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# *IN-VITRO* SEED GERMINATION AND EFFECT OF TDZ AND $A_gNO_3$ ON HIGH FRE-QUENCY SHOOT REGENERATION FROM *RUELLIA TUBEROSA* L., USING COTYLE-DANARY NODE EXPLANTS

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**Keywords:**

*In-vitro* propagation, Micro shoots, *Ruellia tuberosa*, Thiadiazuron and silver nitrate

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**ABSTRACT:** *Ruellia tuberosa,* commonly called Minnie root, has plenty of applications in herbal medicine due to the presence of several bioactive compounds. A simple, efficient, and reproducible *in-vitro* plant regeneration protocol was developed for *Ruellia tuberosa*. The cotyledonary nodal explants were excised from seedlings were inoculated on Murashige and Skoog (MS) medium supplemented with various concentrations of BAP and NAA, respectively. The highest numbers of 9.86 ± 0.15 shoots/explant were obtained in MS medium augmented with BAP (2.0 mg $L^{-1}$) and NAA (1.0 mg $L^{-1}$). The micro shoots induced per explant significantly increased with the supplementation of BAP to auxin-containing medium. The results showed that maximum percentage of multiple micro shoots 89%, with a number (13.80 ± 0.26) shoots / explant was found on MS medium supplemented with BAP (2.0 mg $L^{-1}$) and NAA (1.5 mg $L^{-1}$). Further, the highest frequency of micro shoots was produced on MS medium supplemented with TDZ (0.3 mg $L^{-1}$) and $AgNO_3$ (0.4 mg $L^{-1}$) for large-scale production. The highest frequency of root induction (96%) was recorded on a half-strength MS medium fortified with 1.5 mg $L^{-1}$ IBA. The well-rooted plantlets were transferred into the plastic cups containing peat and soil (2:1) and successively established in the greenhouse. In conclusion, our study demonstrates efficient *in-vitro* regeneration, and in the future, this protocol will be a valuable tool to enhance secondary metabolite production in *Ruellia tuberosa*.

**INTRODUCTION:** The recent researches on *Ruellia tuberosa* have shown the presence of rich secondary metabolites, which are in demand commercially in the pharmacology field. These include traces of Luteolin and appreciable amounts of apigenin and Malvidin 5 Glucoside in the aerial parts.

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Apigenein and Malvidin 5 glucoside are the anthocyanins that are present in flower buds of *Ruellia tuberosa* [1]. Anthocyanins are the most studied class of natural polyphenols that provides color for fruits and flowers.

Piyaporn *et al.*, 2010 [2] conducted research regarding dyeing property of flowers of *Ruellia tuberosa* on cotton fabrics by applying chitosan as a mordant obtained best result. Several studies have also shown that foods rich in anthocyanins are healthy, and they can act against cancer, inflammation, cardiovascular and chronic disorders. Recent advancements in biotechnological techniques such as tissue culture, enzyme and fermentation

technology promote the synthesis and processing of secondary plant metabolites *in-vitro*. In tissue culture, shoot, root and callus suspension cultures are utilized for the production of secondary metabolites [3]. The secondary metabolite production can also be increased by using different biotic and abiotic elicitors. Acanthaceae is a taxon of dicotyledonous blossoming plants containing nearly 250 genera and around 2500 species. Among these species, *Ruellia tuberosa* has been considered as the most qualified species with high restorative qualities are known through the exploration work completed previously. *Ruellia tuberosa* plants can be seen well from March to August and are damaged during the winter season **Fig. 1**. *Ruellia tuberosa's* phytochemical studies disclosed that alkaloids, saponins, triterpenoids, flavonoids, and sterols were present [4]. *Ruellia tuberosa* is extensively used as an effective medicine in treating diuretic, analgesic, gastroprotective disorders [5, 6,] and gonorrhoea [7]. It is also used to treat antidiabetic, antipyretic and antihypertensive. It was reported that in Thai traditional medicine, it was used as a remedy for detoxification of toxic substances [8]. In pharmacological investigations, extracts of elevated parts indicated anticancer, antinociceptive, and anti-inflammatory activities [9]. The methanol root extracts of *Ruellia tuberosa* have demonstrated antifungal and antibacterial activities [10]. The phytochemical examination has revealed cirsimartin, cirsimarin, betulin, and cirsiliol-4-glucoside.

**FIG. 1: PICTURE OF *RUELLIA TUBEROSA* L., DAMAGED DURING WINTER**

Adventitious shoot formation may be an effective clonal propagation technique because it avoids somaclonal changes in the cultures. Vinitha *et al.*, 2013 [11] reported *in-vitro* shoot regeneration by indirect organogenesis, where shoots are formed through callus.

Due to the non-uniform nature of callus tissue[12], genetic mutations are more common in shoots formed through callus, mostly in an extended subculture, than in other forms of tissues [13]. The tissues or explants used also affect the possibility of genetic variation. Axillary buds, Shoot apex, Embryo, Apical meristem, and Cotyledonary node were identified as suitable, as they are modest to regenerate. This is due to the presence of pre-existing meristematic tissues, thereby reducing the difficulty of the regeneration process [14, 15].

Our main objective was to enhance secondary metabolites from *Ruellia tuberosa* by using elicitors in *in-vitro.* Hence the establishment of *Ruellia tuberosa* in *in-vitro* is necessary for the production of secondary metabolites. In our study, we established clonal propagation in *in-vitro* by following different methods for seed germination, multiple shoot production from cotyledonary nodes of *Ruellia tuberosa.*

Earlier regeneration with nodal segments in *Ruellia tuberosa* produced less shoots. In our work, we established the highest frequency of seed germination in a short period of time without callus induction and maximum numbers 60 shoots/ explants were produced in *Ruellia tuberosa*.

## MATERIALS AND METHODS:

**Plant collection:** *Ruellia tuberosa,* seeds were collected from the fields of kamathamoor village, Andhra Pradesh, India, and propagated in Herbal garden, Dravidian university. A specimen of herbarium with an identification voucher number: BS-20 has been authenticated and deposited in the Department of Botany, S. V. University, Tirupathi. Explants needed were collected from these plants to carry out further experiments.

**Seed Germination:** The Capsulated mature brown fruits of *Ruellia tuberosa* were collected in the months of March to August 2017, stored in air-tightened polythene bags. Capsulated seeds when comes in contact with water, seeds are out by popping process. Seeds are sterilized using tap water, washed 3 times, 5% Tween 20 for 15 minutes, followed by distilled $H_2O$ wash for five times. Surface-sterilized seeds were imbibed in distilled water overnight in dark conditions in a 1:20 ratio (5 gm of seeds per 100 ml of water).

**Effect of Pre-treatment of $H_2O_2$ on Seed Germination:** The overnight imbibed seeds are then treated with 6% $H_2O_2$ by submerging for different time intervals 10, 15 or 20 min at 100 rpm in shaking incubator **Fig. 2.** Pre-treatment was carried out by submerging 25 seeds in 50 ml solution at 26 °C temperature. The seeds are again thoroughly rinsed with distilled $H_2O$ five times. The seeds untreated with $H_2O_2$ are taken as control.

