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In Vitro Propagation and *Ex Vitro* Establishment of a Medicinal Plant- *Gynura procumbens* (Lour.) Merr. through Leaf Culture

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Abstract: *An effective protocol for adventitious shoot proliferation was established on MS medium supplemented with different concentrations of cytokinin and auxin using leaf explants from in vitro grown shoots of Gynura porcambens. Highest frequency 86.67% of adventitious shoot formation, maximum number 20.52 of shoots per explant and average 7.3 cm length of shoot were obtained on MS medium supplemented with 4.0 µM 6-Benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA). Shoot multiplication and growth were significantly affected by different types of basal medium, level of concentration of sucrose, and pH. The highest rate (92.31%) of shoot production was achieved in a shoot-regenerating full strength of MS basal medium with 30 gm/l sucrose where pH was adjusted to 6.0. For rooting, the in vitro proliferated and elongated shoots were excised into 2–4 cm long microcuttings, which were planted individually on a root-induction MS medium containing 4.0 µM Indole-3-butyric acid (IBA). Within 4 weeks of transfer to the rooting medium, all the cultured microcuttings produced 2–6 roots. The in vitro regenerated plantlets were transferred to vermicompost, and the survival rate underex vitro was 100.00%.*

Keywords: *Medicinal Plant, Adventitious Shoot Regeneration, Culture Medium, Plant Growth Regulators, Vermicompost.*

1. INTRODUCTION

Gynura procumbens Merr. belonging to the family Asteraceae is a medicinal plant commonly found in tropical Asia countries such as China, Thailand, Indonesia, Malaysia, and Vietnam. Traditionally, it is widely used in many different countries for the treatment of a wide variety of health ailments such as kidney discomfort, rheumatism, diabetes mellitus, constipation, and hypertension. Based on the traditional uses of *G. procumbens*, it seems to possess high therapeutic potential for treatment of various diseases making it a target for pharmacological studies aiming to validate and provide scientific evidence for the traditional claims of its efficacy. *G. procumbens* have been long used as ethnoherbal products to treat various ailments such as diabetes, hypertension, urinary infection and used as anti-inflammatory and anti-allergic agents (Jiratchariyakul *et al.*, 2000). However its phytochemical properties have not been well studied until recently. Bohari *et al.* (2006) reported the extracts of this plant had an enhancing effect on glucose uptake in 3T3 adipocyte cell lines and they suggested that the anti-diabetic action of *G. procumbens* might be mediated through the stimulation of glucose uptake. Iskander *et al.* (2004) discovered that the crude ethanolic extracts of *G. procumbens* showed anti- inflammatory properties and steroid might be one class of anti-inflammatory compounds found in this plant. Zhang and Tan (2000) reported that the leaves extracts of *G. procumbens* had significantly suppressed the elevated serum glucose levels and reduced the serum cholesterol and triglyceride levels in diabetic rats. Akowuah *et al.* (2001) discovered that the n-butanol extracts of this plant could reduce the blood glucose levels in streptozotocin-induced type 2 diabetic rats. Two compounds, 3, 5-di-O-caffeoylquinic acid and 4, 5-di-O-caffeoylquinic acid, identified from this plant were found to inhibit the replication of viruses (Jiratchariyakul *et al.*, 2000).

G. procumbens is commonly used for diabetes treatment in traditional medicine and its hypoglycemic effect has been reported in *in vivo* studies (Hamid *et al.*, 2004; Algariri *et al.*, 2014). One intriguing finding on *G. procumbens*, is its specificity in inducing hypoglycemic effect only in diabetic animals as it has been shown to cause a significant decrease in fasting blood glucose levels and suppression of glucose elevation during glucose tolerance test in diabetic rats but not normal rats (Algariri *et al.*,

2014).The effect of *G. procumbens* treatment on insulin level has been investigated. Hamid *et al.* (2004) has reported the stimulation of insulin secreting cell lines by *G. procumbens* extract. However, the exposure of clonal pancreatic cells with extract of *G. procumbens* did not stimulate insulin secretion (Hassan *et al.*, 2010). These contradicting results might be due to the differing response of different cell lines when treated with *G. procumbens*. Therefore, its effect on insulin secretion has been further tested using *in vivo* studies. However, no significant change has been observed in plasma insulin level in diabetic rats treated with the extracts, implying that the hypoglycemic activity of *G. procumbens* does not rely on insulinotropic activity but may instead be due to its extra-pancreatic effect (Hassan *et al.*, 2010; Lee *et al*., 2012).

