

Effects of Bavistin and Cefotaxime on *in vitro* Contaminant free Shoot Regeneration of *Ruellia tuberosa* L.

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Abstract

In general, antimicrobial agents are often used in micropropagation techniques to obtain contaminant free clones. The objective of the present study was to evaluate the effects of bavistin and cefotaxime on producing contaminant free plants of *Ruellia tuberosa* cultured on MS supplemented with phytohormones. Field grown nodal explants of *Ruellia tuberosa* was used to regenerate entire plants via direct organogenesis. Among the decontaminants tested, the fungicide bavistin along with higher concentration of BAP (2.0 mg/l) and lower concentration of NAA (1.0 mg/l) was the most effective in regeneration and producing contaminant free shoots from cultured explants. This fungicide at 300 mg/l minimised fungal contamination with survival rate of 54%. While the addition of decontaminant cefotaxime at low concentration (200 mg/l) along with same concentration of BAP and NAA stimulated the bud formation and controlled the bacterial contamination. However, its increasing concentration adversely affected the survival rate of *Ruellia tuberosa*. These findings clearly showed that low concentrations of bavistin and cefotaxime were not only non-toxic but also facilitated bud regeneration. The results achieved showed the decisive role not only of the use of successful fungicides and antibiotics, but also of their sufficient doses were very important in reducing contamination and helping multiple shoot proliferation.

Introduction

Acanthaceae (Acanthus family) is a taxon of dicotyledonous flowering plants containing almost 250 genera and about 2500 species. *Ruellia* or wild petunias are a genus of Acanthaceae with 250 species, found in hot tropical and subtropical regions, Mediterranean region, Australia and USA. These are popular ornamental plants and also

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used as medicinal plants. Among all species *Ruellia tuberosa* has been extensively used as diuretic, anti-diabetic (Wulan et al. 2015), antinoceptive and anti-inflammatory (Alam et al 2009), anti-cancer (Dey et al. 2013) and antimicrobial activity (Arirudran 2011). Phytochemical properties of *Ruellia tuberosa* showed the presence of alkaloids, triterpenoids, saponins, sterols and flavonoids. (Khachitpongpanit et al. 2016). It has been externally used in traditional Thai medicine as an anti-inflammatory, anti-septic and antidote to detoxify poisons.

Earlier reports on *Ruellia tuberosa* revealed the presence of apigenin, luteolin, 3, 5-diglucoside, apigenin-7-O-glucuronide, apigenin glucoside, apigenin rutinoside, luteolin glucoside, and flavone glycoside (Wulan et al. 2015). The *Ruellia tuberosa* flowers were also tested for its dyeing property by (Piyaporn et al. 2010).

Secondary metabolites are the natural products of plants that are commercially used in medicines, flavours, fragrances, insecticides, dye, etc., (Berni et al. 2018). Plant Tissue Culture (PTC) has been used as a major platform for the production of secondary metabolites owing to its several benefits (Ming et al. 2016). Plant secondary metabolites production in the plant naturally has been minimized due to various environmental factors which pose unfavourable conditions to the plants to produce secondary metabolites. PTC with its fascinating advantages to produce the required amount of secondary metabolites is an alternative approach to surpassing it and commercially producing the requisite metabolites under controlled conditions over a short period of time (Kolewe et al. 2008). It authorises the bulk propagation of plants under managed environmental conditions without any recurrent constrictions.

Earlier studies on *in vitro* propagation of *Ruellia tuberosa*, Lakshmanan et al. (2015) developed shoots using nodal explants, where there was no protocol for decontamination in *Ruellia tuberosa*, focusing on *in vitro* activities for plant extracts. Vinitha et al. (2013), induced shoots from callus using different plant growth hormones.

The use of pre sterilization method to decontaminate the explants with antibiotics was routinely practised but the major limitation in this plant is the survival of the plants *in vitro*. As the wild explants of *Ruellia tuberosa*, are quickly infection prone, it is essential to establish regeneration protocol to obtain fast and infection free *in vitro* plants. The present study was conducted to optimize different concentrations of bavistin and cefotaxime to minimize the contamination level and to induce multiple shoot regeneration *in vitro*.