**FIG. 2: SEEDS OF *RUELLIA TUBEROSA* AFTER PRETREATMENT WITH $H_2O_2$**

**Effect of Disinfectants on Seed Germination:** Three sterilized procedures are assessed for $H_2O_2$ treated seeds. In the first method, rinsing seeds with 1% (W/V) bavistin for 15 min followed by distilled $H_2O$ wash until the whiteness from the seeds is removed. In the second method, seeds are surface sterilized with 95% (v/v) ethanol for 1 min followed by distilled water wash five times. Thirdly, seeds were cleansed for 2 min with 0.1% freshly prepared $HgCl_2$, followed by distilled water wash five times. Seeds are then cultured on a solid MS medium containing 3% sucrose. Cultures were maintained under 16 h / 8 h light and dark conditions with 26 ºC temperatures.

**Effect of Culture Methods on Seed Germination:** To resolve the culture methods two types of methods was followed; the $H_2O_2$ disinfected seeds are inoculated on basal solid MS medium and a liquid medium with/without shaking in shaking incubator at 90 rpm. The other one is the paper roll method, where seeds are spread on the whatman filter paper and were moistened with water. And the seeds are maintained in 16 h / 8 h light and dark photoperiod at 26 °C temperature.

**Culture Conditions and Explants Preparation:** The 10-day old surface-sterilized Cotyledonary node (CN) segments with root initials excised aseptically and were inoculated on the full strength solid MS Medium with different plant growth hormones concentrations. For all the experiments, MS medium complemented with 3% sucrose is used as basal medium [16].

After adding Plant Growth hormones to the medium, pH was modified to 5.8, before including 1% agar (Bacteriological grade, HiMedia) and autoclaved for 20 minutes at 121 °C temperature. All the cultures were provided by these conditions; the temperature at 22 ± 2 °C under a 16 / 8 h light and dark photoperiod and cool daylight fluorescent lamps at a photon flux rate of 60 µmol $m^{-2}$ $s^{-1}$.

**Experiment I: Shoot Bud Induction in *In-vitro* Cotyledonary Nodes:** In the first experiment, *in-vitro* germinated cotyledonary node explants were transferred on to the shoot bud induction MS medium augmented with various concentrations of Benzyl Amino Purine (0.0, 0.5, 1.0, 1.5 and 2 mg $L^{-1}$ ) and Naphthalene Acetic Acid (0.0, 0.5, 1.0 and 1.5 mg $L^{-1}$) in alone and in combinations. Cultures were incubated under 16 h photoperiod at an intensity of 80 µmol $m^{-2}$ $s^{-1}$ and temperature at 23 ± 2 °C under cool-white fluorescent light.

**Multiplication and Elongation of Shoot Buds:** For further multiplication, the *in-vitro* derived micro shoots obtained from cotyledonary nodes were cultured on BAP (0.5, 1.0, 1.5 and 2.0 mg $L^{-1}$), Kinetin (0.5, 1.0, 1.5 and 2.0 mg $L^{-1}$), and NAA (0.5, 1.0, 1.5, 2.0 mg $L^{-1}$) in alone and in combinations were used for multiplication of shoot buds.

Results were noted after 4 weeks of inoculation. Cultures were kept at 25 ± 2 °C under cool-white fluorescent light at an intensity of 80 µmol $m^{-2}$ $s^{-1}$ with 16 h photoperiod.

**Experiment II: Large Scale Production of Shoots:** *In-vitro* regenerated shoot buds were grown on MS medium complemented by 0.3 mg $L^{-1}$ TDZ (Thiadiazuron) and 0.4 mg $L^{-1}$ $AgNO_3$ (Silver Nitrate) in 250 ml flasks.

Freshly prepared same media is used for subculture in once every 3 weeks. The same method has been repeated for the next three subcultures (each 21 days) to investigate the impact of sub-culture on the development of shoots on a large scale. A number of shoots were analyzed after culturing for 65 days.

**Rooting of Elongated Shoots and Acclimatization:** *In-vitro* rooting studies were conducted by transferring elongated shoots (5-7 cm long) on to half-strength MS basal medium fortified with Indole Butyric Acid (2.0 mg $L^{-1}$) and NAA (1.5 mg $L^{-1}$) concentrations alone and also in amalgamation with KN (1.0 mg$L^{-1}$). For control, few shoots were kept on HF MSB medium without auxin. Rooted shoots and root length percentage were noted for every treatment. To acclimatize, *in-vitro* explants were smoothly splashed with tap $H_2O$ to take away the leftovers of agar and are moved to autoclaved mixture of soil, peat, and perlite (1:1:2) placed in plastic cups. Soil is humidified with MSB liquid medium for one week. The regenerated plants were lastly transferred into greenhouse conditions.

**Data Collection:** The seed germination percentage was calculated after 5, 7, and 10 days of inoculation. There were three replicates of 25 seeds in each treatment. Each seed with plumule is counted as the germinated seed.

**Statistical Analysis:** For each experiment, 10 test tubes with a single explant for shoot-bud initiation and regeneration studies were used. In rooting studies, 3 replicates of jars containing 3 shoots each were used for each treatment. Experiments have been repeated twice, and the results have been recorded. The two factors studied in all experiments were the type of explant and phytohormonal treatment. Factorial variance analysis (ANOVA) was conducted using a complete randomized design (CRD on data using SPSS software), and the means were compared using Duncan's multiple range test (DMRT) at a probability level of $p<0.05$ percent.

## RESULTS:

**Effect of Pre-treatment with $H_2O_2$ on Seed Germination:** We preferred $H_2O_2$ for pretreatment of seeds, as many seeds can be treated at once. The appropriate time was determined by submerging the seeds in 6% $H_2O_2$ for 10, 15, and 30 min **Table 1.** The seeds submerged in $H_2O_2$ for 15 min have been found to have a maximum percentage of germinated seeds (82.6% on the $10^{th}$ day). We also observed a minimum percentage of germinated seeds when held for 10 min (59.3% on $10^{th}$ day) and 30 min (48.6% on $10^{th}$ day). The germination percentage was decreased significantly when seeds were treated for 30 min. In control, seeds that are untreated with $H_2O_2$ have shown the lowest percentage of germination (20% on the $10^{th}$ day). Submersion of seeds in $H_2O_2$ for 15 min has significantly increased the germination percentage when compared to untreated seeds.

**TABLE 1: THE PERCENTAGE OF SEED GERMINATION OF *RUELLIA TUBEROSA* UNDER DIFFERENT PRE-TREATMENTS OF $H_2O_2$**

| $H_2O_2$ Treatment time | Percentage of Seed Germination | | |
|---|---|---|---|
| | $3^{rd}$ day | $7^{th}$ day | $10^{th}$ day |
| Control | 0.00±0.00[a] | 6.66±2.30[a] | 20.00±4.00[a] |
| 10 min | 1.33±1.15[a] | 37.33±3.05[b] | 59.33±3.05[b] |
| 15 min | 29.33±6.11[c] | 68.00±4.00[c] | 82.66±6.11[c] |
| 30 min | 15.53±3.05[b] | 36.00±4.00[b] | 48.66±3.05[b] |

Legend 1: Means followed by same letter within the column were not significantly different according to Duncun's multiple-range test at $p < 0.05$

**Effect of Culture Methods on Seed Germination:** Here, we followed two methods for the germination of seeds. In the first method, we used the paper roll method, where 89.30% of the seed germination was observed **Table 2**. In the second method, plain MS medium was used in two forms solid medium and liquid medium. In a solid medium, we observed only 24.6% of seed germination on the $10^{th}$ day.