This plant is conventionally propagated by cuttings. The conventional method cannot meet the increasing demandof this plant used as the raw material for the preparation of pharmaceutical, dermaceutical and aromatherapeutical products. The *in vitro* culture techniques can be the alternative for the continuous provision of plantlet stocks for large scale field cultivation. By considering the medicinal value and antidiabetic properties of *G. procumbens* was undertaken for develop a suitable micropropagation protocol for the mass production of this plant species. Here, we established an efficient protocol which can be used at a large scale for the clonal multiplication of this plant species using leaf as explants derived from the *in vitro* grown shoots. To our knowledge, there are few reports on axillary shoot proliferation but there are no report on adventitious shoot regeneration of this plant species.

2. MATERIALS AND METHODS

Plant material and culture medium

Leaf explants of *Gynura procambens* were collected from *in vitro* grown shoots, were cut small pieces, and were placed in 250 ml conical flask containing 2.0-6.0 µM BAP in combination with 1.0-2.0 µM NAA or IBA supplemented MS (Murashige and Skoog, 1962) medium prepared with 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma Chemical Co. USA). The pH of the medium was adjusted to 5.7 ± 1 before autoclaving at 121°C for 20 minutes at 1.2 kg/cm^2 pressure.

Shoot induction and proliferation

To find out the suitable basal medium for adventitious shoot regeneration from leaf explants were initially cultured on four different basal medium viz. MS (Full strength of MS medium), ½MS (Half strength of MS medium), ¼MS (Quarter strength of MS medium) and B5 (Gamborg *et al*., 1968) medium supplemented with 4.0 µM BAP with 2.0 µM NAA.

Leaf explants were also used for examining the effects of sucrose concentrations on shoot regeneration. Six different concertation (15.0, 20.0, 25.0, 30.0, 35.0 and 40.0 gm/l) of sucrose in a medium were employed for the experiments. In addition to test the effects of pH on adventitious shoot regeneration from leaf explants of *in vitro* grown shoot were cultured on MS medium containing 4.0 µM BAP in combination of 2.0 µM NAA where pH level of culture medium were adjusted to 5.0, 5.5, 6.0 and 6.5.

Formation of adventitious root, hardening and acclimatization

Microshoots of 1-3 cm length were prepared from usable shoots by snipping off the basal leaves and cultured them individually in 25 × 150 mm culture tubes with 10-15 ml of full strength MS medium supplemented with 2.0-6.0 µM of IBA, NAA, or IAA (Indole-3-acetic Acid). Rooted plantlets were transferred on to the small plastic pots containing sterilized soil mix (garden soil: sand: compost in 2:1:1 ratio) and vermicompost, separately. Transferred plantlets were hardened in growth chamber condition for 20 days and then transferred to outdoor condition. The total number of plants transferred to the pots and the number of surviving plants in the outdoor condition were recorded. All the cultures were maintained at 25 ± 1°C under a 16h light and 8h dark cycle with the light intensity of 2000-3000 lux provided by cool-white fluorescent tubes.

Statistical analysis

Data were recorded after 8 weeks of culture except for rooting experiment when the data were recorded after 4 weeks of incubation. In all the experiments, 12-15 explants were used and each experiment was repeated three times. Mean and standard error were calculated for all numerical data. The mean data of each treatment were compared by using Duncan's Multiple Range Test (DMRT) at P=0.05%.

