Efficient shoot regeneration using leaf explant and simulated microgravity effect on shoot development in *Solanum nigrum* L.

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Abstract
Solanum nigrum L. is an important medicinal plant of India and in the present study, leaves were used as an explant to establish shoot cultures. Murashige and Skoog's[18] (MS) medium supplemented with 20 µM kinetin (Kn) regenerated optimum 93.80 ± 0.89 shoots (100% response) within eight weeks through indirect organogenesis. Further, leaves were placed under simulated microgravity conditions and shoot regeneration was achieved within 16th days in both horizontal and vertical conditions. But horizontal position was better as compared to vertical position and it formed 11.60 ± 0.46 shoots.

The in vitro shoots were successfully rooted in ½MS static medium fortified with sucrose (1%) and indole-3-butyric acid (IBA, 2 µM) which induced 39.40 ± 1.08 roots (100% response) within four weeks. This study can be used for mass-propagation of the species, extraction of valuable metabolites and to observe the effect of microgravity on plants.

Keywords: Clinostat, indirect organogenesis, leaf explant, medicinal plant, Murashige and Skoog's medium.

Introduction
Solanum nigrum L. (Solanaceae), commonly known as blacknight shade, is an important medicinal plant of India. Traditionally, different plant parts are used to cure ailments related to heart, nerves, piles, dysentery etc.[15,23]. Some other studies documented that *S. nigrum* has antioxidant, anti-ulcerogenic, hepatoprotective and diuretic properties[6]. The plant contains various alkaloids of which the most important one is solasodine and it is used against a number of pathogens and predators as well as in the treatment of cancer[5]. As *S. nigrum* is being used by pharmaceutical industries as well as tribal people, the annual requirement is very high and according to National Medicinal Plant Boards, India (NMPB), this is one of the high-traded plants with an annual requirement of 2000-5000 MT (https://www.nmpb.nic.in/medicinal_list).

To fulfil the market demand, it is indiscriminately harvested from wild causing a threat to the species and soon may become endangered[30]. Biotechnology tool like plant tissue culture has proved to be an alternative for conservation of the plant species and provides source material for extraction of valuable metabolites similar to mother plants[22]. An efficient regeneration protocol can be developed using different explants, but leaf is known to be a suitable explant for inducing large number of shoots[13,20,21]. Although previous studies have documented regeneration using leaf explant in *S. nigrum*[4,25,29,30], there is a need to develop an efficient regeneration system in short duration of time.

The plants are known to reduces atmospheric CO_2 and elevate O_2 level, thus growing them on international space station will provide ambient air, sustainable food supply along with positive psychological effects on astronauts[27,28]. Hence, the space agencies like NASA are focusing on usage of higher plants in bioregenerative life-support systems (BLSS) since few decades[7]. As the growth and development of plant organs are under high influence of gravity, growing *in vitro* cultures under simulated microgravity will provide an insight to understand the effect on morphogenesis[14]. Previously, it has been documented that *S. nigrum* can become a model plant for *in vitro* regeneration[11] which makes this species appropriate for conducting microgravity experiment.

Thus, aim of the present study was to achieve rapid and efficient regeneration using leaf explant of *S. nigrum*. The explants were further exposed to simulated microgravity to evaluate its effect on growth and development of shoots.

Material and Methods
Plant material and shoot culture establishment: Healthy leaves of *S. nigrum* were collected from the Arboretum of The Maharaja Sayajirao University of Baroda and kept under running tap water for 1 h followed by washing with labolene (Fisher Scientific, Mumbai, India) for 5 min. Prior to inoculation, they were surface sterilized using bavistin (0.01%) and $HgCl_2$ (Merck, Mumbai, India) (0.1%) solution for 3 min each. Square pieces (1 cm^2) of leaf containing midrib were inoculated abaxially on Murashige and Skoog (MS) medium[18] fortified with sucrose (SRL, Mumbai, India) (3%) and cytokinins (5-20 µM) like 6-benzyladenine (BA) and kinetin (Kn) (SRL, Mumbai, India).

