were higher in *in vitro* regenerated plants than *in vivo* plants. Total phenolics and flavonoid contents and antioxidant and antibacterial activities were also greater in *in vitro* regenerated plants than *in vivo* plants. This regeneration protocol can be used for the genetic transformation and also large scale production of phenolic compounds through *in vitro* regeneration of *C. anguria*.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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## Authors' contributions

M. Thiruvengadam conceived, carried out, and analyzed the data and wrote the manuscript. I.M. Chung analyzed the data, provided the necessary facilities and helped in writing the manuscript.

## References

[1] Nayar NM, Singh R. Taxonomy, distribution and ethnobotanical uses. In: Nayar NM, More TE, editors. Cucurbits. Enfield, NH: Science Publishing Inc.; 1998. p. 1–18.
[2] Ju HJ, Jeyakumar J, Kamaraj M, Praveen N, Chung IM, Kim SH, et al. High frequency somatic embryogenesis and plant regeneration from hypocotyl and leaf explants of gherkin (*Cucumis anguria* L.). Sci Hortic 2014;169:161–8. http://dx.doi.org/10.1016/j.scienta.2014.02.023.
[3] Senthil Kumar S, Kamaraj M. Analysis of phytochemical constituents and antimicrobial activities of *Cucumis anguria* L. against clinical pathogens. Am Eurasian J Agric Environ Sci 2010;7:176–8.
[4] Abdullahil Baque M, Hahn EJ, Paek KY. Growth, secondary metabolite production and antioxidant enzyme response of *Morinda citrifolia* adventitious root as affected by auxin and cytokinin. Plant Biotechnol Rep 2010;4:109–16. http://dx.doi.org/10.1007/s11816-009-0121-8.
[5] Moyo M, Amoo SO, Aremu AO, Gruz J, Šubrtová M, Doležal K, et al. Plant regeneration and biochemical accumulation of hydroxybenzoic and hydroxycinnamic acid derivatives in *Hypoxis hemerocallidea* organ and callus cultures. Plant Sci 2014;227: 157–64. http://dx.doi.org/10.1016/j.plantsci.2014.08.003.
[6] Amoo SO, Aremu AO, Van Staden J. *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. Plant Cell Tissue Organ Cult 2012;111:345–58. http://dx.doi.org/10.1007/s11240-012-0200-3.
[7] Baskaran P, Moyo M, Van Staden J. *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. S Afr J Bot 2014;90:74–9. http://dx.doi.org/10.1016/j.sajb.2013.10.005.
[8] Thiruvengadam M, Praveen N, Kim EH, Kim SH, Chung IM. Production of anthraquinones, phenolic compounds and biological activities from hairy root cultures of *Polygonum multiflorum* Thunb. Protoplasma 2014;251:555–66. http://dx.doi.org/10.1007/s00709-013-0554-3.
[9] Thiruvengadam M, Rekha KT, Jayabalan N, Praveen N, Kim EH, Chung IM. Effect of exogenous polyamines enhances somatic embryogenesis via suspension cultures of spine gourd (*Momordica dioica* Roxb. ex. Willd.). Aust J Crop Sci 2013;7:446–53.
[10] Sivanandhan G, Mariashibu TS, Arun M, Rajesh M, Kasthurirengan S, Selvaraj N, et al. The effect of polyamines on the efficiency of multiplication and rooting of *Withania somnifera* (L.) Dunal and content of some withanolides in obtained plants. Acta Physiol Plant 2011;33:2279–88. http://dx.doi.org/10.1007/s11738-011-0768-y.
[11] Arun M, Subramanyam K, Theboral J, Ganapathi A, Manickavasagam M. Optimized shoot regeneration for Indian soybean: The influence of exogenous polyamines. Plant Cell Tissue Organ Cult 2014;117:305–9. http://dx.doi.org/10.1007/s11240-014-0431-6.
[12] Scholten HJ. Effect of polyamines on the growth and development of some horticultural crops in micropropagation. Sci Hortic 1998;77:83–8. http://dx.doi.org/10.1016/S0304-4238(98)00139-3.