3. Result and Discussion

Shoot Regeneration

Most of the leaf explants of *G. porcambens* elongated within 20 days of incubation. Direct shoot regeneration was observed after 5 weeks of culture incubation. Adventitious shoot proliferation occurred from the cut margin of the leaf explants. Figure 1 shows the percentage of explants that responded formation of shoots, average number of shoots, and average length of shoots per explant. Most of the media induced shoot proliferation through direct organogenesis, and the primordia always regenerated directly from the leaf without forming callus (Fig.2A). The range of the percentage of shoot regenerating explants was 86.67 ± 2.5 to 64.29 ± 1.7%. The data showed significant diff erences ($p < 0.05$) among the various hormonal treated culture media. In this experiment the maximum percentage (86.67 ± 2.5%) of shoot regenerating explants was observed on MS medium with 4.0 µM BAP and 2.0 µM NAA, which also induced the average highest total number (20.52 ± 0.8) of shoots and the average highest length (7.3 ± 0.5 cm) of shoots (Fig. 2B-C). Similar result was found in *Alstroemeria* (Lin *et al*., 1997), *Phellodendron amurense* (Azad *et al*., 2005), *Torenia fournieri* (Kanchanapoom *et al*., 2009), *Plectranthus barbatus* (Thangavel *et al*., 2011). Among the BAP-IBA combinations, the maximum frequency (78.57 ± 2.2%) of shoot bud formation and total number (16.31 ± 1.1) of shoot per explant were obtained at 4.0 µM BAP with 2.0 µM IBA. The results obtained in the present study are in agreement with this regarding Malus hupehensis var. pinyiensis (Wanmei*et al*., 2014). Among different growth regulator supplements, NAA seems to be more effective in shoot differentiation than IBA. High concentrations of cytokinin and auxin in the medium inhibited shoot formation.

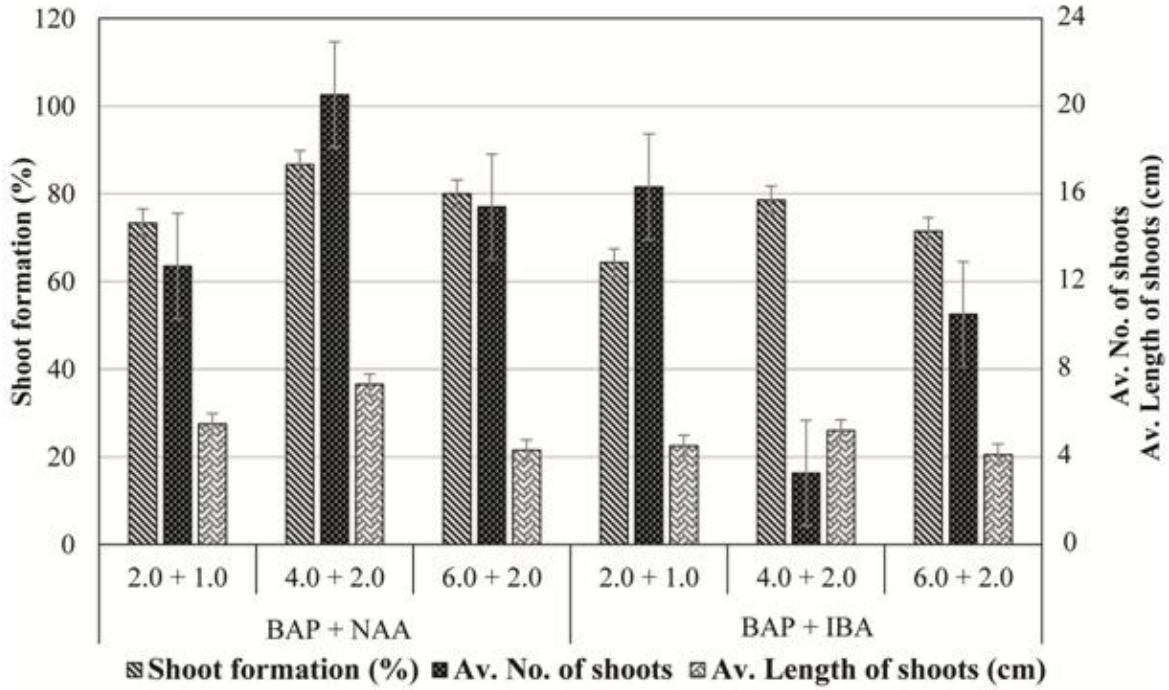

Figure 1. *Effects of explants on MS medium containing different concentrations of auxins and cytokinin on axillary shoot proliferation of Gynura procumbens.*

Effects of Medium

The leaf explants significantly affected shoot proliferation on full strength MS medium supplement with 4.0 µM BAP with 2.0 µM NAA than other three media (½MS, ¼MS and B5) tested (Fig. 3). Maximum 85.71 ± 1.31 % explants produced highest 22.2 ± 0.21 shoots with highest average length 7.2 ± 0.11 cm on MS medium. The second highest frequency 76.92% of shoot proliferation 12.5 ± 0.21 average number of shoot and 5.5 ± 0.52 cm length of shoot were obtained on ½MS medium. Considerable shoot proliferation were observed on B5 medium. The lowest performance of shoot proliferation, number of shoot, and shoot length were found on ¼MS medium. This study revealed that full strength MS medium was preferred for adventitious shoot proliferation from leaf explants of