Effect of simulated microgravity: Leaves were inoculated on optimized medium having Kn (20 µM) and kept on a one-axis clinostat in horizontal and vertical position (HCS and VCS respectively) at 1 rpm in clockwise direction. Static position for both served as a control (HC and VC respectively). The samples were fixed in FAA solution [Formalin: acetic acid: ethanol (70%): D/W in 10:5:70:15

ratio], dehydrated in isopropanol-alcohol series and embedded in paraffin wax. 15 µm sections were cut using a rotator microtome, stained with heamatoxylene and mounted in DPX. The photographs were obtained using Leica MC120 HD microscope (Leica Microsystems, Mumbai, India).

***In vitro* rooting:** Eight weeks old healthy shoots (> 3 cm) were excised, dipped in sterile D/W and bavistin (0.01%) solution for 1 min each. The shoots were placed in ½MS static and liquid medium fortified with sucrose (1%) and different concentrations (1-4 µM) of indole-3-butyric acid (IBA; SRL, Mumbai, India).

Media and culture conditions: pH of the media was adjusted at 5.80 using NaOH/HCl (1 N) and agar (0.8%) (SRL, Mumbai, India) was used as the solidifying agent. Media were sterilized in autoclave at 121 ºC (15 psi) for 30 min. The cultures were maintained at 26 ± 2 ºC under 16/8 h (dark/light) photoperiod with a photosynthetic photon flux density (40 µmol m^{-2} s^{-1}) of cool white fluorescent lights (Philips India Ltd., India).

Statistical analysis: All the experiments were repeated twice and 10 replicates were maintained for regeneration and rooting studies and 5 replicates for microgravity studies. Mean and standard error (SE) were calculated and the means were further analysed using ANOVA ($\alpha = 0.05$) followed by Tukey's test using GraphPad Prism 6.01.

Results and Discussion

Shoot culture establishment: Leaf explants of *S. nigrum* failed to show any morphogenic response on basal MS medium, but fortifying it with cytokinins differentiated shoots. Variation in shoot number was recorded at different concentrations of BA and total 90.40 ± 1.10 shoots were regenerated within eight weeks at 10 µM concentration. Replacing BA with Kn increased the shoots number as optimum 93.80 ± 0.89 shoots (100% response) were formed at 20 µM (Table 1). In this media, callus formation was observed on the periphery and midrib at lower surface of explant within first week (Fig. 1a).

This greenish yellow callus turned nodular and differentiated shoot buds from lower surface during second week (Fig. 1b). These buds elongated into shoots by 16th day and further proliferation of callus and shoot buds were observed till fourth week (Fig. 1c).

When this morphogenic callus was transferred to a fresh medium, it further proliferated and covered whole lamina and differentiated healthy shoots by sixth week (Fig. 1d) which continued till eight weeks (Fig. 1e).

Table 1
Effect of cytokinins on shoot regeneration from leaf explant of *S. nigrum* (8 weeks)

PGRs (µM)		No. of shoots/explant* (Mean ± SE)	Response (%)
BA	Kn		
0	0	0 m	0
5	0	76.80 ± 1.01 ef	100
10	0	90.40 ± 1.10 ab	100
15	0	84.10 ± 1.40 cd	100
20	0	79.80 ± 0.98 de	100
0	5	62.20 ± 1.11 kl	100
0	10	71.20 ± 0.73 fghi	100
0	15	92.70 ± 0.92 a	100
0	20	93.80 ± 0.89 a	100
5	5	62.10 ± 1.15 kl	100
5	10	86.30 ± 0.71 bc	100
5	15	61.10 ± 0.96 kl	100
5	20	58.70 ± 0.98 l	100
10	5	68.70 ± 1.22 hij	100
10	10	74.70 ± 0.80 efgh	100
10	15	79.40 ± 0.99 de	100
10	20	76.70 ± 0.92 efgh	100
15	5	73.10 ± 1.32 fgh	100
15	10	75.30 ± 0.88 efgh	100
15	15	76.20 ± 0.82 efgh	100
15	20	69.70 ± 1.06 ghij	100
20	5	64.60 ± 1.20 jk	100
20	10	66.20 ± 1.14 ijk	100
20	15	75.20 ± 1.43 efgh	100
20	20	73.20 ± 1.48 fgh	100