Materials and Methods

Ruellia tuberosa was collected from the fields of the Kamathamoor village, propagated in the herbal garden in Dravidian University, Andhra Pradesh, India. 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.

Nodal segments were obtained from field-grown plants, used as explants. These explants were washed under running tap water for 10 min followed by soaking in detergent solution 5% (v/v) teepol (Qualigens, India Ltd.) for 10 min and again rinsed under running tap water. The explants were surface sterilized using 0.4% (w/v) bavistin followed by 70% (v/v) ethanol for 60 sec and washed thoroughly with sterile double distilled water 3-4 times prior to inoculation.

For all the experiments, MS medium containing 3% sucrose (HiMedia, Mumbai) was used as basal medium. All growth hormones were added to the medium before autoclaving and the pH of the media was adjusted to 5.8, before including 1% agar (HiMedia, India) and autoclaved for 20 min at 121°C at 15 lb pressure. All the cultures were kept in growth chambers at 22 ± 2°C under a 16h/8 h (light/dark) photoperiod at a photon flux rate of 60 μmol m⁻² s⁻¹ provided by cool daylight fluorescent lamps.

Various concentrations of BAP (0.0, 0.5, 1.0, 1.0, 1.5 and 2 mg/l) alone and in combinations of NAA (0.0, 0.5, 1.0 and 1.5 mg/l) were used to analyse their effects on shoot regeneration of *Ruellia tuberosa*. Each experiment was conducted thrice with 20 replicates. The number of regenerated shoot per explant and shoot length were calculated for each treatment.

To control the contamination and to investigate efficiency of decontaminants on *Ruellia tuberosa* nodal explants, two types of decontaminants with different concentrations were used. Bavistin (100, 200, 300 and 400 mg/l) and cefotaxime (100, 150, 200, 250 and 300 mg/l) were supplemented to MS medium along with the BAP (2.0 mg/l) and NAA (1.0 mg/l).

Among the two decontaminants used percentage of survival, contaminated and necrotic explants were calculated using the following formula:

$$\% \text{ of survival, contaminated and necrotic explants} = \frac{\text{Number of explant survived, contaminated or necrotic}}{\text{Total number of explants inoculated}} \times 100$$

In vitro rooting experiments were carried out by the transfer of elongated shoots (5-6 cm long) to a half-strength MS basal medium supplemented by combinations of IBA and NAA at three different concentrations (0.5, 1.0 and 1.5 mg/l). The well-rooted plantlets were gently rinsed with tap water to remove the remnants of agar and transferred to plastic cup filled with a mixture of autoclaved peat, perlite and soil (1 : 1 : 2), moistened with MSB liquid medium and held in a mist chamber with 80-90 per cent relative humidity at day/night temperatures of 22 ± 2°C below 16 h photoperiod for four weeks. The acclimatized plantlets were eventually moved to the greenhouse conditions.

Experiments are conducted in a fully randomised block design (RBD) and all experiments are conducted thrice with 20 replicates. Each data value was the mean ± SD of 20 independent determinations. The experimental results were statistically analysed

by one-way ANOVA using SPSS software version 16.0 and means compared by DMRT ($p < 0.05$).