**TABLE 2: EFFECT OF CULTURE TYPES ON PERCENTAGE OF SEED GERMINATION IN *RUELLIA TUBEROSA***

| No. of days | Percentage of Seed Germination | | | |
|---|---|---|---|---|
| | Solid Medium | Liquid Medium | | Paper Roll Method |
| | | Without Agitation | With Agitation | |
| $3^{rd}$ | 0.00±0.00 | 10.00±2.00 | 41.33±5.03 | 10.66±4.61 |
| $7^{th}$ | 11.33±1.15 | 24.00±4.00 | 78.66±1.52 | 72.00±4.00 |
| $10^{th}$ | 24.66±3.05 | 40.00±4.00 | 91.60±1.51 | 89.33±2.30 |

In the liquid MS medium, we also followed two systems to improve seed germination, *i.e.,* without agitation and with agitation in a shaking incubator. In liquid plain MS medium without agitation, only 40% of seed germination was recorded, whereas with continuous agitation, nearly 91.60% of seed germination was achieved.

We found that there was a significant increase in seed germination in liquid medium with continuous agitation provided at 26 ºC temperature. Therefore the seeds pretreated with $H_2O_2$ for 15 min were cultured on a liquid medium with agitation for further, subsequent experiments.

**Effect of Disinfectants on Seed Germination:** To reduce contamination during seed germination, the $H_2O_2$ pretreated seeds were disinfected with three sterilants **Table 3**. Seeds sterilized with 0.1% $Hgcl_2$ and 95% ethanol have shown the highest percentage of seed germination, 60.66% and 42.66%, respectively, than seeds treated with 1% Bavistin (17.33%). Sterilization with 0.1 percent $HgCl_2$ was more effective in preventing contamination compared to untreated seeds (13.3 %) indicated the need for disinfectant treatment for *Ruellia tuberosa* seeds. The remaining percentage of seeds has been contaminated and disposed of.

**TABLE 3: THE PERCENTAGE OF SEED GERMINATION USING DIFFERENT STERILIZATION METHODS IN *RUELLIA TUBEROSA***

| Type of Disinfectant | Percentage of Seed Germination | | |
|---|---|---|---|
| | 3rd day | 7th day | 10th day |
| Untreated | 0.00±0.00 | 6.66±2.30 | 13.33±2.30 |
| Bavastin | 2.66±2.30 | 9.33±2.30 | 17.33±2.30 |
| 95% ethanol | 6.66±2.30 | 22.66±4.61 | 42.66±2.30 |

**Shoot Bud Induction in *In-vitro* Cotyledonary Nodes:** Cotyledonary leaf node cultured on MS medium devoid of any Plant growth hormones resulted in single shoot primordial per explant within 2 weeks. Even though the single node has shown the bud formation, the node with petiole has more effect on the formation of more buds in less time than node without a petiole.

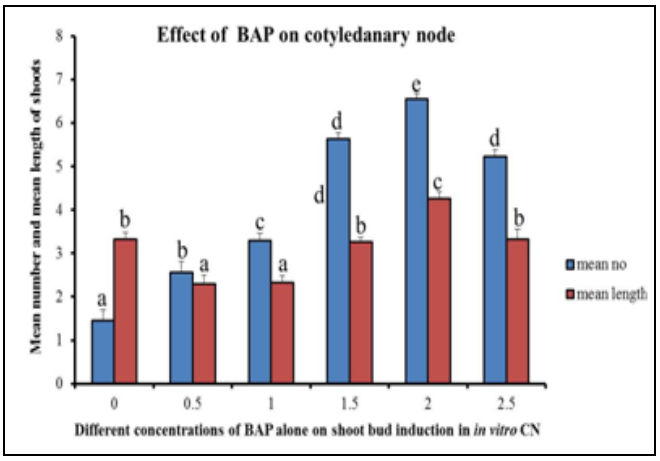


**FIG. 3: EFFECT OF DIFFERENT CONCENTRATIONS OF BAP ALONE ON COTYLEDONARY NODES OF *RUELLIA TUBEROSA:*** *In-vitro* cotyledonary nodes cultured on full strength MS medium supplemented with different concentrations of BAP (0, 0.5, 1.0, 1.5, 2.0 and 2.5) alone for multiple shoot bud induction, in which 2.0 mg/l has shown maximum mean a number of shoots 6.56 ± 0.11 with a mean length of 4.26 ± 0.15 showing 74% of the response. Means followed by the same letter within the column not significantly different at ($p \leq 0.05$).

In first experiment, the effectiveness of BAP alone and in a combination of NAA was assessed for multiple shoot bud induction from cotyledonary nodes on full strength MS medium.

Explants grown on basal MS medium in the absence of any growth hormones were taken as control. Individually, when BAP (2.0 mg $L^{-1}$) was supplemented to the culture medium, it resulted in a frequency of 74% response with a mean number of 6.56 ± 0.11 shoots/explant and mean shoot length of 3.90 ± 0.10cm per explant **Fig. 3**.

While Shoot multiplication was observed very well on BAP alone, there is no increase in shoot length after two subcultures. The concentration and amalgamations of BAP and NAA used affected the average shoot bud number per cotyledonary node and also the mean length of shoots.

Formation of green callus at the base is simultaneous with multiplication of shoot buds after one week of primary culture on BAP and NAA where callusing didn't affect shoot bud multiplication in *in-vitro* Cotyledonary node.

The cotyledonary nodes treated with BAP (2.0 mg $L^{-1}$) in combination with NAA (1.0mg $L^{-1}$) have shown 93.6% of response with a significant mean number 9.86 ± 0.15 of shoots and with a mean

length of 4.56 ± 0.11 per explant **Fig. 4**. The increase in NAA concentration from 0.5 to 1.0 mg $L^{-1}$ at the same level of BAP has significantly increased the induction of the shoot bud **Fig 5A, B & C.**

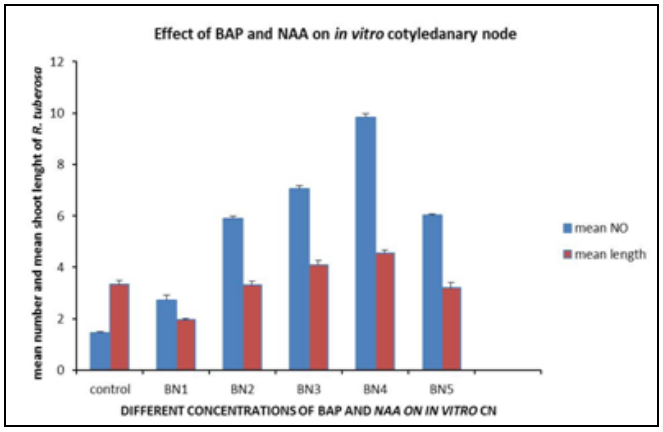


**FIG. 4: *IN-VITRO* COTYLEDONARY NODES CULTURED ON FULL STRENGTH MS MEDIUM SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF BAP** (0, 0.5, 1.0, 1.5, 2.0, and 2.5) and in combinations with naa (0.5, 1.0, 1.5, and 2.0 mg/l) for multiple shoots induction.