*Means (n = 10) followed by same letter are not significantly different ($p \leq 0.05$) using Tukey's test

Figure 1: Shoot regeneration from leaf explant of *S. nigrum* in MS medium supplemented with Kn (20 µM)- (a) callus induction after first week, (b) induction of shoot buds at the end of second week, (c) proliferation of shoot buds and formation of shoots at the end of fourth week, (d) further proliferation and elongation of shoots during sixth week and (e) healthy shoots at the end of eight weeks

Further, when both the cytokinins were added together in the media, they failed to increase shoot number as it was slightly decreased to 86.30 ± 0.71 in presence of BA (5 µM) with Kn (10 µM) (Table 1). Shoot regeneration from leaf explant of *S. nigrum* was carried out in presence of cytokinins. Cytokinins are one of the key factors for *in vitro* regeneration due to their role in regulation of proteins synthesis, which controls cell division and shoot formation[8,10]. Eight weeks observations revealed that the large numbers of shoots were regenerated in the present study, which is rapid and efficient as compared to earlier reports for the same plant[4,25,29,30].

Among both the hormones tried, it was noted that Kn proved better as compared to BA, which is in line with earlier report on *S. nigrum*[10] as well as in other species like *Tylophora indica*[9], *Justicia gendarussa*[1] and *Ionidium suffruticosum*[24].

Effect of simulated microgravity: In this experiment, *S. nigrum* leaves were placed in horizontal and vertical positions under simulated microgravity conditions (on clinostat) to observe its effect on shoot differentiation. Under control conditions, swelling was observed within 5th day in both horizontal as well as vertical positions (Fig. 2a, c), but no visible difference was observed in anatomy (Fig. 2b, d). Further callus development was observed on periphery and lower surface on the 10th day in both positions (Fig. 2e, g) and the same was observed in anatomy slides (Fig. 2f, h).

These callus tissues proliferated and differentiated shoot buds (Fig. 2i, k) which was observed as a meristemoids in anatomy of callus in control condition on 13th day (Fig. 2j, l). These buds formed shoots along with proliferation of callus on 16th day (Fig. 2m, o). Anatomy of callus confirmed

that the parenchyma cells adjacent to vascular tissues differentiated continuously and give rise to new meristemoids. Formations of leaf primordias were observed along with the development of vascular strands (Fig. 2n, p). Total 17.40 ± 0.46 shoots were formed in HC whereas 14.40 ± 0.73 shoots were formed in VC position (Table 2). Similarly, less callus mass and shoot bud formation was observed when the explants were placed under simulated microgravity conditions. The leaves swelled within five days in both HCS and VCS (Fig. 3a-d).

Formation of callus was observed from cut ends and on lower surface on the 10th day, but it was comparatively less than control (Fig. 3e-h). The callus started to differentiate shoot buds (Fig. 3i, k) which was confirmed as visible meristemoids under microscope by 13th day (Fig. 3j, l).

This callus was further proliferated with simultaneous elongation of shoot buds into shoots (Fig. 3m, o) and the same was observed as increased number of meristematic centres and its differentiation into apical meristems in anatomy (Fig. 3n, p). Total 11.60 ± 0.46 and 10.40 ± 0.83 shoots were formed for HCS and VCS respectively which was less as compared to control (Table 2).