Results and Discussion

Nodal segments of *Ruellia tuberosa* cultured on MS medium without any plant growth hormones resulted in single shoot primordial after four weeks of inoculation. It was observed that regeneration is too late from the time of inoculation without PGRs. Multiple shoot buds were induced at the sides of internodes on nodal segments cultured on medium supplemented with various PGRs were visible by 2 weeks of culture. Medium supplemented with BAP alone at 2.0 mg/l produced shoots with mean number of 3.9 ± 0.05 per explant (Fig. 1a). Although shoot formation of buds were seen, no further growth/elongation or increase in shoot number was seen after two weeks. George (1993) reported that BAP improves the formation of shoots and releases lateral buds from dormancy. BAP alone demonstrated better shoot regeneration but shoot elongation was prohibited at this higher concentration (Fig. 2a). *Gentiana kurroo* (Sharma et al. 1993) and *Chlorophytum borivilianum* (Ashraf et al. 2014) are the earlier reports that support the effectiveness of BAP on shoot induction and less impact on shoot elongation. Saha et al. (2007), in Bottle gourd noted the greater amount of release of ethylene in medium with BAP (2.0 mg/l), where shoot regeneration is contributed but shoot elongation is prohibited. Ethylene also serves as an endogenous regulator of morphogenic processes, which also includes organ size (Bleecker et al. 1998).

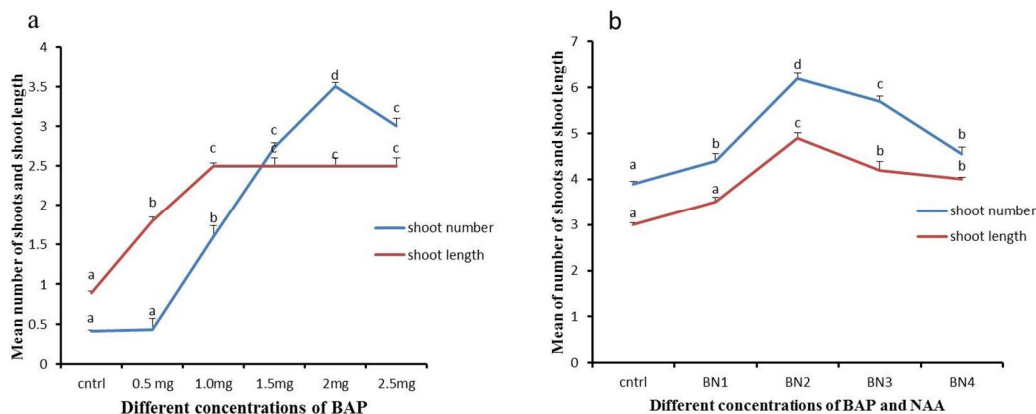


Fig. 1a-b: Effects of different concentrations of BPA alone on shoot number and shoot length of *Ruellia tuberosa* nodal explants. b. Effects of different concentrations of BAP and NAA on shoot number and shoot length of *Ruellia tuberosa* nodal explants. BN1 (BAP 2.0 mg/l, NAA 0.5 mg/l), BN2 (BAP 2.0 mg/l, NAA 1.0 mg/l), BN3 (BAP 2.0 mg/l, NAA 1.5 mg/l), BN4 (BAP 2.0 mg/l, NAA 2.0 mg/l).

To increase the number of shoots, media containing BAP (2.0 mg/l) in combination with different concentrations of NAA were used. Increase in concentration of NAA from 0.5 to 2.0 mg/l at the same level of BAP did significantly build up the shoot-bud induction (Fig. 1b). The maximum number and length of shoots was achieved on media containing 2.0 mg/l of BAP and 1.0 mg/l NAA (Fig. 2b). There is a substantial increase in the regeneration of the shoots when BAP was paired with NAA; this combination was found to be main combinations in many plant species like *Echinocereus cinerascens* (Elias et al. 2015), *Echinop skebericho* (Enyew and Feyissa 2019) and Banana (Gebeyehu 2015).