**Shoot Multiplication and Elongation in *In-vitro* Derived Micro Shoots:** Micro Shoot clusters formed from above BAP and NAA were subdivided into single shoots and were sub-cultured in combinations of BAP with KN and NAA to further increase shoot number **Table 4**. When used in combinations, BAP (2.0 mg $L^{-1}$) with KN (2.0 mg $L^{-1}$) and NAA (1.5 mg $L^{-1}$) showed a significant difference in the increase in shoot multiplication. The maximum mean number of shoots was 5.80 ± 0.20 and mean length was 4.26 ± 0.15 cm, with 74% of response on MS medium complemented with BAP (2.0 mg $L^{-1}$) alone **Fig. 5D**. No further multiplication was observed on increasing BAP beyond 2.0 mg $L^{-1}$. The plants were very well survived for two weeks along with multiplication and elongation, after that sub-culturing on to the fresh medium resulted in stunted growth and release of phenols where the survival rate was diminished greatly.

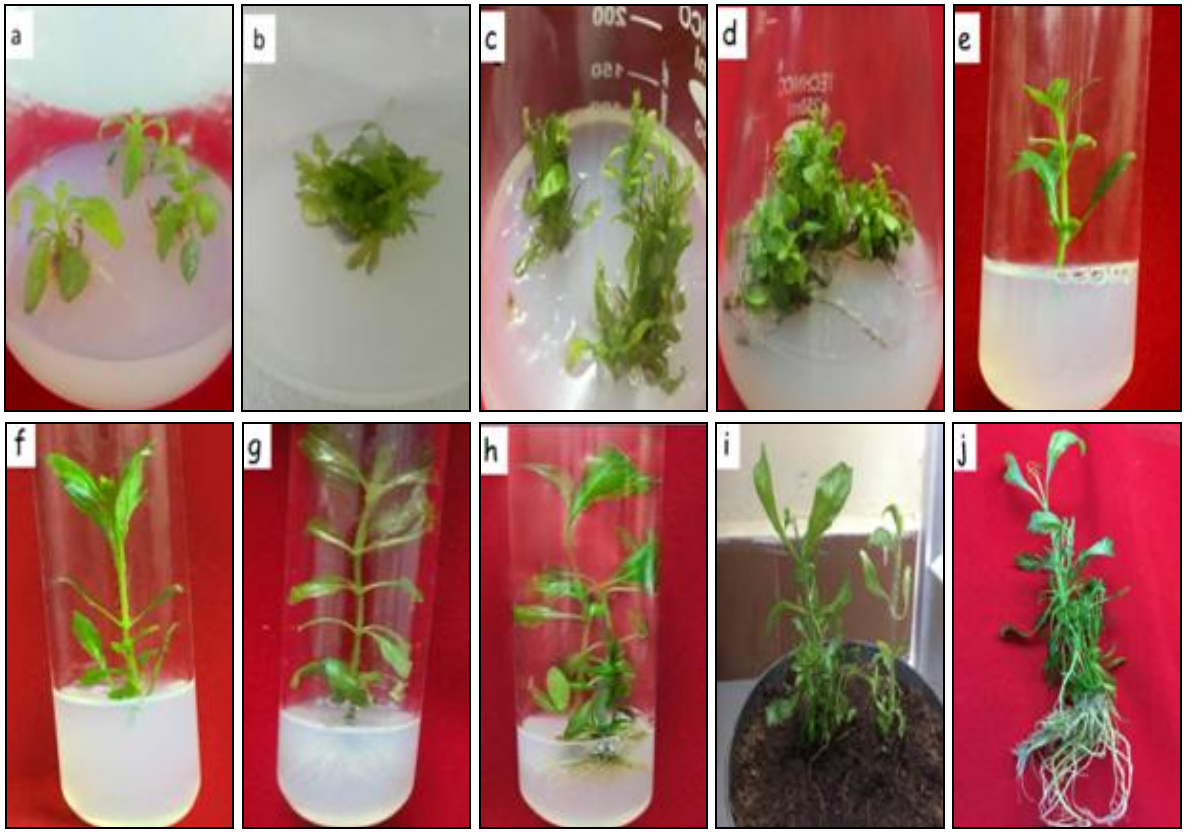


**FIG. 5A: CONTROL MEDIUM WITHOUT GROWTH HORMONES, B) SHOOT BUD INITIATION ON MS MEDIUM WITH BAP 2.0 Mg/L, IN *IN-VITRO* COTYLEDONARY EXPLANT OF *RUELLIA TUBEROSA*, C)** Shoot bud initiation on MS medium containing 2.0 mg/l BAP and 1.0 mg/l NAA in micro shoots, d) Shoot bud initiation on MS mediun containing 2.0 mg/l BAP and 2.0 mg/l KN in micro shoots, e) Shoot bud elongation on MS medium with BAP 2.0 mg/l, in *in-vitro* cotyledonary explant of *Ruellia tuberosa*, f) Shoot bud elongation on MS medium containing 2.0 mg/l BAP and 1.0 mg/l NAA in micro shoots, g) Root formation on half-strength MS medium with 1.5 mg/l NAA, h) Root formation on half-strength MS medium with 1.5 mg / l IBA, i) Hardening and acclimatization of *Ruellia tuberosa*, j) *In-vitro* grown complete plant of *Ruellia tuberosa* with elongated shoots and roots.

**TABLE 4: EFFECT OF VARIOUS CONCENTRATIONS OF BAP ALONE AND IN COMBINATIONS WITH KN AND NAA ON DEVELOPMENT OF MULTIPLICATION AND ELONGATION OF COTYLEDONARY NODE DERIVED MICROSHOOTS CULTURED ON FULL STRENGTH MS MEDIUM**