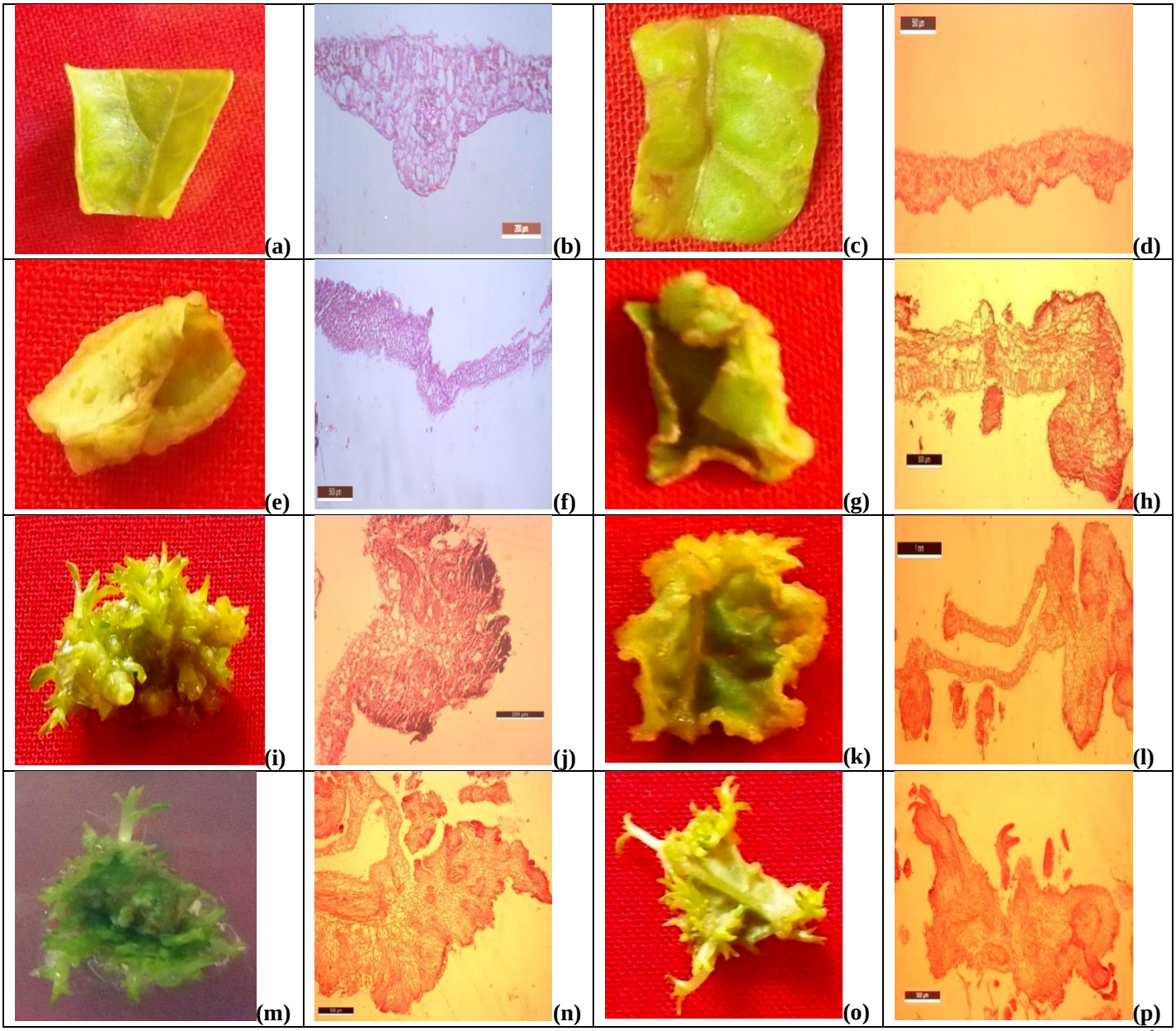

Figure 2: Shoot regeneration in horizontal (HC) and vertical (VC) control conditions- (a-d) swelling of leaf after 5th day in (a, b) HC and (c, d) VC, (e-h) callus induction after 10th day in (e, f) HC and (g, h) VC, (i-l) formation of shoot buds from callus as meristamatic centres (arrow) in anatomy after 13th day in (i, j) HC and (k, l) VC, (m-p) proliferation of shoot buds and their elongation into shoots as shoot primordial formation (arrows) in anatomy at the end of 16th day in (m, n) HC and (o, p) VC