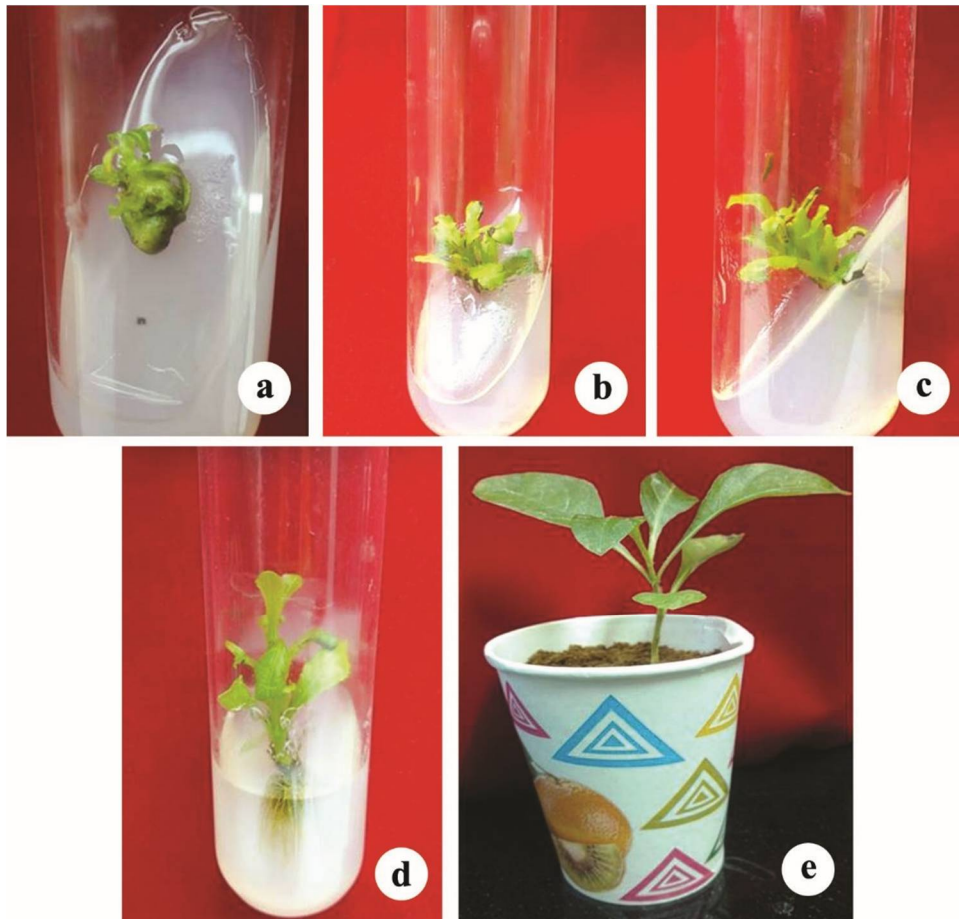


Fig. 2(a-e). Different stages of *in vitro* shoot regeneration, root formation and acclimatization of *Ruellia tuberosa*. a. Shoot bud initiation of nodal explant of *Ruellia tuberosa* on media containing only BAP at 2.0 mg/l. b. multiple shoot formation and shoot elongation on media containing BAP at 2.0 mg/l and NAA (1.0 mg/l). c. complete regeneration of shoots of *Ruellia tuberosa*. d. Root formation on IBA 1.0 mg/l + NAA 1.0 mg/l e. Acclimitization of plantlet.

The increase in concentration of NAA at 0.5 to 1 mg/l had a major effect on regeneration. In nodal explants of *Phalaenopsis* cv. 'Surabaya' Balilashaki et al. (2015) reported that BAP at 5.0 mg/l and NAA at 2.0 mg/l produced 8.70 numbers of shoots due to the synergistic effects of cytokinin and auxin. In present study the higher concentration of BAP (2.0 mg/l) and lower concentration of NAA (1.0 mg/l) produced significant number of shoots (Fig. 2c). In kinnow nodal explants, higher concentration of BAP (3.0 mg/l) and lower concentration of NAA (0.5 mg/l) showed the maximum shoot regeneration (Sharma et al. 2012). At 1.0 mg/l, NAA in combination with BAP 2.0 mg/l developed a maximum of 6.23 ± 0.25 shoots with a 4.9 ± 0.19 cm increase in elongation. The developed shoots were transferred to rooting media containing IBA 1.0 mg/l + NAA 1.0 mg/l (Fig 2d). Two weeks after root formation, the plants are transferred to pot containing peat, perlite and soil (1 : 1 : 2). Plants were supplemented with half strength liquid MS medium for one week (Fig. 2 e).