G. procambens while B5 medium showed a little effect in terms of shoot proliferation. Full strength MS medium has been proved best for adventitious shoot proliferation in many other medicinal species, such as *Malus hupehensis* var. pinyiensis(Jin *et al*., 2014), *Hygrophila polysperma* (Karatas *et al*., 2013), *Plectranthus barbatus* (Thangavel *et al*., 2011), Lilium (Bacchetta *et al*., 2003), and *Adhatoda vasica* (Azad *et al*., 2003). Similar results were also observed in some other woody trees, like *Phellodendron amurense* (Azad *et al*., 2004), *Accia catechu* (Kaur *et al*., 1998), *Accia nilotica* (Abbas *et al*., 2010), and *Colutea istria* (Hegazi and Gabr, 2010).

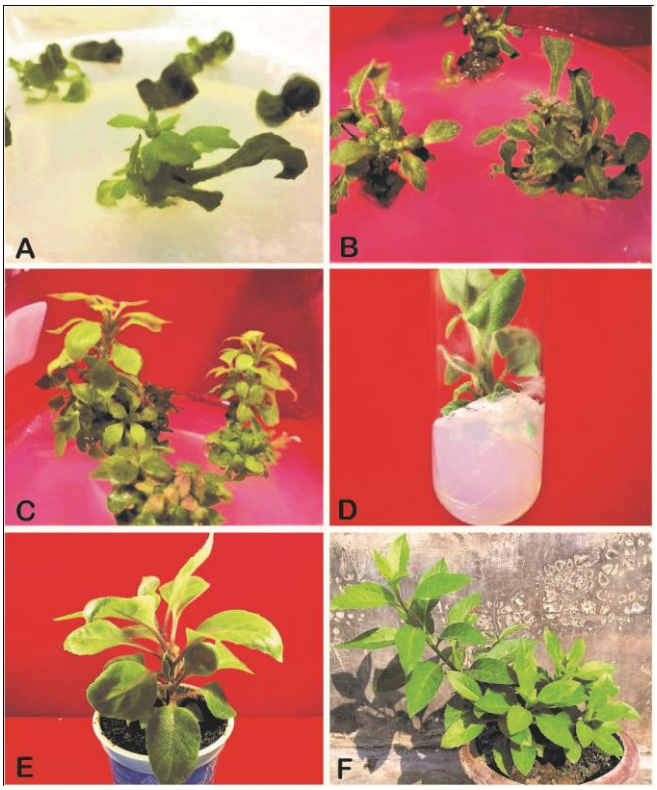

Figure 2. *A-F: In vitro regeneration of plantlet from leaf explants of Gynura procumbens.*

A: *Proliferation of adventitious shoots from leaf explantscultured on MS medium containing 4.0 µM BAP with 2.0 µM NAA after five weeks of culture incubation.*

B-C: *Proliferation and development of multiple shoots from leaf segments on MS medium containing 4.0 µM BAP plus 2.0 µM NAA after eight weeks of culture.*

D: *Formation of roots from the regenerated shoots cultured on MS medium supplemented with 4.0 µM IBA.*

E-F: *Growth of transplant on the vermiculite after 6 weeks (E) and twelve weeksof transfer under ex vitro conditions (F).*

Effects of Sucrose Concentrations

Shoot development from excised leaf may vary among species and genotypes upon the level of optimum sucrose concentration (Nowak *et al*., 2004). The percentage of shoot formation, average number and length of shoots per culture increase gradually with the increase of the sucrose concentration in medium up to 30 gm/l. However, the performance of shoot development gradually decreased with an increase in the sucrose concentration to above 30 gm/l. The medium with 30 gm/l sucrose showed the highest percentage of shoot formation (92.31%), average number of shoots per culture (20.1 ± 0.2) and average length of shoots per culture (6.7 ± 0.8 cm) (Fig. 4). An efficient carbon sources for enhanced shoot growth and development has been examined in tissue cultures of some plant species, such as *Echinacea purpurea* (Nilanthai and Yang, 2013), *Allium chinense* (Xu *et al*., 2008), *Elaeocarpus robustus* (Rahman *et al.,* 2004) and *Paederia foetida* (Amin *et al.,* 2003). Our study revealed that MS medium with 30 gm/l sucrose showed the best result for shoot formation in *G. porcambens*. Lower concentrations of sucrose have been shown to be less effective for shoot formation. On the other hand, the detrimental effect of a high sucrose concentration on shoot formation implies that the osmotic level in the medium may be inhibitory to further shoot development. Thus, high concentrations of sucrose seem to inhibit shoot growth and development. This observation are supported by another report (Nowak *et al*., 2004).