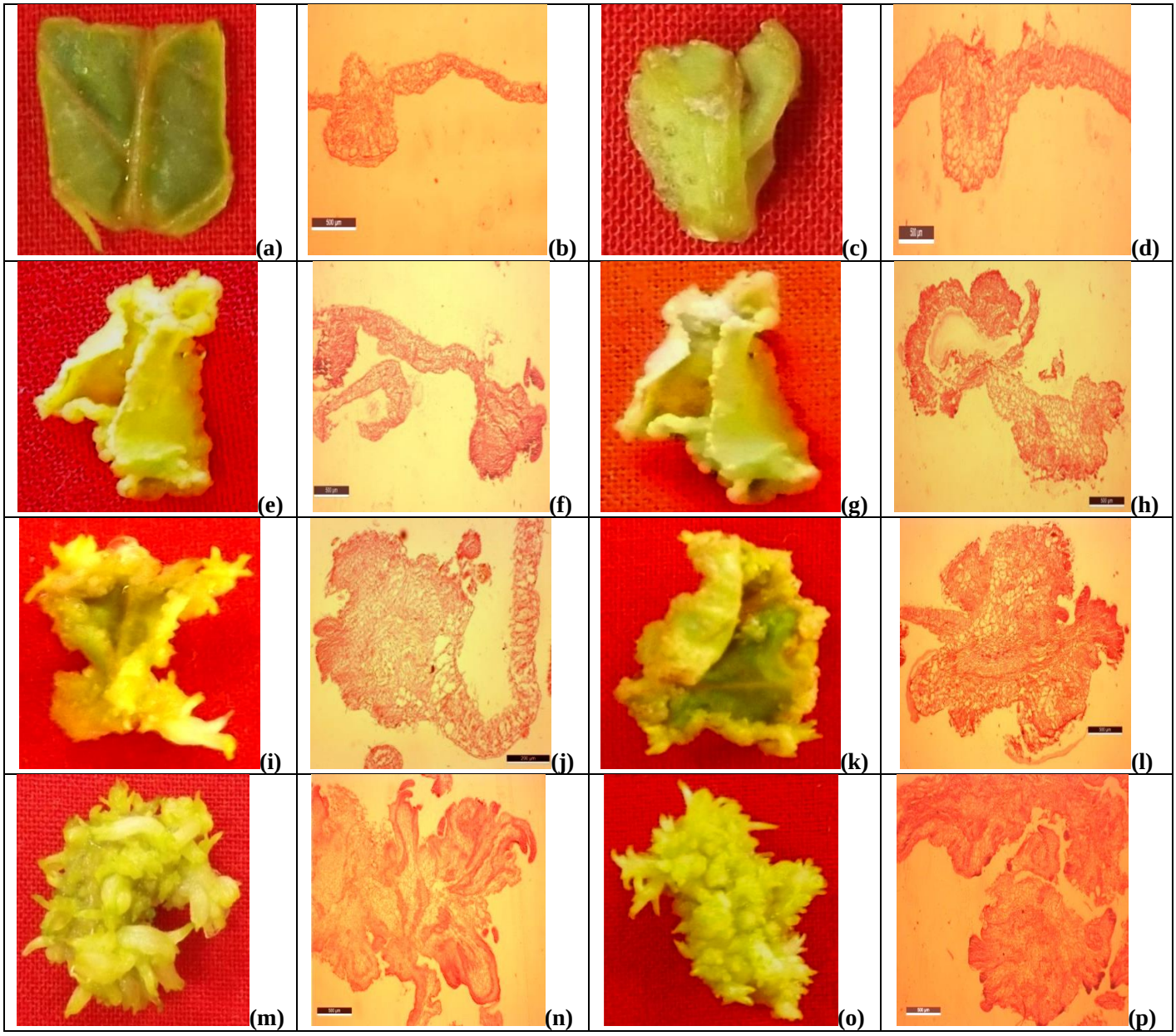

Figure 3: Shoot regeneration in horizontal (HCS) and vertical (VCS) simulated microgravity conditions- (a-d) swelling of leaf after 5th day in (a, b) HCS and (c, d) VCS, (e-h) callus induction after 10th day in (e, f) HCS and (g, h) VCS, (i-l) formation of shoot buds from callus as meristamatic centres (arrow) in anatomy after 13th day in (i, j) HCS and (k, l) VCS, (m-p) proliferation of shoot buds and their elongation into shoots as shoot primordial formation (arrows) in anatomy at the end of 16th day in (m, n) HCS and (o, p) VCS

The shoot formation depends on growth and differentiation of cells as well as their divisions and expansion[2]. When *S. nigrum* leaves were placed on different positions (horizontal and vertical), it affected the regeneration efficiency and horizontal positions evoked better response as compared to vertical positions. This is in corroboration with previous study on same plant in which it was also documented that changing orientation of explant affected the regeneration frequency[4].

Growth and morphogenesis of plants are influenced by factors like light, temperature and gravity, of which gravity has a profound influence on its growth[3]. When comparing growth of *S. nigrum* cultures between control and microgravity conditions, it was similar between both the conditions and this is in line with studies on *Jatropha curcas*[19,26]. However, when callus size and shoot number were considered, it was less under microgravity conditions and similar observations were reported for rapeseed and carrot cells[12] as well as in *Arabidopsis* hypocotyls[17].

***In vitro* rooting:** Eight weeks old shoots were then taken up for *in vitro* rooting in static and liquid ½MS medium fortified with different IBA concentrations. In basal medium, it formed 8.60 ± 0.47 and 8.40 ± 0.45 roots under static and liquid conditions respectively. Further augmentation of IBA increased the number of roots.

Table 2