| BAP | KN | NAA | Percentage of Response | Mean No of Shoots Per Explant | Mean Length of Shoots (cm) |
|---|---|---|---|---|---|
| 0 | - | - | 47.66±2.51[a] | 1.3±0.17[a] | 1.70±0.17[a] |
| 0.5 | - | - | 56.00±2.80[c] | 1.9±0.10[b] | 2.63±1.15[c] |
| 1.0 | - | - | 63.33±2.88[c] | 2.00±0.01[b] | 3.43±0.20[d] |
| 1.5 | - | - | 67.00±2.00[c] | 2.36±0.32[c] | 3.67±0.20[d] |
| 2.0 | - | - | 74.66±1.53[d] | 5.80±0.20[d] | 3.90±0.10[e] |
| 2.5 | - | - | 53.66±1.52[b] | 2.90±0.11[c] | 2.33±0.17[b] |
| 0 | - | - | 47.66±2.51[a] | 1.3±0.17[a] | 1.70±0.17[a] |
| 0.5 | 2.0 | - | 82.67±2.51[c] | 2.43±0.26[b] | 3.23±0.25[b] |
| 1.0 | 2.0 | - | 82.30±2.52[c] | 2.56±0.11[b] | 3.26±0.30[b] |
| 1.5 | 2.0 | - | 87.00±2.00[d] | 4.16±0.28[c] | 4.23±0.25[a] |
| 2.0 | 2.0 | - | 89.33±0.57[d] | 6.46±0.30[e] | 5.73±0.15[d] |
| 2.5 | 2.0 | - | 75.60±1.15[b] | 3.06±0.11[d] | 3.23±0.25[b] |
| 0 | - | - | 47.66±2.51[a] | 1.3±0.17[a] | 1.70±0.17[a] |
| 0.5 | - | 1.5 | 82.00±0.00[c] | 2.70±0.20[b] | 3.16±0.28[b] |
| 1.0 | - | 1.5 | 87.00±2.00[e] | 4.83±0.20[c] | 3.96±0.05[c] |
| 1.5 | - | 1.5 | 85.66±1.15[d] | 6.96±0.05[e] | 4.40±0.17[d] |
| 2.0 | - | 1.5 | 89.66±0.57[f] | 13.80±0.26[f] | 6.06±0.11[e] |
| 2.5 | - | 1.5 | 73.17±1.73[b] | 5.20±0.20[d] | 4.16±0.28[d] |

Legend 2: Means followed by same letter within the column was not significantly different at Duncun's multiple range test at ($p \leq 0.05$).

**TABLE 5: SCHEMATIC REPRESENTATION OF NUMBER OF WEEKS REQUIRED FOR MULTIPLE SHOOT PROLIFERATION FROM *IN-VITRO* DERIVED SEEDLINGS OF *RUELLIA TUBEROSA***

| FG and CN Explants | Number of Weeks |
|---|---|
| Seed germination | 10 days |
| MS basal medium for CN explants | 10 days |
| Shoot bud formation (BAP+NAA) | 3 weeks |
| Shoot multiplication and development ( MS medium, BAP, NAA, KN) | 3 -4 weeks |
| Three subsequent subculture Shoot elongation from regenerated shoots (MS medium+TDZ+$AgNO_3$) | 90 days |
| Root formation (NAA 1.0 mg/l, IBA 1.5 mg/l ) | 14 days |
| Hardening | Four weeks |

The other combination of cytokinins was used for multiplication of micro shoots. The optimised concentration of BAP (2.0 mg $L^{-1}$), beside with different concentrations of KN was used. On MS Medium fortified with KN (2.0 mg $L^{-1}$) and BAP (2.0 mg $L^{-1}$) the mean shoot number of 6.46 ± 0.30 with a response of 89% was observed. Intensification in a concentration of KN above 2.0 mg $L^{-1}$, no further multiplication has been shown. Relative to BAP alone, the response percentage and also the shoot number was significantly higher. Also the leaves formed on this combination were green, large in size and the number of leaves was more compared to BAP alone. Even though shoots with mean length of 4.23 ± 0.25 were observed, after the second subculture the stunted growth was witnessed after transferring on to the same fresh medium. Secondly the combination of BAP (2.0 mg $L^{-1}$) and NAA (1.5 mg $L^{-1}$) gave a maximum mean number of 13.80 ± 0.26 shoots and mean length of 7.90 ± 0.30 cm per explant with mean percentage response of 89.66 ± 0.57. An increase in applications of NAA (0.5 - 2.0 mg $L^{-1}$) along with optimized BAP has shown better response in shoot multiplication and also in shoot length. The shoot number and shoot length were significantly higher compared to BAP alone and in KN combinations. An increase in the concentration of BAP and also NAA beyond 2.0 mg/$L^{-1}$ doesn't show any effect on further multiplication after two weeks also. During *in-vitro* multiplication, shoot length varied among different combinations used. On MS medium without PGR, the shoot length was 1.7 cm **Table 4**. The interaction effects of BAP at 2.0 mg $L^{-1}$ and NAA at 1.0 mg $L^{-1}$ have shown a maximum shoot length of 7.9 cm. The shoot length was significantly higher in the BAP and NAA combinations than in the BAP alone and the BAP

and KN combinations as well **Fig 5 E & F** It was found that with a higher concentration of BAP, the number of shoots was higher with a relatively shorter length of the shoot. The callus formation was found at the base of the explant in the combination of BAP and NAA, but does not affect the multiplication of the shoot.

During *in-vitro* multiplication, we found in our observations that the time taken for induction and multiplication of shoot buds on BAP alone and BAP with KN combinations was more than 60 days without subculture. The addition of auxin to the medium decreased the time required for induction of shoots. BAP at 2.0 mg $L^{-1}$ and NAA at 1.0 mg $L^{-1}$ have shown multiplication of shoots within 40 days of inoculation.

The use of glassware also influenced the elongation and multiplication of the shoots. Throughout test tubes, the shoots produced were in shorter clusters, where as in bottles, the shoots produced spread freely and the elongation between the shoots can be clearly seen. The principle disadvantage observed on these different combinations was callusing at the shoot proliferation part and browning of plants due to release of the phenols in to the medium after one week of the sub-culturing, resulting in diminishing the survival rate.

**Experiment II: Large Scale Production of Shoots from *in-vitro* Derived Micro Shoots:** Because of the release of phenols into the medium, plants acquired on the combination of BAP and NAA became brown after three subcultures and influenced the development and maintenance of plants *in-vitro*. The survival rate of *in-vitro* plants has been significantly reduced due to plant browning.

To solve this issue, we used TDZ to multiply the shoots. The produced explants showed stunted development and there is also no prevention of browning in the proliferation portion of the shoot. To solve this $AgNO_3$ was added along with TDZ, which prevented the browning of the shoots and also improved the rate of shoot multiplication. Cultures have repeatedly been sub-cultured on the same fresh media to increase production. For the multiplication of shoots different concentrations of TDZ alone and in combination with $AgNO_3$ were cultured **Fig. 6**.