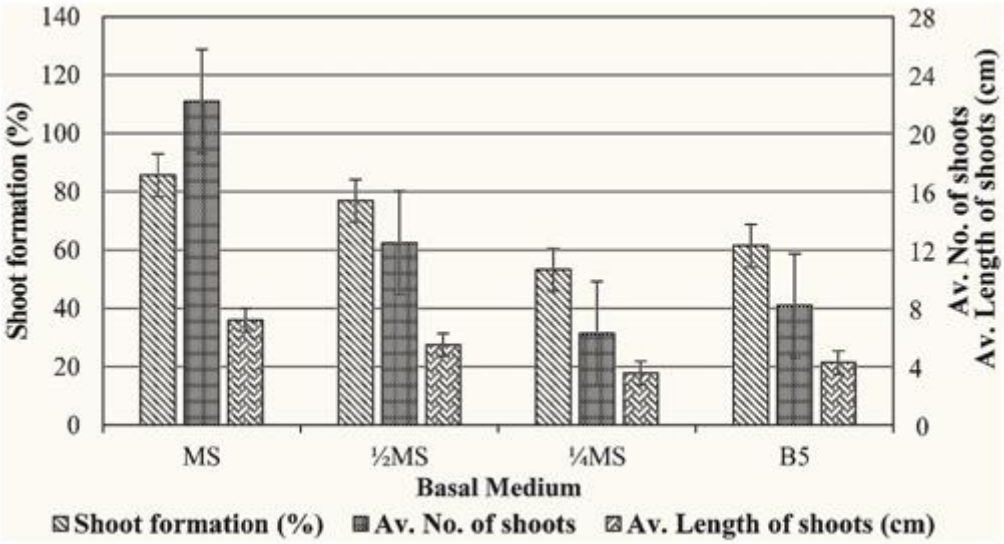

Figure 3. *Effects of different basal media containing 4.0 µM BAP with 2.0 µM NAA on in vitro shoot multiplication from leaf explants of Gynura procumbens.*

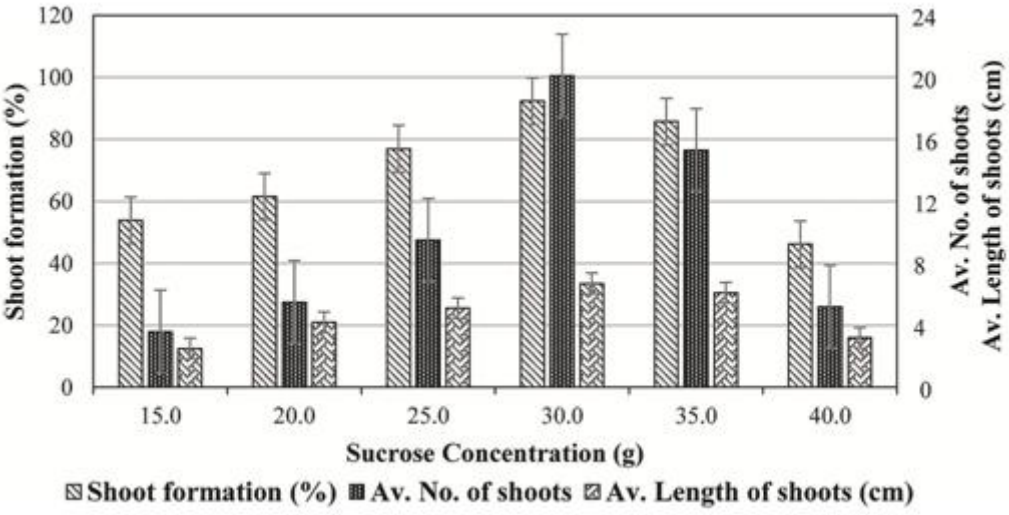

Figure 4. *Effects of sucrose concentrations on adventitious shoot proliferation from leaf segments on MS medium containing 4.0 µM BAP plus 2.0 µM NAA of Gynura procumbens.*