[13] Thiruvengadam M, Chung IM, Chun SC. Influence of polyamines on *in vitro* organogenesis in bitter melon (*Momordica charantia* L.). J Med Plants Res 2012;6: 3579–85.
[14] Vasudevan A, Selvaraj N, Ganapathi A, Kasthurirengan S, Ramesh Anbazhagan V, Manickavasagam M, et al. Leucine and spermidine enhance shoot differentiation in cucumber (*Cucumis sativus* L.). In Vitro Cell Dev Biol Plant 2008;44:300–6. http://dx.doi.org/10.1007/s11627-008-9135-0.
[15] Jeyakumar JJ, Kamaraj M, Thiruvengadam M. Efficient plant regeneration from petiole explants of West Indian gherkin (*Cucumis anguria* L.) via indirect organogenesis. J Plant Biochem Biotechnol 2014;23:307–15. http://dx.doi.org/10.1007/s13562-013-0215-9.
[16] Murashige T, Skoog F. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 1962;15:473–97. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.
[17] Gamborg OL, Miller RA, Ojima K. Nutrient experiments of suspension culture of soybean root callus. Exp Cell Res 1968;80:150–8.
[18] Thiruvengadam M, Chung IM. Selenium, putrescine, and cadmium influence health-promoting phytochemicals and molecular-level effects on turnip (*Brassica rapa* ssp. *rapa*). Food Chem 2015;173:185–93. http://dx.doi.org/10.1016/j.foodchem.2014.10.012.
[19] Thiruvengadam M, Rekha KT, Jayabalan N, Yang CH, Chung IM. High frequency shoot regeneration from leaf explants through organogenesis in bitter melon (*Momordica charantia* L.). Plant Biotechnol Rep 2010;4:321–8. http://dx.doi.org/10.1007/s11816-010-0151-2.
[20] Sarowar S, Oh HY, Hyung NI, Min BW, Harn CH, Yang SK, et al. *In vitro* micropropagation of a *Cucurbita* interspecific hybrid cultivar — A root stock plant. Plant Cell Tissue Organ Cult 2003;75:179–82. http://dx.doi.org/10.1023/A:1025043130423.
[21] Thiruvengadam M, Rekha KT, Jayabalan N. An efficient *in vitro* propagation of *Momordica dioica* Roxb. ex Willd. Philipp Agric Sci 2006;89:165–71.
[22] Stipp LCL, Mendes BMJ, Piedade SMDS, Rodriguez APM. *In vitro* morphogenesis of *Cucumis melo* var. inodorus. Plant Cell Tissue Organ Cult 2001;65:81–9. http://dx.doi.org/10.1023/A:1010684922210.
[23] Couée I, Hummel I, Sulmon C, Gouesbet G, Amrani AE. Involvement of polyamines in root development. Plant Cell Tissue Organ Cult 2004;76:1–10. http://dx.doi.org/10.1023/A:1025895731017.
[24] Zhu C, Chen Z. Role of polyamines in adventitious shoot morphogenesis from cotyledons of cucumber in vitro. Plant Cell Tissue Organ Cult 2005;81:45–53. http://dx.doi.org/10.1007/s11240-004-2773-y.
[25] Chi GL, Lin WS, Lee JEE, Pua EC. Role of polyamines on *de novo* shoot morphogenesis from cotyledons of *Brassica campestris* spp. *pekinensis* (Lour) Olsson *in vitro*. Plant Cell Rep 1994;13:323–9. http://dx.doi.org/10.1007/BF00232630.
[26] Shilpa K, Selvakkumar C, Senthil AK, Lakshmi BS. *In vitro* root culture of *Ocimum sanctum* L. and evaluation of its free radical scavenging activity. Plant Cell Tissue Organ Cult 2010;101:105–9. http://dx.doi.org/10.1007/s11240-009-9661-4.
[27] Dias ACP, Tomás-Barberán FA, Fernandes-Ferreira M, Ferreres F. Unusual flavonoids produced by *callus* of *Hypericum perforatum*. Phytochemistry 1998;48:1165–8. http://dx.doi.org/10.1016/S0031-9422(97)00963-1.
[28] Abbasi BH, Khan MA, Mahmood T, Ahmad M, Chaudhary MF, Khan MA. Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. Plant Cell Tissue Organ Cult 2010;101:371–6. http://dx.doi.org/10.1007/s11240-010-9692-x.
[29] Al Khateeb W, Hussein E, Qouta L, Alu'datt M, Al-Shara B, Abu-Zaiton A. *In vitro* propagation and characterization of phenolic content along with antioxidant and antimicrobial activities of *Cichorium pumilum* Jacq. Plant Cell Tissue Organ Cult 2012;110:103–10. http://dx.doi.org/10.1007/s11240-012-0134-9.