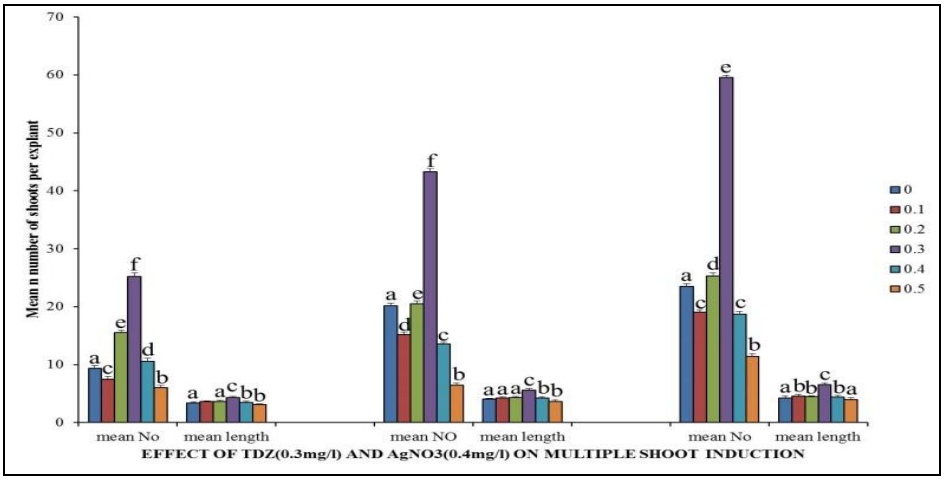


**FIG. 6: EFFECT OF VARIOUS CONCENTRATIONS OF TDZ AND $AGNO_3$ ON MULTIPLE SHOOT INDUCTION OF COTYLEDONARY NODE DERIVED IN VITRO MICRO SHOOTS OF *RUELLIA TUBEROSA*.** Cotyledonary node derived *in-vitro* micro shoots cultured on full strength MS medium supplemented with TDZ (0.1 mg/l to 0.5 mg/l) and $AgNO_3$ (0.1 mg/l to 0.5 mg/l), means followed by same letter within the columns were not significantly different at ($p \leq 0.05$).

Among all TDZ-alone concentrations ranging from 0.1 mg/$L^{-1}$ to 0.3 mg/$L^{-1}$, the maximum amount of shoot formation was observed at a concentration of 0.3 mg $L^{-1}$ with a mean of 9.4 ± 0.47 shoots / explant **Fig. 7**. The increase in TDZ concentrations from 0.3 mg $L^{-1}$ to 0.5 mg $L^{-1}$ suppressed the multiplication of shoots.

The optimal concentration of TDZ was found to be 0.3 mg $L^{-1}$. Explants cultured on MS Medium containing TDZ alone at 0.3mg $L^{-1}$ was taken as control. Since there is no prevention of browning and increase in the length of the shoot, different concentrations of $AgNO_3$ were used to overcome this problem along with TDZ of the different

concentrations for $AgNO_3$ (0.1mg/$L^{-1}$ to 0.5mg/$L^{-1}$) studied, 0.4 mg/$L^{-1}$ caused multiple shoot development along with shoot elongation substantially. When micro shoots grown on TDZ at 0.3 mg $L^{-1}$ in combination with $AgNO_3$ at 0.4 mg $L^{-1}$ generated a maximum number of shoots with a mean number of 25.2 ± 0.67 in the primary culture **Fig 7A**. Increase in concentrations of TDZ and $AgNO_3$ at 0.5mg $L^{-1}$, didn't show any further multiplication significantly. The percentage of shoot multiplication increased at the lower concentrations of TDZ and $AgNO_3$, thereafter, shoot multiplication was gradually declined with an additional rise in the application of TDZ and $AgNO_3$.

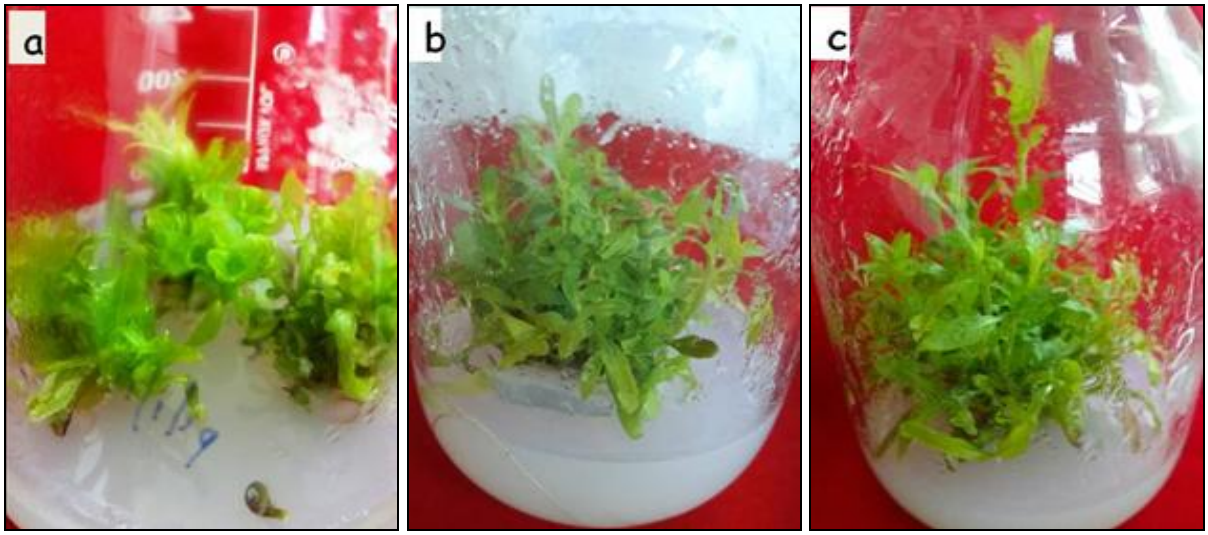


**FIG. 7: (A) FIRST SUBCULTURE OF RUELLIA TUBEROSA ON TDZ 0.3 MG/L AND AGNO3 0.4 mg/l WITH MEAN NUMBER OF 25.2 SHOOTS PER EXPLANTS, B) SECOND SUBCULTURE OF *RUELLIA TUBEROSA*** on TDZ 0.3 mg/l and $AgNO_3$ 0.4 mg/l with mean number of 43.3 shoots per explants, c) Third subculture of Ruellia tuberosa on TDZ 0.3 mg/l and $AgNO_3$ 0.4 mg/l with mean number of 59.57 shoots per explants.

Micro-shoots cultured on MS medium complemented by 0.3 mg/$L^{-1}$ TDZ and 0.4 mg $L^{-1}$ $AgNO_3$ were repetitively sub-cultured for the regeneration of multiple-shoot. This process was carried out to increase the number of multiple shoots per culture. Repeated subculture of shoots over first three passages may enable the continuous development of healthy callus-free shoots devoid of any sign of decline. At first subculture, when cultured on media augmented with TDZ (0.3 mg $L^{-1}$) and $AgNO_3$ (0.4 mg $L^{-1}$), the maximum number of shoots was 25.2 ± 0.67 with a mean shoot length of 4.30 ± 0.2 cm per explant. The shoot number was increased but shoot length was varied among the regenerated shoots. After three weeks, the shoots formed were further freshly sub-cultured with the same media composition, *i.e.* the second subculture produced an average of 43.3 ± 0.53 shoots with an average length of 5.57 ± 0.35cm per explant **Fig. 7B**. The shoot number and the length of the shoots increased significantly from the first subculture to the second subculture. In the third subculture, *i.e.*, after 90 days of culturing, we observed an increase in the shoot number and elongation at a maximum of 59.57 ± 0.40 and 6.53 ± 0.21 per explant, respectively with a survival rate of 85-90% **Fig. 7C**. The shoots produced are green and healthy, with more leaves per shoot.