Effects of pH

In vitro multiple shoot development depends upon some other factors rather than plant growth regulators. The pH of culture medium is an important factor for the i*n vitro* proliferation and healthy culture growth. Leaf segments taken from *in vitro* culture that grew on medium containing 4.0 µM BAP with 2.0 µM NAA were used in the present study. Leaf segments were cultured on MS medium adjusted to five different levels of pH viz. 5.0, 5.5, 6.0 and 6.5 but supplemented with only one concentration of cytokinin (4.0 µM BAP). Among these pH levels, the highest percentage of explant showing proliferation was observed on the media adjusted to pH 6.0.and that was 84.62%.The second highest percentage of explant showing proliferation was observed on media having 5.5 pH and it was 76.92%. The lowest frequency of explant showing proliferation was observed on the media where pH was adjusted to 5.0 where the proliferation frequency ranged from 46.15% (Fig. 5). Average number of usable shoot per culture was highest in medium having pH 6.0 and the value was 18.3 ±0.5. From the present investigation it was revealed that both lower (5.0) and higher (6.5) pH levels hindered multiple shoot proliferation. Comparatively less acidic pH (6.5) gave harder gel which might have adverse effects on regeneration and proliferation of shoots. *In vitro* proliferation of *Azadirachta indica* (Gautam *et al*., 1993), *Plantago ovata* (Barna and Walklu,1988) and *Smilax zeylanica* (Jha *et al*., 1987) shoots were increased significantly when the pH the culture media was adjusted at 5.8 before autoclaving.

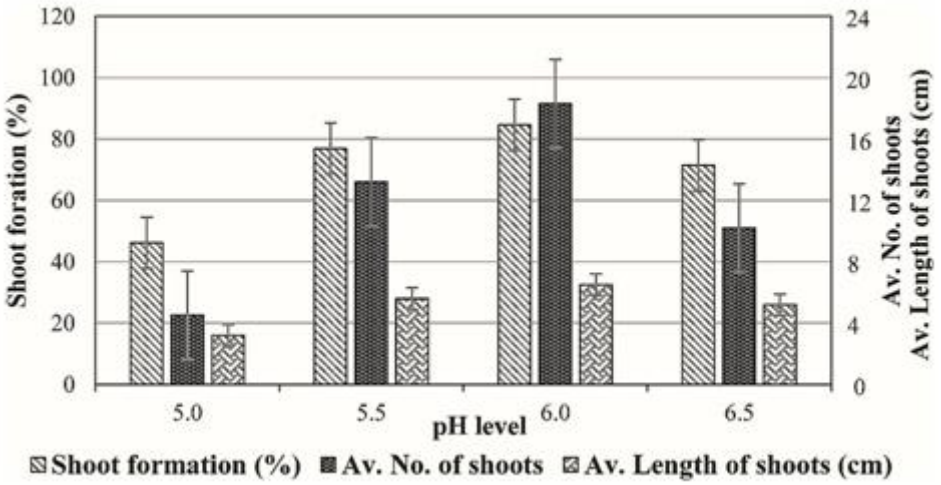

Figure 5. *Effects of pH level on adventitious shoot proliferation from leaf segments on MS medium containing 4.0 µM BAP plus 2.0 µM NAA of Gynura procumbens.*

Rooting

Root formation was enhanced considerably when either IBA, NAA or IAA at different concentrations were added to MS medium. IBA supplemented medium remarkably influenced the rate of root induction than NAA or IAA (Fig. 6). Among the different concentrations of IBA tested, highest frequency (92.31%) with maximum average number 5.2 ± 0.2 of roots per shoot and longest 6.3 ± 0.3 cm roots having considerable lateral roots were obtained with 4.0 µM IBA (Fig. 2D) whereas, 4.0 µM NAA produced considerable root formation where highest frequency was 76.92, average number of roots 4.3 ± 0.3, and average length of roots 4.5 ± 0.5 cm. On the other hand IAA was found to be less effective than IBA or NAA regarding rooting of micro-shoots where the roots were thin in nature. Poor rooting was observed when micro-shoots (2-4 cm in length) were cultured on auxin free MS medium (data not shown). In this study, IBA was proved to be best auxin as comparable to NAA or IAA with regard to all rooting parameters. There are many authors reported that IBA has been found suitable for rooting in many species like *Gynura porcambens* (Majumder *et al*., 2016), *Acacia nilotica* (Dhabhai *et al*., 2010), *Bauhinia variegata* (Mathur and Mukunthakumar, 1996), *Phellodendron amurense* (Azad et. al., 2005, 2009), and *Pterocarpus marsupium* (Husain *et al*., 2008).