Nodal explants cultivated on MS medium containing bavistin (300 mg/l) is the most efficient in terms of regeneration frequency and reducing the fungal contamination and bacterial contamination in explants from 91 to 65 per cent with survival rate of $54 \pm 00.0\%$ (Fig. 3a). Bavistin (300 mg/l) along with BAP (2.0 mg/l) and NAA (1.0 mg/l) showed maximum shoot regeneration; with increased shoot number (7.16 ± 0.15) (Fig. 4a). The survival rate is improved in the medium supplemented with bavistin. The length of shoots was significantly increased from 4.9 to 5.5 cm compared to BAP and NAA combination. The increase in further concentration resulted in decrease in shoot number and browning of plants. The survived shoots were healthy and green. Release of phenolic substances and necrosis is seen in the medium with the survived shoots after five weeks.

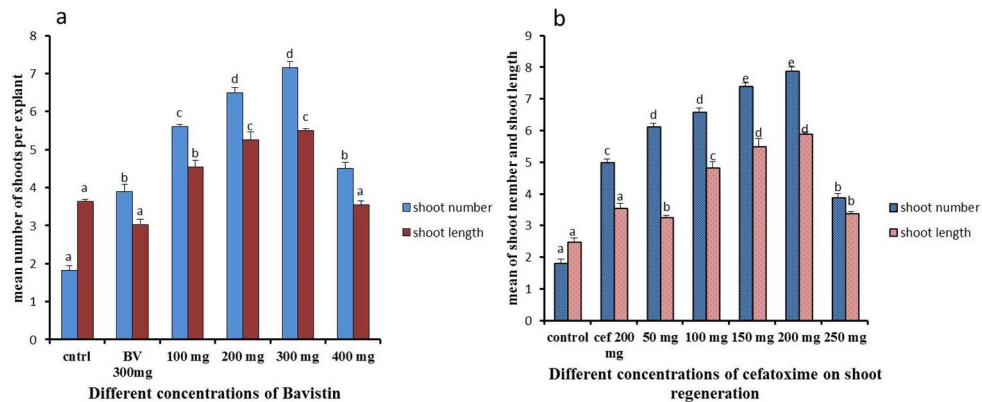


Fig. 3a-b. a. Effects of different concentrations of Bavistin along with BAP (2.0 mg/l) and NAA (1.0 mg/l) on shoot regeneration and shoot length of *Ruellia tuberosa* nodal explants. b. Effects of different concentrations of Cefotaxime along with BAP (2.0 mg/l) and NAA (1.0 mg/l) on shoot regeneration and shoot length of *Ruellia tuberosa* nodal explants.

Antimicrobial agents are usually supplemented in plant tissue culture media to minimise or remove contamination that is either existent in the original explant or

present as physical faults. In the present research, bavistin was used to control fungal contamination and to facilitate enhanced shoot proliferation. Positive impact of bavistin on decontamination and shoot proliferation was recorded indifferent plants viz. *Bacopa monnieri* (Ramesh et al. 2009), *Mentha piperita* (Sujana et al. 2011), and *Centella asiatica* (Panathula et al. 2014).

Bavistin seemed to have a much greater cytokinin function as its molecular structure has been reflected to have some likeness to that of kinetin, adenine, and many other adenine-based cytokinins (Tripathi and Ram 1982). In current study, use of bavistin in MS medium has shown significant effect on controlling fungal contamination and increased shoot proliferation. The increase in the regeneration of the shoot may be due to its cytokinin like activity.

In the present study, cefotaxime, along with PGR, was used to investigate the effect of regeneration, control of contamination and to increase the survival rate. The obtained data indicated that cefotaxime at 200 mg/L has a major impact on controlling the bacterial contamination and helping in the shoot initiation, proliferation and increasing the survival percentage by minimising the fungal contamination (Fig. 3b). Maximum survival rate, number of shoots per explant (7.90 ± 0.25) and the shoot length (5.9 ± 0.17 cm) was obtained at the concentration of 200 mg/l cefotaxime. Increased concentration of cefotaxime results in decreased number of shoots (3.2 ± 0.15) and length of shoots (3.9 ± 0.05 cm), respectively (Fig. 4b).