***In-vitro* Rooting and Acclimatization:** The root induction frequency, along with root number and mean length, was recorded after 14 days of culture and affected significantly by the concentration of auxins. MS Medium (Full strength) lacking of auxins didn't respond for rooting rather resulted in browning of explants. The decreased concentration of cytokinin and increased concentration of auxin significantly increased the root induction. Shoots formed in clusters were alienated and cultured on a half-strength rooting medium composed of various concentrations of BAP in combinations of IBA, and NAA **Fig. 5 G & H**. Formation of roots without auxins have been taken as a control. IBA has been found to be more potent in root induction than NAA. The augmentation of MS half-strength medium with BAP at 1.0 mg $L^{-1}$ and IBA concen-

trations of 1.0 mg $L^{-1}$ and 1.5 mg $L^{-1}$ produced 11.83 ± 0.29 to 15.17 ± 0.29 number of roots per explant respectively **Fig. 8**. In the same manner, NAA at applications of 1.0 mg $L^{-1}$ and 1.5 mg $L^{-1}$ produced up to 6.80 ± 0.26 and 9.20 ± 0.15 number of roots per explant respectively. Increasing of IBA concen-trations didn't induce any further rooting. The maximum root length was 8.20 ± 0.2 cm and 5.25 ± 0.2 cm per explant in both IBA and NAA in the supplemented medium at 1.5 mg/$L^{-1}$. An increase in NAA concentration did not affect in root number or root length. In the control medium, roots formed were looking hard in texture and less in number with a maximum length of 5.37 ± 0.21whereas in NAA and IBA medium, roots formed were thin and formation of hairy lateral roots were observed. Medium containing IBA at 1.5 mg $L^{-1}$ was found to be the most suitable concentration for induction of roots with 85% response. The well-rooted shoots were moved to peat, perlite, and soil plastic cups (1:1:2). For a week, liquid MS medium was supplied, and the healthy plants were transferred to the greenhouse afterward **Fig 5 I & J**.

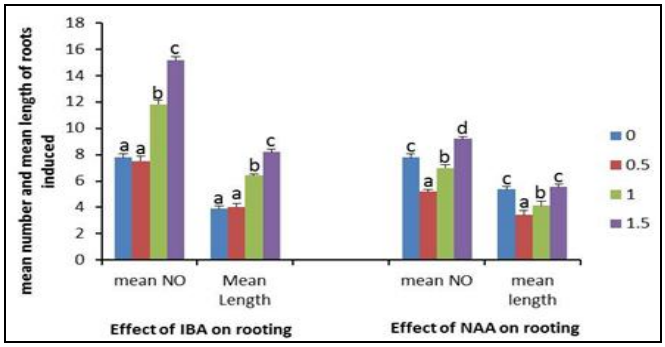


**FIG. 8: EFFECT OF VARIOUS CONCENTRATIONS OF TWO AUXINS (NAA AND IBA) ON INITIATION OF ROOTS FROM *IN-VITRO* DERIVED ELONGATED SHOOTS OF *RUELLIA TUBEROSA***. IBA at 1.5 mg/l has shown maximum number of roots than NAA. Means followed by same letter within the figure was not significantly different at ($p \leq 0.05$).

**DISCUSSION:** Pre-treatment with $H_2O_2$ has shown major effect on germination compared to untreated seeds. Seeds treated with $H_2O_2$ for 15 minutes have shown 82.6% of germination efficiency on 10th day. By triggering ABA catabolism and $GA_3$ biosynthesis, hydrogen peroxide acts as a signalling core for both seed dormancy regulation and germination. In *Arabidopsis*, by enhancing ABA catabolism and $GA_3$ biosynthesis, $H_2O_2$ can promote germination [17].

Several reports said that pre-treatment of seeds with $H_2O_2$ increased the germination efficiency in plants like *Gossypium hirsutam*[18], *Pea* [19], *Zea mays* [20].

Besides pre-treatment with $H_2O_2$ we also investigated different culture methods on seed germination development. We obtained the highest percentage (91.60% on 10th day) of seed germination in a liquid medium with continuous shaking in shaking incubator. Du Hyun Kim 2019 [21], reported in *Prunus yedoensis* that treatment of seeds of with $GA_3$ along with vigorous shaking in shaking incubator increased the seed germination by 71% and also stated that adequate shaking incubation can resolve the impermeability of the seed coat to intake of water and hormones.

In the 3-sterilant disinfection process, 0.1% of $HgCl_2$ disinfection for 5 min was shown to be effective in reducing the percentage of conta-mination compared to 95% of ethanol and 1% of Bavistin. $HgCl_2$ is said to be best phyto toxic agent that prevents the growth of bacteria and fungi on exposing at low concentrations for a short period of time. Several reports have been made on surface sterilization with $HgCl_2$ in seed germination, *Cucumis sativus* [22] and *Aconitum heterophyllum* [23]. Seeds germination percentage was highest in our study at 0.1% concentration for 5 min of exposure time, preventing the fungal contamination.

Even though previously *in-vitro* regeneration of *R. tuberosa* was established, when we followed the same method with the field-grown internodes of *R. tuberosa*, we have encountered a contamination problem that has dramatically affected the rate of survival *in-vitro* plants. It was difficult to maintain plants in *in-vitro* using field grown internodes as there is contamination, browning and no proper growth of plants.

**Shoot Bud Induction in *in-vitro* Cotyledonary Nodes:** No previous studies have been reported the multiplication of shoots from the cotyledonary nodes of this species. Our main objective was achieved in this study by establishing multiple shoot regeneration in *in-vitro* from cotyledonary nodes of *Ruellia tuberosa.* From previous studies, it was found that the cytokinins presence was requisite for shoot proliferation. In our study, when

cytokinin BAP from 0.5 mg/$L^{-1}$ to 2.0 mg $L^{-1}$ alone was supplemented on MS medium, the multiplication of shoots was significantly the highest. In several plant species, the effect of BAP on the induction of multiple shoots from cotyledonary explants was reported, including *Jatropha curcas* [24], *Crambe abyssinica* [25], *Arachis hypogea* [26].

When cotyledonary nodes were supplied with BAP at 2.0 mg $L^{-1}$, the induction of shoot buds was increased significantly with mean of 6.56 ± 0.11 shoot buds/explant. BAP alone at 2.0 mg $L^{-1}$ gave maximum mean of 5.80 ± 0.20 numbers of shoots per explant in *in-vitro* derived micro shoots. In both cotyledonary nodes and micro shoot explants, BAP alone at higher levels from 0.5 mg $L^{-1}$ to 2.0 mg $L^{-1}$ has enhanced the multiplication. In *Simarouba glauca* [27] reported a high rate of multiplication with BAP alone at 2.0 mg $L^{-1}$ due to BAP being readily metabolized than any other synthetic PGR [28]. Among all the concentrations tested, the optimum concentration of BAP was found at 2.0 mg $L^{-1}$ for multiplication. And beyond optimum level, there is no further increase in multiplication rate observed. This is parallel to the results by Venkatachalam *et al*., 2018 [29] in *Cucumis sativus* cotyledonary node explants where increasing BAP concentration 2.0 mg $L^{-1}$ beyond optimum level lowered the shoot induction efficiency.