Effect of positions and microgravity on shoot regeneration (16 days)

Positions	No. of shoots/explant* (Mean ± SE)
HC	17.40 ± 0.46 a
HCS	11.60 ± 0.46 bc
VC	14.40 ± 0.73 b
VCS	10.40 ± 0.83 c

*Means (n = 5) followed by same letter are not significantly different ($p \leq 0.05$) using Tukey's test

However, it was recorded that overall static media was proved to be beneficial as compared to liquid media for rooting of *S. nigrum* shoots. An optimum 39.40 ± 1.08 roots (100% response) were formed in static medium fortified with 2 µM IBA (Fig. 1f) whereas the number was decreased to 19.80 ± 0.88 in liquid media at same IBA level, but the % response remained same (Table 3).

In vitro rooting of shoots requires addition of auxins and IBA is one such hormone which has been used for rooting in other medicinal plants like *Hemidesmus indicus*[21] and *Bacopa monnieri*[13].

In the present study, optimum roots were formed in presence of 2 µM IBA which is in accordance with earlier report on *S. nigrum*[25]. The beneficial effect of IBA on rooting is because of its slow movement and degradation which facilitates its localization near the rooting site and thus increases the rooting response[16].

Conclusion

Thus, an efficient shoot regeneration protocol was developed in the present study from leaf explant of *S. nigrum*, which will aid in propagation of the species and will reduce the threat on wild population. The leaves were able to differentiate shoots under simulated microgravity conditions and thus it can be considered as a model plant for spaceflight experiments.

Microgravity is known to alter the genetic expression of plants and further studies should focus on investigating the genetic changes and differential gene expression. The developed shoot cultures will be assessed for their biosynthetic potential to synthesize metabolites and can be used as an alternative to wild plants.

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