In earlier reports like Tomato 2019, *Solanum viarum* (Dunal) (Mahadev et al. 2014), Chinese cabbage (Meng et al. 2014) and *Allium cepa* (Passi et al. 2018) cefotaxime was used to suppress endophytic bacteria and to induce shoot regeneration. β -Lactams do not inhibit plant growth because their antibacterial activity mechanism is based on the blocking of peptidoglycan biosynthesis, one of the components of the bacterial cell wall (Gerszberg and Grzegorzczuk-Karola 2019). Cefotaxime demonstrated shoot regeneration at 200 mg/l in this study, out of all the concentrations used, with a survival rate of 36.6 per cent. Despite the fact that bavistin had a higher survival rate, it had shown necrosis after four weeks, cefotaxime was added to the medium to improve the survival rate. The highest shoot number and shoot length was achieved on cefotaxime 200 mg/l along with BAP (2.0 mg/l) and NAA (1.0 mg/L). These results are similar with Oliveira et al 2010 where plant regeneration from mature tissue of citrus cultivars is effective when supplemented with 500 mg/l cefotaxime and BAP and NAA. The present study showed an important role of the decontaminants used and its concentration on the frequency of shoot bud development. This remains consistent with the findings of Tambarussi et al. (2015).

The foremost cause for supplementing decontaminants was to control the contamination and to increase the survival rate of the inoculated plants. The survival rate was calculated for the fungicides used (Fig. 5). The plants cultured with BAP alone and in combination of BAP and NAA without decontaminants have shown less survival percentage of 13.3 ± 0.30 and contaminated plants of 91.6 ± 2.8 per cent. Even though

shoot regeneration was seen after two weeks of inoculation with BAP and NAA, within four weeks plants were prone to contamination and the remaining survived plants too became necrotic. When the cultures were supplemented with BAP and NAA along with bavistin (300 mg/l), we observed increase in survival rate by $54.0 \pm 5.0\%$ and contaminated plants by $46.6 \pm 4.5\%$. There is significant increase in shoot number and survival rate compared to plants without bavistin (Fig 4c). The percentage of necrotic plants in the medium containing bavistin was reduced to 30.0 ± 2.8 percent. Survival rate was increased up to 36% when plants developed in bavistin. In *curcuma amada*, (Benerjee et al. 2011) reported that bavistin containing synthetic seeds produced five times better regeneration to contamination free shoots with 75% survival rate. Ramesh et al (2009) in *Bacopa monneiri*, reported seeds encapsulated with bavistin in MS medium resulted in contamination free shoots with 5.2 mean numbers of shoots. Increase in concentration above 300mg/L decreased shoot regeneration and also resulted in browning of explants.