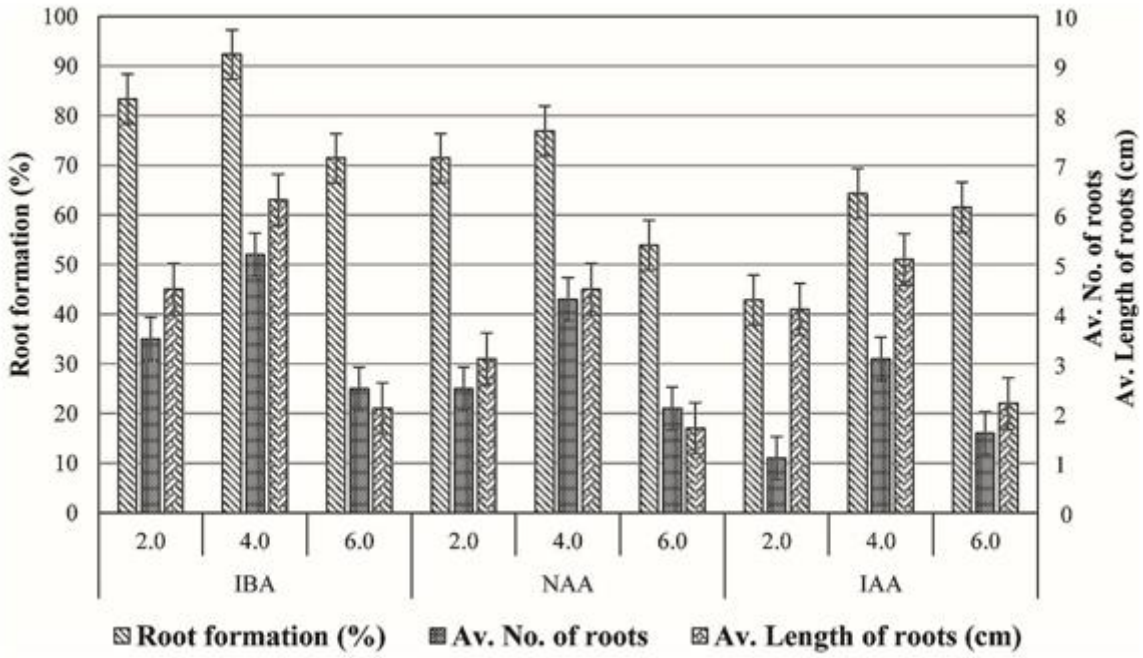

Figure 6. *Effects of auxins on adventitious rooting on MS medium of in vitro derived micro-shoots of Gynura procumbens.*

Hardening and Acclimatization

After successful rooting of micro-shoots, attempts were taken to establish regenerated plantlets onto soil. Plantlets had been transferred to small plastic pots containing soil mix (garden soil: compost: sand, 2:1:1) and Vermicompost separately, maintained under humid *ex vitro* condition in the growth room (Fig. 2E).The *in vitro* derived plantlets acclimated better under *ex vitro* condition when they were maintained in growth room for 20 days before transferring them to outdoor condition (Fig. 2F). Finally, 95% transplanted plantlets were survived and acclimated well under *ex vitro* condition after 25 days of transplantation.

The results revealed that survival rate, plant height, and number of leaf of plantlets after 20 days of transplantation were significantly influenced by vermicompost and soil mix (Fig. 7). Mean survival rate 100%, plant height 7.2 cm and number of leaves 15.4 in vermicompost were significantly greater than that of soil mix where survival rate, plant height, number of leaves were 85.7%, 4.5 cm, and 10.2, respectively. These agreements were supported in Lycopersicum esculentum (Azarmi *et al*., 2008; Joshi *et al*., 2010; Goel and Kaur, 2012), Stevia (Zaman *et al*., 2015), *Phaseolus vulgaris* (Singh *et al*., 2011), *Solanum lycopersicum* (Vaidyanathan and Vijayalakshmi, 2017).