The mean of 5.70 ± 0.20 shoots per explant was produced on medium fortified with BAP (2.0 mg $L^{-1}$) and KN (2.0 mg $L^{-1}$) concentrations. The mean number of shoots obtained on BAP and KN combination was not significantly higher than the shoots obtained on the medium with BAP (2.0 mg $L^{-1}$) alone. Such poor regeneration may be due to the production of ethylene and also to the superiority of BAP over KN for the initiation of shoots reported in a number of plants such as *Ceropegia bulbosa* [30], *Turnera ulmifolia* and *Morinda coreia* [31, 32] and *Salvadora oleoides* [33]. Surprisingly the leaves produced in this combination were green and large in size compared to leaves on BAP alone. In the present case, BAP along with NAA was more efficient in comparison to KN in relation of shoot proliferation and elongation in both cotyledonary nodes and *in-vitro* derived micro shoots. The MS medium fortified with cytokinin BAP and auxin NAA induced maximum shoot generation.

The optimized levels of BAP, when augmented with NAA, the higher rate of shoot multiplication was observed in both explants, mutually. When higher concentrations of BAP (2.0 mg $L^{-1}$) augmented with NAA (1.0 mg $L^{-1}$) have shown maximum response with an average of 9.86 ± 0.15 and 13.80 ± 0.20 shoots/explant in cotyledonary nodes and micro shoots, respectively.

In many species, the combination of BAP and NAA with high concentrations has improved the proliferation of shoots like *Brassica oleracea* [34], *Canna indica* [35], *Drosera burmanni* [36]. Such findings are significant in view of the fact that interactions between cytokines and auxins regulate development processes such as, cell growth, differentiation and organogenesis in plant tissue culture. Increased growth parameters on the MS medium supplemented by BAP and NAA, result from positive signals in latent meristematic cells to produce new shoots by maintaining juvenescence in plant tissues [37]. In our analysis, BAP and NAA induced shoot multiplication and also formed callus at the base of the explant; this is in agreement with Kumar *et al.,* (2017) [38] where it was stated in *S. oleoides* that the BAP and NAA combination formed callus at the base of the explant did not affect shoot multiplication.

It was found that with a higher concentration of BAP, the shoot number was higher with a relatively shorter length of shoot. The cause may be the repression of apical dominance at higher levels of cytokinin. Madhulatha *et al.,* (2004) [39] stated a comparable negative relationship amid the shoot number and shoot length of Nendran in *in-vitro* regeneration. The main drawback of the BAP and NAA combination was a reduced survival rate due to the release of phenols after three subcultures in *Ruellia tuberosa*. Some reports revealed the toxic effects of high concentrations of cytokinin (0.5-10 mg $L^{-1}$) in shooting media, which would blockage cell division, lead to apoptosis, lag elongation, and further root formation [40, 41].

**Large Scale Production of Shoots from *In-vitro* Derived Micro Shoots:** To overcome the problem of toxicity that occurred due to higher concentrations of BAP, TDZ was used for multiplication of shoots. In a number of plants, it has been stated that TDZ is a cytokinin having

immense potential in shoot organogenesis, including legumes [42, 43]. The lower concentrations of TDZ (0.3 mg $L^{-1}$) in the full strength MS medium significantly increased (more than 90%) the number of shoots/explant. TDZ alone at 0.3 mg $L^{-1}$ produced 9.4 ± 0.47 of shoots/explant on full strength MS medium. An increase in TDZ concentration above 0.3 mg $L^{-1}$ doesn't exhibit any further multiplication; hence it is effective when applied at lower concentrations. Simultaneous findings have been published by Kumar *et al.,* 2016 [44] and Hussain *et al.,* 2019 [37] where TDZ at lower concentrations is more successful in increasing shoot multiplication. The highest number of shoots was formed in media with TDZ alone; this may be due to the ability of TDZ to suppress the activity of cytokinin oxidase, which exaggerates the level of endogenous cytokinins [43]. As TDZ has shown poor shoot length and minimal browning of the tissue, $AgNO_3$ was added to the medium, which resulted in large production of shoots after three subcultures.

Our results revealed the formation of multiple shoots on media augmented with TDZ (0.3 mg $L^{-1}$) and $AgNO_3$ (0.4 mg $L^{-1}$) combination. In this study, the use of $AgNO_3$ with TDZ had a vital effect on enhancing shoot regeneration in *R. tuberosa*. When TDZ is applied in combination with $AgNO_3$ the shoot multiplication with increased shoot length and browning also controlled at final sub culture. At third subculture, the maximum mean of 59.57 shoots per explant with mean length of 6.9 cm were observed in the combination of TDZ and $AgNO_3$. Among all the concentrations tested, $AgNO_3$ at 0.4 mg $L^{-1}$ induced the maximum number of shoots per explant. At low concentrations, $AgNO_3$ was found to cause hindered senescence triggering in enhanced growth of proliferated shoots [45]. The shoots developed by this combination of plant growth regulators have shown normal green and healthy growth. The positive effect of $AgNO_3$ in a combination of different cytokinin was also seen in plants like *Hordeum vulgare* [46], *Peanut* [47], *Polygonum tinctorium* [48].

Repeated subculture is typically used to increase the rate of multiplication of the shoot bud. In *Stevia rebaudiana,* Venkatachalam *et al.,* 2012 [49] efficaciously used this method to surge the number of shoots. At each subculture, multiplication of number of shoots along with shoot length was noticed without a sign of decline in shoot number. This might be of inhibition of apical dominance in repetitive shoot subculture, which has led the basal dormant meristematic cells to increase the number of regenerative shoots at this stage [50]. Also, the effectiveness of $AgNO_3$ in up regulating cytokinin biosynthesis genes and to induce a less oxidised cellular environment in favouring shoots multiplication [51].

***In-vitro* Rooting and Acclimatization:** For rooting of elongated shoots, IBA and NAA are supplemented in a half-strength MS medium. IBA at 1.5 mg $L^{-1}$ was found to be the better rooting medium for *Ruellia tuberosa* than NAA. IBA at a greater concentration of 1.5 mg $L^{-1}$ has given a maximum of 15.17 numbers of roots with 8.2 cm of root length. This is parallel to the findings stated by Khanam and Anis, 2018 [52] in *Allamanda cathartica*, that IBA was more successful in rooting than NAA and IAA. Also Revathi *et al.,* 2018 [53], in *Oldenlandia corymbusa* reported that a higher concentration of IBA (2.0 mg $L^{-1}$) had shown effective rooting in half-strength MS medium.

**CONCLUSION:** In conclusion, in our study, we present an efficient and reproducible protocol for the first time to the best of our knowledge for multiple shoot regeneration from cotyledonary explants of *Ruellia tuberosa.* This protocol demonstrated the effective methods of seed germination in a short period of time. And also revealed the significance of TDZ and $AgNO_3$ in large-scale production of *Ruellia tuberosa*. Thus, this method may be advantageous for the cultivation of *Ruellia tuberosa* under *in vitro* conditions. This protocol will be remarkably beneficial for various interventions in biotechnology and for the improvement of this species.

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