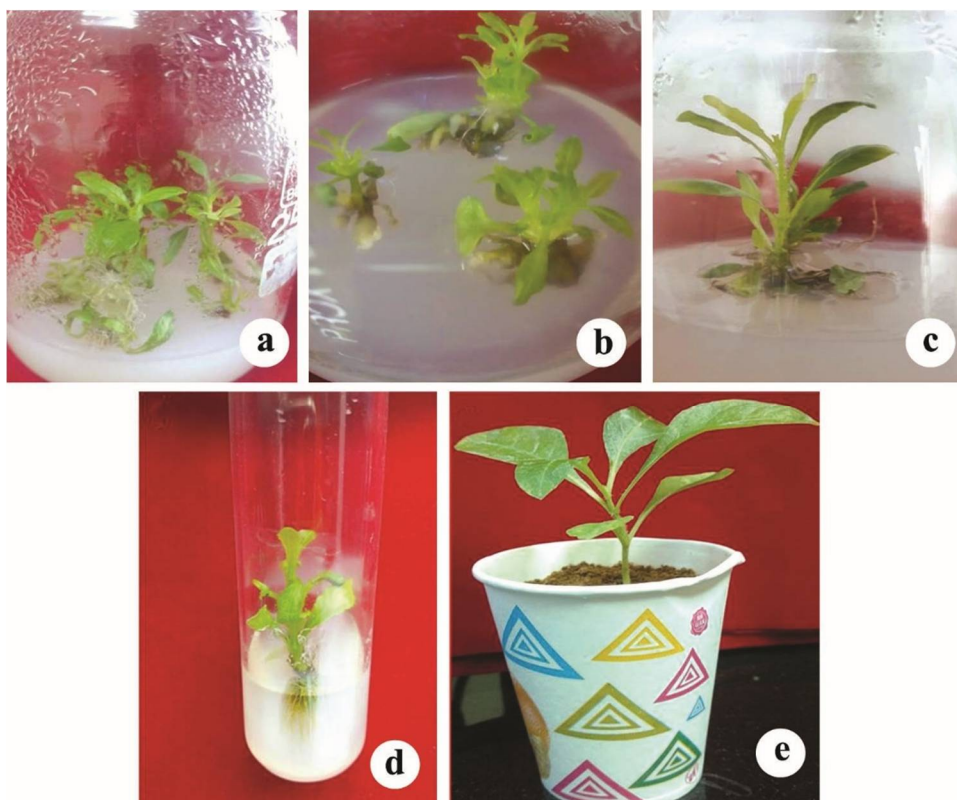


Fig. 4a-e. Regeneration of *Ruellia tuberosa* on Bavistin, Cefatoxime and PGRs. a. Shoots formed on media with bavistin 300 mg/l along with BAP 2.0mg/l and NAA 1.0 mg/l. b. shoots formed on media with cefotaxime at 200 mg/l along with BAP 2.0mg/l and NAA 1.0 mg/l. c. complete shoot formation of *Ruellia tuberosa*. d. Root formation on media augmented with NAA 0.5 mg/l and IBA 1.5 mg/lg. e. Acclimatization of plantlet.

Secondly, cefotaxime at 200 mg/l augmented MS medium along with BAP and NAA significantly decreased the level of contamination to $65.0 \pm 5.0\%$ and increased the survival rate to $36.6 \pm 2.8\%$. After four weeks of culture, the necrosis in plants was also decreased to $41.6 \pm 0.0\%$. But the survival rate and regeneration of shoots were not significant compared to the bavistin. Although shoot regeneration was observed in a cefotaxime added culture medium, necrosis in plants resulted in decreased survival rate, rendering cefotaxime unfavourable at higher concentrations. It is similar to the results in Tomato plants (Gerszberg and Grzegorzczak-Karola 2019), and where at higher concentrations cefotaxime decreased the shoot bud frequency and showed phytotoxicity. In contrast to our previous results in *Allium cepa* (Passi et al. 2018) reported that cefotaxime increased the survival rate by 53.17% and produced the bacterial free cultures. In this study increase in concentration of cefotaxime (50-200 mg/l) has shown successful bacterial decontamination, the survival rate and shoot bud formation was not prominent after four weeks. These results are similar to the sugarcane (Kenneth et al. 2011) where at higher concentrations of cefotaxime bacterial decontamination was achieved by 100% but it also lead to the death of shoot buds. In contrary to our results the positive influence of cefotaxime was observed in Melon (Naderi et al. 2016).