These studies showed that increases of plant growth at vermicompost in the potting medium could probably be due to improvement in the physico-chemical properties of the container medium, increase in enzymatic activity**,** increases in microbial diversity and activity, nutritional factors and plant growth regulators (Atiyeh *et al*., 2000a,b; Arancon *et al*., 2004a,b). Results obtained from this experiment revealed that growth parameters such as survival rate, plant height number of leaf were significantly affected by applying vermicompost. Azarmi*et al*. (2008) reported positive effects of vermicompost on the growth and yield in tomato, especially increases flower blossom, shape and weight of fruits. Mishra *et al*. (2005) showed that vermicompost had beneficial effects on growth and yield of rice, especially caused significant increase of many growth parameters, seeds germination, chlorophyll concentration and yield. Similar results were reported by Najar *et al*. (2015), who mentioned that brinjal (*Solanum melongena*) plant growth and yields in field soils amended with compost were significantly greater than those in the untreated plots.

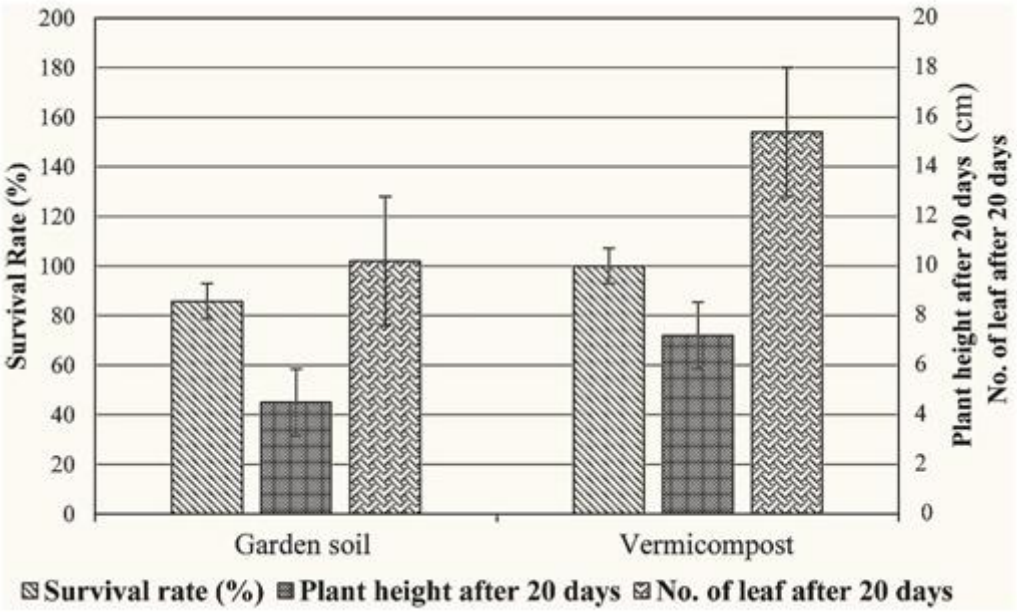

Figure 7. *Effects of vermicompost and soil mix for acclimatization of in vitro derived plantlets of Gynura procumbens under ex vitro condition.*

4. CONCLUSIONS

Traditionally, *G. procumbens* leaves have been known to possess high medicinal values and was used to treat illnesses such as eruptive fevers, rash, kidney diseases, migraines, constipation, hypertension, cancer and diabetes mellitus. Our observation indicates that due to the lack of knowledge, nursery owners are not interested to grow seedlings of these non-conventional plants. The method of

production of *G. probumbens* is not popular. So, that the people are not using these commercially important plants sustainably. Thus, the Biodiversity is being damaged and the *G. procumbens* plant users and businesspersons are being in difficulties for their need. The sustainable use and the rapid production are badly needed. The conventional multiplication method is not sufficient for plantation program. Then multiplication through modern technique is urgently required. Tissue Culture technique in the proposed project is to develop a protocol for producing a large number of disease free plants in a short period irrespective of seasonal variations. These plants will be used for developing *ex situ* conservation, cultivation, and nursery development at home garden. Rapid propagation of *G. procumbens* plants through tissue culture and other conventional method will ensure the availability of disease free plantlets throughout the country. Moreover the tissue culture derived plantlet are easy to transfer from place to place.

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