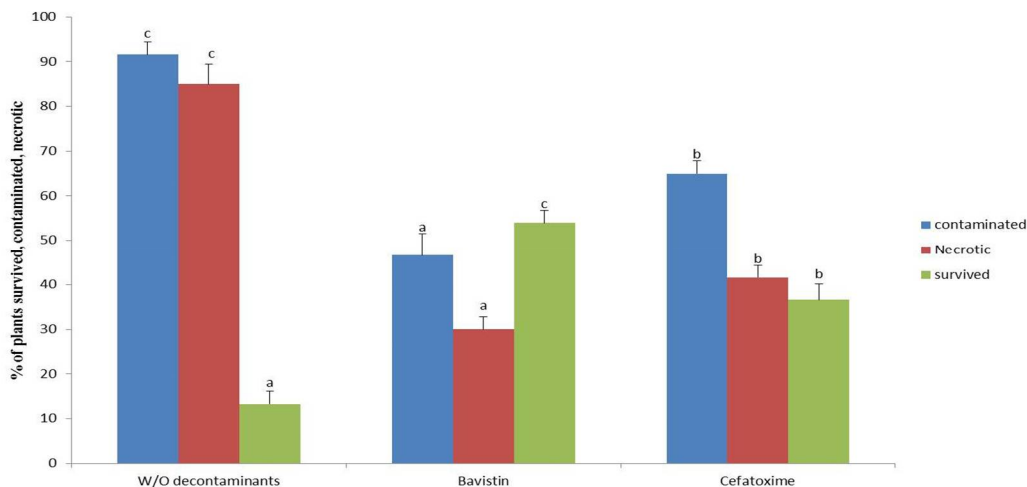


Fig. 5. Percentage of plants survived, contaminated and necrotic after treatment with bavistin and cefotaxime in *Ruellia tuberosa* nodal explants. Mean \pm SD with in a bar followed by same letters are not significantly different according to DMRT $p \leq 0.05$.

The individual roots were separated and placed on rooting medium. The elongated shoots placed on auxin free medium showed no significant increase in root formation. Among different concentrations used, IBA 1.0 mg + NAA 1.0 mg supplemented in half strength MS medium showed significant root formation along with root length (Fig. 4d). IBA at 1.5 mg/l has shown 24.7 ± 0.6 numbers of roots with 5.76 ± 0.2 cm root length (Fig.

6). The similar findings were reported in Passi et al. (2018) where supplementation with NAA and IBA in medium increased the root number and root length.

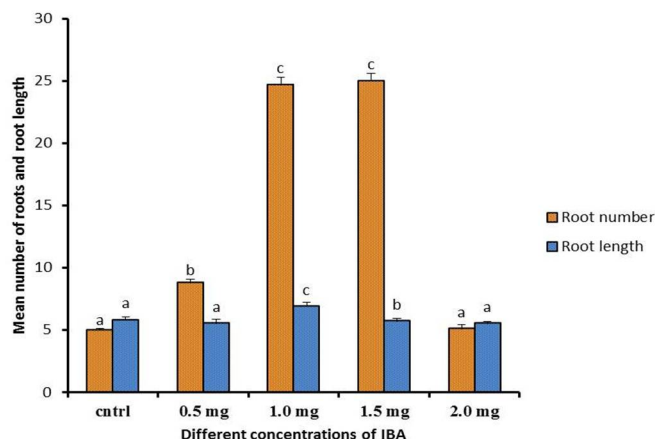


Fig. 6. Effect of different concentrations of auxins on root number and root length of *Ruellia tuberosa*. Mean \pm SD with in a bar followed by same letters are not significantly different according to DMRT $p \leq 0.05$.

The rooted plants were then transferred to peat, perlite and soil (1 : 1 : 2). Plants were supplemented with half strength liquid MS medium for one week (Fig. 4e). After two weeks, plants were successfully acclimatized in green house conditions.

Conclusion

In this analysis it can be concluded that BAP and NAA increased the number of shoots, worked synergistically to achieve the optimal number of shoots along with the elongation of *Ruellia tuberosa*. It is possible to obtain contaminant free plants of *Ruellia tuberosa* by using decontaminants in MS medium containing bavistin along with BAP and NAA and cefatoxime along with BAP and NAA. The two decontaminants bavistin and cefatoxime used in this study helped in producing contaminant free plants of highest shoot regeneration frequency, where the survival rate was best observed in bavistin than cefatoxime. Hence the regeneration system developed in this study is expected to facilitate our efforts to produce large number of contaminant free plants of *Ruellia tuberosa* using bavistin and cefatoxime as decontaminants.

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